Debmalya Barh
Editor

Omics Approaches in Breast Cancer

Towards Next-Generation Diagnosis, Prognosis, and Therapy

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Springer

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My loving Gul: Shaurya Shree

Foreword

The modern trends of disease diagnosis, prognosis, and therapy are changing from conventional to rationally designed personalized approaches. "Omics" based next-generation technologies have boosted this revolution. The fact is true in the case of breast cancer also, which is one of the most common cause of morbidity and mortality in women worldwide.

This book *Omics Approaches in Breast Cancer*: *Towards Next-Generation Diagnosis*, *Prognosis and Therapy*, edited by Debmalya Barh, is one of the endeavour efforts that presents omics based research outcomes and their real-time applications in breast cancer diagnosis, prognosis, and therapy. The book is designed in such a way that blends the basic topics such as biology, conventional diagnosis and treatment approaches along with the latest advances in tackling breast cancer using next-generation omics based technology outcomes. The book has highlighted several latest technology and fields in the subject area such as metabolomics, nutrigenomics, RNAomics, stem cell and cellomics, pharmacogenomics, animal and *in silico* models, exhaled volatile biomarkers, minimal/non-invasive molecular biomarkers, targeted therapy, personalized medicine, and gene therapy of breast cancer among others. Male breast cancer, although it is very rare, is also included in the book. There are 27 chapters in this book written by 65 breast cancer specialists across the world. Therefore, the book provides state-of-the-art contents, real-life personal experiences, and future prospects of breast cancer research.

For the benefit of readers, topics are grouped under two sections where the first section describes various omics technologies and their outcomes in breast cancer research and the second section is having chapters that contain the applications of the latest omics technologies in next-generation breast cancer diagnosis, prognosis, and therapy. In the first part, the book contains chapters on: Overview of omics technologies applied in breast cancer research; Omics of hereditary breast cancer; Oncogenes and tumor suppressor genes as biomarkers in breast cancer; Breast cancer genomics; Epigenomics approaches in breast cancer; Breast cancer neutrigenomics; Implications of long non-coding RNAs in breast cancer pathogenesis, diagnosis and therapy; micro-RNA as clinical biomarkers for diagnosis and treatment of breast cancer; Breast cancer proteomics; Metabolomics in breast cancer; Lipidomics in breast cancer; Breast cancer stem cells and cellomics; Omics of male breast cancer; Omics approaches in chemoresistant and metastatic

breast cancer; Animal and *in silico* breast cancer disease models; and Systems biology and integrative omics approaches in breast cancer. In the application part, the book includes chapters on: Gynaecological aspects and considerations for women suffering from breast cancer; Currently available imaging technologies and their applications in early diagnosis and prognosis of breast cancer; Non-invasive or minimal invasive molecular biomarkers for risk assessment, screening, detection, diagnosis, and prognosis of breast cancer; Circulating tumor cells for diagnosis and prognosis of breast cancer; Molecular diagnosis of metastasizing breast cancer using liquid biopsy; Volatile biomarkers in breast cancer; Classical and targeted therapy in breast cancer; Personalized medicine in breast cancer; Gene therapy in breast cancer; and Clinical trials endpoints in breast cancer.

It is a great effort by Debmalya Barh to cover almost all aspects of breast cancer in this omics era in this book. The broad-coverage, latest information, and rich contents of the book in the field of breast cancer is the uniqueness of the book that will definitely help in next-generation diagnosis, prognosis, and therapy of breast cancer. I highly recommend the book for readers who are working in the field of breast cancer research, diagnosis, and treatment.

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Preface

Breast cancer is the leading cause of cancer specific deaths in women worldwide. Screening and early diagnosis provides better patient management, improves treatment efficacy, and reduce mortality. With the advent of next-generation "omics" technologies; several early markers, novel targets, and personalized targeted therapeutics are now available that are either under development or in use. However, the new cases are increasing rapidly and control of the incidences and the mortality rate is not coming down significantly due to several factors associated with the disease. Therefore, identification of the disease biology at a deeper level and search for the gold standard molecular markers for screening, early diagnosis, prognosis, and novel therapeutics irrespective to the type of breast cancer will continue till we identify them. In this "omics" era, a large amount of data have been generated and analyzed in various aspects of breast cancer to achieve these ultimate goals. However, the outcomes of these data in an organized form is not readily available so far, so that, the summery and the advancements in these fields can be glanced in a single resource.

This book entitled *Omics Approaches in Breast Cancer*: *Towards Next-Generation Diagnosis*, *Prognosis and Therapy* is introduced to fill these gaps by providing all basic and latest developments in various "omics"-based breast cancer research outcomes and applications in a single volume. The book also contains basic topics such as types of breast cancers, conventional treatment strategies, currently used diagnostic tools, etc. so that readers can get the entire spectrum of breast cancer.

The book is a successful effort of more than 65 experts (scientists, clinicians, pharmacists, etc.) from nearly 20 countries who are either working on various "omics" aspects of breast cancer biology or developing breast cancer biomarkers and therapeutics or treating breast cancer for last several decades. Therefore, the book reflects richest and up-to-date contents, personal and real-life experiences, and most importantly, provides the future directions of breast cancer research.

Omics Approaches in Breast Cancer: *Towards Next-Generation Diagnosis*, *Prognosis and Therapy* contains 27 chapters covering most of the aspects of female breast cancer "omics" and is divided into 2 parts. While Part I contains applied technologies and outcomes of various "omics" approaches in breast cancer, Part II provides real-life applications of "omics"-based research outcomes in breast cancer diagnosis, prognosis and therapy along with basic clinical and therapeutical aspects of the disease. A special chapter is also

included on male breast cancer to describe up-to-date "omics"-based outcomes on this rare type of breast cancer.

Part I starts with an introductory chapter (Chap. 1) by Dr. Cusati and colleagues to provide an overview of "Omics technologies applied in breast cancer research". In Chap. 2, the "Omics of hereditary breast cancer" has been described by Dr. Catherine's group. Dr. Uctepe et al. provide the insights on how the oncogenes and tumor suppressor genes can act as biomarkers in breast cancer in the Chap. 3. Chapter 4 by Dr. Kumar and Dr. Mandal gives a detail account on various aspects of "Breast cancer genomics". In the next chapter (Chap. 5), Dr. Yaykasli and colleagues have provided the epigenomics approaches in breast cancer. How the nutrients and genes interplay in breast cancer have been covered by Dr. Dwivedi and colleagues in Chap. 6. In Chap. 7, the implications of long non-coding RNAs in breast cancer pathogenesis, diagnosis and therapy have been discussed by Dr. Juracek and colleagues. The subsequent chapter (Chap. 8) by Dr. Shafi's team gives a detail account on how microRNA can be utilized as clinical biomarkers for diagnosis and treatment strategies in breast cancer. In Chap. 9, Prof. Minafra has demonstrated a detail account on "Breast cancer proteomics". The next chapter (Chap. 10) by Dr. Calomarde et al. covers the "Metabolomics in breast cancer". The "Lipidomics in breast cancer" written by Dr. Kamili and Dr. Byrne is included in Chap. 11. "Breast cancer stem cells and cellomics" by Dr. Demir and colleagues in Chap. 12 has provided the emerging field of potential stem cell therapeutic aspects in breast cancer. In this book, breast cancer generally means female breast cancer. However, there are cases where males are also diagnosed with breast cancer. The book has included a chapter (Chap. 13) on "Omics of male breast cancer" by Dr. Nur Unal et al. to give up-to-date "omics"-based strategies, outcomes, and other aspects of this very rare type of breast cancer. In Chap. 14, omics approaches in chemoresistant and metastatic breast cancer have been discussed by Dr. Aguilera. In the next two chapters (Chaps. 15 and 16), animal and *in silico* breast cancer disease models and their various aspects have been described by Dr. El-Abd and Dr. Munshi's groups, respectively. The last chapter (Chap. 17, by Dr. Hernández-Lemus) under Part-I provides systems biology and integrative omics approaches in breast cancer to give the latest developments in this area.

Part II of this book provides information on how the "omics"-based research outcomes are used in real-life diagnosis, prognosis and therapy of breast cancer. The section starts with a chapter (Chap. 18) by Dr. Robinson and Dr. Ali that provides the basic clinical or gynaecological aspects and considerations for women suffering from breast cancer. The next chapter (Chap. 19) developed by Dr. Mar Gil and colleagues gives the detail account of currently available imaging technologies and their applications in early diagnosis and prognosis of breast cancer. In Chap. 20, Dr. Verma and I have demonstrated various molecular biomarkers that are either under development or in practice for risk assessment, screening, detection, diagnosis, and prognosis of breast cancer. Next three chapters deal with very important aspects in breast cancer diagnosis and prognosis through minimal- or non-invasive strategy. Dr. Van Pham in Chap. 21 has provided how the circulating tumor cells can be used for diagnosis and prognosis of breast cancer. In the

same direction, Dr. Dwivedi and colleagues in Chap. 22 have described the molecular diagnosis of metastasizing breast cancer using liquid biopsy, and in Chap. 23, Dr. Barash and Dr. Haick have demonstrated the emerging potential of exhaled volatile biomarkers in breast cancer. Chapters 24, 25, and 26 cover therapeutic aspects of breast cancer. Dr. Ch Yiannakopoulou in Chap. 24 has given a detail account of classical therapy and drug targets along with targeted therapy in breast cancer. In the next chapter (Chap. 25), Dr. Ch Yiannakopoulou and me have discussed the pharmacogenomics or personalized medicine and their therapeutic implications in breast cancer. The current status and future prospects of gene therapy in breast cancer has been discussed in Chap. 26 by Dr. Büyükköroğlu and her colleagues. The last chapter (Chap. 27) is on clinical trial, and in this chapter Dr. George and Dr. Selvarajan have described the essentiality of optimum end points in breast cancer clinical trials to select the right drug having the best efficacy and minimal toxicity.

I believe that this book and its up-to-date contents and broad coverage will be worthwhile to cutting-edge breast cancer research, diagnosis, and clinician communities. I highly appreciate your comments and suggestions to improve the next edition of the book.

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About the Editor

Debmalya Barh (MSc, MTech, MPhil, PhD, PGDM) is the founder of the Institute of Integrative Omics and Applied Biotechnology (IIOAB), India, a virtual global platform for multidisciplinary research and advocacy. He is a biotechnologist and an active researcher in integrative omics-based biomarkers, targeted drug discovery, and personalized medicine. He works with over 400 well-regarded researchers from more than 40 countries and has to his credit over 125 high-impact international publications, several books, and book chapters. He is a globally branded editor for editing omics-related research reference books and an editorial and review board member for a number of highly reputed international journals. Due to his significant contributions to the field and in promoting biomedical sciences using unique research strategies, in the year 2010 he was recognized by *Who's Who in the World* and in 2014 he entered the *Limca Book of Records,* the Indian equivalent of the *Guinness Book of World Records*.

Abbreviations

2DE	Two-dimensional electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
ABC	ATP binding cassette transporter family
aCGH	Array-comparative genome hybridization
ACOG	American Congress of Obstetricians and Gynecologists
ACP	American College of Pathology
ACR	American College of Radiology
ACS	American Cancer Society
ADP	Adenosine diphosphate
AE	Adverse event
AI	Aromatase inhibitor
AIDS	Acquired immunodeficiency syndrome
ALA	Alphalinolenic acid
ALDH	Aldehyde dehydrogenase activity
AMBP	α-1-microglobulin/Inter-α-trypsin inhibitor light chain precursor
ANN	Artificial neural network
A-T	Ataxia-telangiectasia
ATAC	Arimidex, Tamoxifen Alone or in Combination (trial or study)
ATM	Ataxia-telangiectasia mutated
BBN	Bayesian belief network
BC	Breast cancer
BCC	Breast cancer cells
BCI	Breast cancer index
BCRAT	Breast cancer risk assessment tool
BCSC	Breast cancer stem cells
BIG	Breast International Group (1–98 study/trial)
BIP	Immunoglobulin heavy chain binding protein
BI-RAD	Breast Imaging Reporting and Database
BLBC	Basal-like breast cancer
BM	Bone marrow
BMAC	Breath methylated alkanes contour
BSE	Breast self-exam
CAFs	Cancer-associated fibroblasts
CAGE	Cap analysis of gene expression
CAN	Copy number aberrations
CAV1	Caveolin-1
CD	Cluster of differentiation (molecules or markers)

cDNA	Complementary DNA
CGH	Comparative genomic hybridization
CMTC	ClinicoMolecular Triad Classification
CNV	Copy number variations
COMT	Catechol-O-methyltransferase
CT	Computerized tomography
CTC(s)	Circulating tumor cell(s)
CTOP	Cancer therapy outcome predictor
DCIS	Ductal carcinoma in situ
DCR	Disease control rate
DEP	Dielectropheresis
DFS	Disease-free survival
DIC	Ductal infiltrating carcinoma
DIGE	Difference in-gel electrophoresis
DNMTs	DNA methyltransferases
DTCs	Disseminated tumor cells
ECD	Ectodomain
ECGC	Epigallocatechin-3-gallate
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMEA	European Agency for the Evaluation of Medical Products
EMT	Epithelial/mesenchymal (or epithelial to mesenchymal) transition
EORTC	European Organization for Research and Treatment of Cancer
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor(s)
ERRα	Estrogen-related receptor α
ERα	Estrogen receptor α
Erβ	Estrogen receptor β
ESI	Electron spray ionization
ESR1	Estrogen-receptor 1
FA	Fanconi anemia
FASN	Fatty acid synthase
FAST	Fiber-optic array scanning technology
FC	Flow cytometry
FDG	2-deoxy-2-[18F]fluoro-d-glucose
FFA	Free fatty acids
FFPE	Including formalin-fixed paraffin-embedded (samples)
FGFR	Fibroblast growth factor receptor
FISH	Fluorescence in situ hybridization
FK-228	Romidepsin
FM	Fluorescence microscopy
FNA	Fine-needle aspiration
FTDT	Finite difference (modelings)
GASP-1	G-protein coupled receptor associated sorting protein 1
GC	Gas chromatography
GES	Gene expression signatures
GLA	Gamma-linolenic acid

GNP	Gold nanoparticles
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GRNs	Gene regulatory networks
GS	Glutamine synthetase
GSEA	Gene Set Enrichment Analysis
GWAS	Genome-wide association studies
HATs	Histone acetyltransferases
HBOC	Hereditary breast and ovarian cancer
HDAC	Histone deacetylase
HDMs	Histone demethylases
HER2	Human epidermal growth factor 2
HIC1	Hypermethylated in cancer 1
HIV	Human immunodeficiency virus
HME	Human mammary epithelial (cells)
HMTs	Histone methyltransferases
hNMSC	Human normal mammary stem cell (signature)
HNPCC	Hereditary non-polyposis colon cancer
HPLC	High-performance liquid chromatography
HPV	Human papillomavirus
HR	Homologous recombination
HR-MAS	High-resolution magic angle spinning
HSV	Herpes simplex virus
HT	Hormonal therapy
HTOTs	High-throughput omic technologies
IDC	Infiltrating ductal carcinoma
IDFS	Invasive disease-free survival
IEL	Intraepithelial lesion
IGF	Insulin-like growth factor
IHC	Immunohistochemical/immunohistochemistry
ILC	Infiltrating lobular carcinoma
ITC	Isolated tumor cells
IVD	In vitro diagnostic
IVF	In vitro fertilization
LC-MS/MS	High-performance liquid chromatography and tandem mass spectrometry
LD	Linkage disequilibrium
LN	Lymph node
lncRNA	Long non-coding RNA
LSINCT5	Long stress-induced non-coding transcript 5
LV(s)	Latent variable(s)
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MAPs	Microtubule-associated protein
MATES	Multidrug toxin extrusion proteins
MBC	Male breast cancer
MBP	Methyl CpG binding protein
MEK	Mitogen-activated protein kinase

miRNA	MicroRNA
MPA	Metabolic phenotypic analysis
MRGs	Master regulator genes
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Mass spectrometry
MSP	Methylation specific PCR
mTOR	Mammalian target of rapamycin
MudPit	Multidimensional protein identification technology
MUFAs	Monounsaturated fatty acids
NCBI GEO	National Center for Biotechnology Information Gene Expression Omnibus
NCI	National Cancer Institute
NCNN	National Comprehensive Cancer Network
ncRNA	Non-coding RNA
NGS	Next-generation sequencing
NIH	National Institutes of Health
NLST	National Lung Screening Trial
NMR	Nuclear magnetic resonance
NPV	Negative predictive value
OA	Oleic acid
ODE	Ordinary differential equations
ORR	Objective response rate
OS	Overall survival
PAM50	Prediction Analysis of Micro-array 50
PARPs	Poly ADP-ribose polymerase inhibitors
PB	Peripheral blood
PC	Principle component
PCA	Principle component analysis
pCR	Pathologic complete response
PCR	Polymerase chain reaction
PET	Positron emission tomography
PFS	Progression-free survival
PHGDH	Phosphoglycerate dehydrogenase
PLS-DA	Partial least squares discriminant analysis
PNN	Probabilistic neural network
ppb	Parts per billion
ppt	Parts per trillion
PR	Progesterone receptor
PTMs	Post-translational modifications
PtNP	Platinum nanoparticles
PTR	Proton transfer
PUFAs	Polyunsaturated fatty acids
QM-MSP	Quantitative multiplex-methylation specific PCR
QOL	Quality of life
qRT-PCR	Quantitative real-time polymerase chain reaction
RASSF1A	Ras association domain family protein 1
RECIST	Response Evaluation Criteria in Solid Tumors

RIP	RNA immunoprecipitation
RNAi	RNA interface
ROLL	Radionuclide lesion localization
ROS	Reactive oxygen species
RT	Reverse transcription
SAGE	Serial analysis of gene expression
SAHA	Vorinostat
SELDI-MS	Surface-enhanced laser desorption/ionization mass spectrometry
SERM	Selective estrogen receptor modulator
SLN	Sentinel lymph node
SLNB	Sentinel lymph node biopsy
SNPs	Single nucleotide polymorphisms
SNRI	Serotonin noradrenalin reuptake inhibitor
SNS	Single-nucleus sequencing
SP	Side population (technique/cells)
SPME	Solid phase micro-extraction
SRA	Steroid receptor RNA activator
SSRI	Serotonin reuptake inhibitor
TCA	Tricarboxylic acid
TCGA	The Cancer Genome Atlas
TERRA (or Tel RNA)	Telomeric repeat-containing RNA
TGF-β	Transforming growth factor-β
TKI	Tyrosine kinase inhibitor
TLC	Thin-layer chromatography
TOF	Time of flight
TRAM	Transverse rectus abdominus myocutaneous
TTF	Time to treatment failure
T-UCRs	Transcribed-ultraconserved regions
UGTs	Glucurosyltransferases
UPSIO	Ultrasmall paramagnetic iron oxide
VEGF	Vascular endothelial growth factor
VOCs	Volatile organic compounds
VOMs	Volatile organic metabolites
XIST	X-inactive specific transcript
Zfas1	Zinc finger antisense

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Part I

Omics Approaches in Breast Cancer

Omics Technologies Applied in Breast Cancer Research

1

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Abstract

Omics are technologies used to quantify cellular components on a large scale. Studying the genome with high-throughput research tool gene expression profiling has produced a wide knowledge of the molecular level process of different pathologies, particularly about cancer. Omics technologies have been linked from the beginning to breast cancer research, achieving the exposure of cancer heterogeneity, its genomic complexity, and the molecular events that drive cancer biology. This whole set of genetic information brought the understanding of breast cancer as a heterogeneous disease with diverse morphologies, molecular characteristics, and clinical behavior; therefore omics technologies are currently being used to identify gene signatures in a need for more accurate diagnosis, prognosis, and treatment. A valuable quantity of genetic information from breast cancer and multiple research groups is being publicly stored, and analyzing and integrating these will achieve a complete and deep understating of the pathogenesis of this disease and help to drastically improve its clinical outcome.

Keywords

Breast cancer • Omics technologies • High throughput • Gene expression profiling • Molecular profiling technologies • Genomics • Epigenomics • Proteinomics • Transcriptomics

Introduction

Nowadays breast cancer is being regarded as several diseases with the same name, due to its numerous subtypes and its different histological, biological, and molecular characteristics. This variability brings different responses to the therapy applied, and therefore a change in its previous

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prognosis [1]. Tumor size, histological grade, lymph node status, and hormone receptor status expression have classically been used in order to set a treatment strategy and prognosis. Nevertheless, these prognostic factors lack in accuracy because there are many genetic and molecular factors of breast cancer disease that are not fully understood yet [2]. It seems like some patients are over-treated, out of fear for the still unexpected and uncontrolled relapses. It is very significant to find new markers in breast cancer that may improve detecting and classifying this disease, as well as predicting and tracking patient response [3, 4].

The Birth of the Omics Era

In 1920 the botanist Hans Wrinkler used a new word to describe the entire genetic material of an animal or plant: *genome*, a combination of the words *gene* and chromosome. Some scientists interpreted the suffix "ome" as a collective from a unit, with gene being the unit, and genome collectively, so then this suffix was applied to create more words. The first example is the use of *proteome* as a word to describe the whole set of protein derivate from genome. At the end of 1990, the word genomics was beginning to be used to describe the study and application of the information obtained from the genome, which resulted in the suffix "omics." This new genetic language has started the omics age, in which the word omics now refers to the comprehensive analysis of biological systems [5].

Omics are high-throughput technologies used to quantify cellular components on a large or wide scale as a genome or proteome. This technological breakthrough has brought cancer genomics, or the study of tumor genome, via various profiling data such as DNA copy number, DNA methylation, transcription, and genome sequencing of cancer cells. This allows identification of genetic pathways of cancer, bringing a greater understanding of the biology of cancer and leading to the discovery of novel diagnostic, prognostic, and therapeutic options. Omics technologies have exposed the genomic complexity, cancer heterogeneity, and the molecular events that drive cancer biology, hence recognizing them as the pathways to creating improved therapies specific for cancer phenotypes [6].

Summarizing, we can use omics technologies to refer to the study and application of the information obtained from the genes involved in tumors, using genetic resources such as the number of copies of DNA or RNA, the transcription, and methylation, with the possibility to scrutinize cancer cells from gene to metabolite [7]. We will describe the main technologies that study each step of the central dogma of biology, gene–DNA–RNA–protein, which in the same order are genomics–epigenomics–transcriptomics–proteinomics, showing how they are applied in breast cancer research, although there are many more omics technologies. Figure 1.1 explains the relationship between gene, DNA, RNA, and protein (the central dogma of molecular biology) with all the information obtained about them through omics technologies.

Genomics

Genomics may be described as the comprehensive analysis of genes and their DNA structure and function or as the scientific discipline looking for information about the entire genome. Before the existence of omics technologies, DNA was sequenced gene by gene, but improving informatics technology and integrating them to the biology have allowed the sequencing of the entire genomes of many types of organisms and the classification and storage of DNA sequences in genomic databases. Variations of the genes or DNA sequences can be created through gene amplification, gene deletion, or gene rearrangement [8].

Copy number aberrations (CNA) or copy number variations (CNV) are the names that encompass the alterations of the organization and amount of DNA within a cell. Genetic alterations are generated by deletions, duplications, inversions, or translocations of chromosomes; moreover these modifications are capable of being inherited. CNA or CNV can disturb the normal

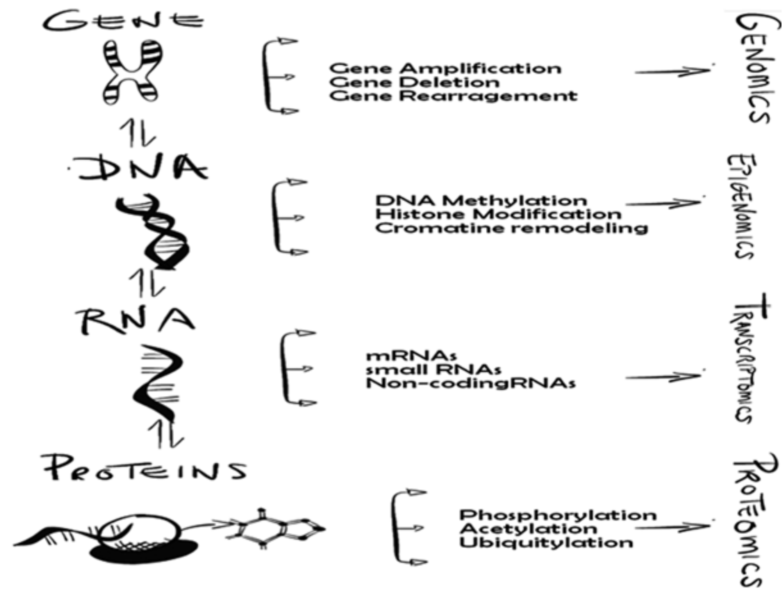

Fig. 1.1 Relationship between gene, DNA, RNA, and protein (the central dogma of molecular biology) with omics technologies

transcriptional activity of a cell, increasing or decreasing it, changing the normal expression of genes [9].

Gene amplification is one of the mechanisms in which a gene can be overexpressed and cause the activation of oncogenes; therefore it is related to disease progression and poor prognosis. It is created by an increase in the copy number of a loci or restricted area of a chromosome arm. Amplification occurs recurrently on some chromosomal locations, indicating the common activation of some oncogenes during tumor development [10]. The HER2 is the most studied breast cancer oncogene; it is located on chromosome 17q21.1 and encodes a transmembrane protein that is similar to the epidermal growth factor the HER1. A normal cell presents two copies of the HER2 gene and about 50,000 copies of the protein, whereas by gene amplification in a breast cancer cell, there can be more than one copy of the HER2 gene and more than one million copies of the protein [11].

High-throughput technologies are needed to discover, monitor, and quantify these DNA modifications. The most widely used technique involves the microarrays that simultaneously monitor the expression levels of thousands of genes inside different samples. This technique has advanced rapidly in recent years. It involves a surface fixed with either a tissue or a panel of different DNA or RNA molecules, proteins, or antibodies that have the possibility of linking up with a corresponding DNA/RNA/protein/antibody from a sample. Genomic microarrays, also called array comparative genomic hybridization (array-CGH), are used to study and quantify chromosomal abnormalities, microdeletions and microduplications, and copy number aberrations (CAN), at a wide level in an entire genome. Array-CGH has a refined process and resolution, which enables evaluation of the genome of any cell, while chromosomal-CGH allows the identification of gene regions where there is DNA gain or DNA loss [1, 12].

Chromosomal-CGH studies have identified that the loss of 16q is one of the most consistent DNA aberrations found in infiltrating lobular carcinoma (ILC). Etzell et al. studied genomic alterations in lobular carcinoma in situ (LCIS). Using comparative genomic hybridization, they found loss of chromosome 16q was in 88 % of cases [13]. Mastracci et al. studied the genetic profile of atypical lobular hyperplasia (ALH) and lobular carcinoma in situ (LCIS), using microarray comparative genomic hybridization (CGH). They found a common alteration in both ALH and LCIS—the loss of 16q21-q23.1 [14]. This common genetic alteration highlights the

Table 1.1 Confirmation of the intrinsic subtypes with several studies

Study	Main technique	Other technique	Novel finding
Sørlie et al. 2001 [18]	cDNA microarrays	Hierarchical clustering	Division of luminal A and B subtypes
Sørlie et al. 2003 [19]	cDNA microarrays	Hierarchical clustering	Association of BRACA1 with basal subtype
Sørlie et al. 2003 [19]	cDNA microarrays	Hierarchical clustering	Correlation of ER status with intrinsic subtypes
Abd El-Rehim et al. 2004 [21]	Tissue microarray	Immunohistochemistry	Correlation with cytokines expression
Carey et al. 2006 [22]	Microarrays	Immunohistochemistry	Population-based distribution of subtypes
Hu et al. 2006 [65]	Microarrays	Hierarchical clustering	Prediction of survival

possibility of a relationship between ILC and LCIS as they may be different forms of the same disease, and it seems that lobular carcinoma in situ (LCIS) is a precursor to infiltrating lobular carcinoma and therefore a marker of risk for breast cancer. This serves as an example of the application of genomic studies in order to discover breast cancer initiation, progression, and metastasis [13, 14].

Breast cancer gene expression profiling permits the collection of all the information about a whole set of genes in a tumor cell, including variations, gene expression, and the way those genes interact with each other and with the environment. The associations of specific genes with a common characteristic of expression or phenotypes are called gene profiles or gene signatures. Gene expression profiling has been used to create a complete new classification of breast carcinomas. The different kinds of clinical breast cancer have been correlated with diversity in gene expression profiles, which usually are studied using DNA microarray techniques [1]. Perou et al. using DNA microarrays in samples of breast cancer and normal breast tissue studied the presence of sets of genes that they called intrinsic genes, because these genes were found repeatedly inside the same patient biopsies but were not found repeatedly in all samples from other patients. A new group of genes associated with different breast cancer types were discovered, showing four distinct molecular subgroups: ER+/luminal-like, basal-like, HER2 enriched, and normal breast-like [15]. This new classification is being studied and recognized as distinct diseases with different treatment options [16, 17]. Table 1.1 summarizes the main findings of several studies that confirm the existence of the intrinsic subtypes, the technology used, and the novel finding that each study provided to this new breast cancer classification.

Novel Classification of Breast Cancer Based on Genome Profile: The Intrinsic Subtypes

Luminal Type

This type responds to endocrine therapy and has the best prognosis of all subtypes although it shows only limited chemosensitivity. Sorlie et al. studied a subdivision of luminal-like carcinoma Type A and Type B with characteristic molecular profiles and different prognoses (better for the A type than the B) [18]. In luminal B type, the HER2 expression is greater and responds only slightly better to chemotherapy than luminal A, which seems to have lower risk for relapse [18, 19].

Basal-Like

Defined by the expression of cytokeratins without ER and HER2 expression, it has shown poor survival independent of nodal status and size and is the subtype most common when the patient has BRCA1 [16, 19, 20].

HER2 Enriched

This type is characterized by the overexpression of the HER2 gene, and it has no expression of genes characteristic of the luminal subtypes. Not all breast cancers with HER2 positive by immunohistochemistry are classified as HER2-enriched type by molecular profiling. The basal-like and the Her2 enriched seem the ones with worst prognoses, the luminal A the best, and the luminal B in the intermediate [18, 19].

Breast cancer tumors had been separated by expression of hormone receptor status defining biologically distinct phenotypes that are used on clinical basis for prognosis and treatment. Genomics links phenotypes to genotypes of breast cancer by means of the study of the intrinsic subtypes and their clinical characteristics. The relationship between breast cancer molecular subtypes and prognosis has been studied extensively by gene expression profiling in order to find the differences that are used in clinical basis using hormone receptor status, HER2 status, and other classical characteristics. Among the intrinsic subtypes, luminal A and B have been identified as the ones with high expression of hormone receptor, while the HER2-enriched and basal-like subtypes have low expression of hormone receptors. In addition, low claudin, interferon rich, androgen receptor, and normal-like are the other subtypes studied in new trials [19]. There are many studies that find a relationship between traditional prognostic factors with gene expression signatures [21–23].

Genomic Testing

The molecular subtypes in breast cancer have shown different tumor characteristics, tumor aggressiveness, and different response to certain types of chemotherapy. They all have the ability to progress to metastases, but with differences regarding their leaning toward relapse into different organs. Smid et al. used microarrays and analyzed them with statistical significance analysis of microarrays (SAM). Studying the preference for organ-specific metastases of each of the intrinsic subtypes, they found that the rate and location of relapses are correlated to different molecular subtypes and metastases conserve genetic similarities with their primaries [24].

Following the intrinsic gene research of today, a new classification of breast cancer exists using omics technologies. New trials show the possibility of more subtypes, but this pathway is barely started, and currently it is not possible to exactly compare the new subtypes with the classical ones, and it is necessary for more studies to set up many new targeted therapies for these new molecular subtypes [11, 15].

Applying this new genetic information on the cluster of genes related to breast cancer, and using the new subtypes or intrinsic types of breast cancer, there is now a connection between the lab and the patient with the creation of several molecular assay tests that use genomics to calculate risk assessment and prognosis, creating a personalized diagnosis, prognosis, and therapy. This genomic test calculates certain clinical outcomes, such as patient risk of relapse if avoiding chemotherapy or if treated only with hormonal therapy and patients having more global risk of relapse. Although these tests searched different genes, when they are applied within the same patient data, they show similar results on prognosis of breast cancer. The MammaPrint and Oncotype DX are the tests most used and studied [1, 3].

The MammaPrint analyzes 70 genes and shows which patients have a low-risk molecular profile and therefore can avoid chemotherapy. It also identifies those women with good clinical factors but who carry poor prognosis genes [1, 3, 4]. Van de Vijver used inkjet-synthesized oligonucleotide microarrays to study the 70-gene expression profile that is associated with prognosis in patients with breast cancer and validate the classification system to predict the likelihood of distant metastases within 5 years [25].

Oncotype DX uses the intrinsic subtype model with 21 genes and is used in estrogen and progesterone receptor-positive and lymph node-negative breast cancer, to identify patients who can avoid adjuvant chemotherapy [1, 3]. Paik et al., using reverse transcription polymerase chain reaction

(RT-PCR), studied 21 selected genes and found the correlation with the likelihood of distant recurrence in patients with node-negative disease and treated with tamoxifen, validating then a recurrence score [26, 27].

Although these tests are beginning to be used in daily medical practice, there is still no consensus about their reliability or guidelines for use, because there is little information about the impact that these tests may have on clinical outcomes in the long term. Where there seems to be agreement is that the information provided by these tests must be taken into account as additional information to that obtained by conventional methods [16, 17].

Regarding the possibility of properly selecting the patient who should use chemotherapy and the one who should avoid it, as personalized medicine should be able to do, it seems necessary to relate a set of genes and its regulation systems with a clinical outcome [4, 11]. In order to accomplish more scientific evidence, prospective clinical trials—MINDACT for MammaPrint and TAILORx for Oncotype Dx— are trying to improve their clinical application [28–30].

One of the main drawbacks of applying past genomics research on a daily clinical basis is the lack of validation because these trials were based on small samples. That is why the latest research is focused on using more biopsy samples and more genetic analysis than the first trials, creating a genomics landscape of breast cancer [6]. Using larger samples in order to integrate breast cancer genome and transcriptome, and search for molecular drivers and gene expression, seems to be more reliable than obtaining information through the analysis of smaller samples [6, 31]. The creation of biobanks allows the storage of multiple samples of different types of cancer, from different patients, and treatments using different techniques of conservation. The existence of a useful biobank offers multiple and extensive databases using many categories, such as family samples, pre- and posttreatment samples, and population characteristics, to classify and identify the samples. To improve the biobank utilization, ethical issues must be addressed regarding the use of the samples for different studies [32].

One example of storage of new public oncogenome data is the Cancer Genome Atlas (TCGA), which is "a comprehensive and coordinated effort to accelerate the understanding of the molecular basis of cancer through the application of genome analysis technologies, including large-scale genome sequencing." This atlas is created with the efforts of the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) that are applying omics technologies to profile DNA, RNA, protein, and others that can be related to this disease. This data is being stored within several samples of cancer, creating a new list of genes, mutations, and molecules.

Now there are tons of genetic data related to breast cancer, but there is lack in the final connection with the actual patient [6, 11]. Curtis et al. used almost 2,000 samples of breast cancer to analyze their genome and transcriptome within copy number variants, single nucleotide polymorphism, and acquired somatic copy number aberrations, and they found new subgroups to create a novel molecular stratification of the breast cancer population [33]. The Cancer Genome Atlas network 2012 used the analysis of large amounts of data through the TGGA platform studying the DNA copy number arrays, DNA methylation, exome sequencing, messenger RNA arrays, microRNA sequencing, and reverse-phase protein arrays from breast cancer, showing the existence of four main breast cancer classes but associating them with several gene mutations and subgroups of proteins expression. They also found many molecular commonalities between basal-like breast tumors and high-grade serous ovarian tumors [34].

Epigenomics

Epigenetic modifications are the alterations found in DNA not affecting its primary structure, such as DNA methylation, histone modifications, and chromatin or nucleosomal remodeling; these alterations are heritable and reversible. Therefore epigenomics is the study of the complete set of those epigenetic modifications [7, 35].

DNA methylation, histone modifications, and chromatin or nucleosomal remodeling mutually interact with each other to regulate gene expression. The modifications obtained through them sometimes end up with aberrant transcription disrupting the normal regulation of a cell. The epigenetics processes modulation of chromatin structure to form a loosely packed DNA that is transcriptionally active, called euchromatin, or a tightly packed DNA that is transcriptionally inactive, the heterochromatin. Therefore, these processes can either turn on or turn off the gene expression.

Cancer seems to be driven by these epigenetic alterations, which cause aberrant cell regulation that result in a change in expression patterns of genes implicated in cellular proliferation, survival, and differentiation. Therefore, in breast cancer cells, DNA methylation, histone modifications, and chromatin or nucleosomal remodeling can lead to initiation, promotion, and maintenance of carcinogenesis and treatment failure. Understanding epigenetic alterations that initiate and maintain gene silencing and oncogene activation in cancer cells is necessary in order to find clinical applications of epigenomics [36–38].

DNA Methylation

DNA methylation is the addition of a methyl group into a region of DNA with participation of the DNA methyltransferase enzymes. They create genomic instability and rearrangement, activation of oncogenes, and inactivation of tumor suppressor genes, resulting in a cancerous cell [36]. Inside a cell there may be a loss of DNA methylation (hypomethylation) or there can exist an excess of DNA methylation (hypermethylation). Currently genes that are aberrantly methylated in breast cancer can be identified; with DNA methylation mapping technologies, it would be possible to identify DNA methylation patterns within a tumor subgroup that are correlated to breast cancer subtypes, clinical stages, and prognosis.

Methods for DNA methylation analysis can be divided into global methylation analysis and gene-specific methylation analysis. The global methylation analysis measures the overall level of methyl cytosines. The gene-specific methylation analysis uses enzymes to digest DNA to process it and quantify and identify methylated genes. One of the processes used is the polymerase chain reaction (PCR), a main technique for omics that amplifies a DNA sequence using an enzymatic reaction to obtain thousands to millions of copies of it. Methylation-specific PCR (MSP) is able to identify methylated sequences or hypermethylated zones inside of the genome [39, 40].

Dejeux et al. studied DNA methylation of certain genes from samples of patients with locally advanced breast cancer before the neoadjuvant treatment with doxorubicin. To quantify the modification, they used DNA methylation analysis by pyrosequencing. They found aberrant methylation in 9 of 14 genes studied, and three of the variations found are possible biomarkers for prognosis for patients with this disease and the prediction of response of this neoadjuvant drug [41].

Hsu et al. studied the methylation of BRCA1 promoter by methylation-specific PCR. They found a significant correlation within triple-negative cancer and with poor overall survival and disease-free survival in patients with breast cancer with methylated BRCA1 promoter [42].

Histones

Histones are protein components of chromatin that package and order the DNA into structural units called nucleosomes. The nucleosome is the fundamental unit of chromatin and it is composed of a structure of four histones around which the DNA is wrapped. Histone modifications and chromatin or nucleosomal remodeling are mediated by various enzymes, and it seems that a deregulation of normal DNA arrangement patterns turns on modifications that generate a new histone code, resulting in chromatin regions for transcription activation or repression, leading to changed gene expression as tumor suppressor genes and oncogenes [37].

The study of histone modifications is more difficult than finding DNA methylation and needs high-throughput techniques such as proteomic techniques that are described in more detail in the section "Proteomics." One such technique is stable isotope labeling with amino acids in cell culture (SILAC), based on mass spectrometry [43]. Cuomo et al. used SILAC technology to quantify and study histone modification changes in breast cancer cells and compared them to normal breast tissue histone modifications. They found significant changes for histone modifications in cancer cells that could represent biomarkers or what they called "breast cancer-specific epigenetic signature" [39].

Immunohistochemistry (IHC)

Another combined omics technique, IHC uses the linkage between antigen and antibody to localize specific antigens in microarray tissues by labeling antibodies with fluorescent material [44]. Elsheikh et al. used this technology with breast cancer samples in order to detect specific histone marks. They found significance between low and high levels of individual histone marks with a range of clinical and pathologic variables of the disease; also they could relate histone modifications with tumor type beside a relationship among histone modifications with biological markers and phenotypic groups of breast cancer such as estrogen receptor, progesterone receptor, expression of luminal cytokeratins, and others [45].

Transcriptomics

Transcription is the process by which the DNA is copied into RNA. The RNA can be translated into peptides or proteins and used as a guide for the synthesis of proteins by ribosome. Also RNA can work as gene regulation and enzymatic activity [46]. Transcriptome is the collection of all cellular RNA, and transcriptomics is the study of the whole transcriptome, measuring and classifying all the RNA within a cell. Transcriptome includes different kinds of transcript, such as a messenger RNA (mRNA), noncoding RNA, and small RNA; the structure of genes where the transcriptions are located; and the expression of each transcript under different conditions [47]. Transcriptomic techniques profile all types of transcript, find the start and end sites of genes to locate where transcriptions occur, and understand the posttranscriptional modifications, enabling to decode all the RNA sequences in order to map and quantify it [46].

Gene Expression

Gene expression is the study of the activity of a gene that can be quantified by the measurement of DNA transcription into mRNA and mRNA into proteins [12]. Knowing oncogene expression provides additional information about the genetic processes that drive cancer. Various high-throughput technologies enable the measurement and classification of the transcriptome as different types of microarrays (cDNA and oligonucleotide microarrays) and next-generation sequence techniques [46].

Complementary DNA (cDNA)

cDNA is the name used for a fragment of DNA that is complementary to another mRNA fragment. cDNA can be formed in the laboratory by extracting mRNA and then using mRNA as a matrix for formation of cDNA sequence. cDNA microarrays study gene transcription or expression through a solid surface with several fragments of cDNA attached in fixed locations that would match and link up with their complimentary mRNA in a process called hybridization. If the mRNA is chemically or fluorescently labeled, it is possible to measure the fluorescence intensities from each location on the array.

An oligonucleotide is a short sequence of nucleotides. An oligonucleotide microarray is a microarray that uses synthetically created DNA oligonucleotide sequences that are chosen to obtain better hybridization characteristics [1, 46]. Yao et al., trying to define genetic changes involved in the progression of breast cancer and

using cDNA array, identified two overexpressed genes (H2AFJ and EPS8) from samples of ductal carcinoma in situ, invasive breast carcinomas, and lymph node metastases. They also observed that the overall frequency of copy number alterations was higher in invasive tumors than in ductal carcinoma in situ. This study demonstrates that cDNA arrays are useful tools to identify gene expression aberrations that can in the future be used as markers for targeted therapy for breast cancer [12].

Among the novel or next-generation sequencing technologies is the RNA-seq. It is achieved by taking the RNA that is studied and converting it into a library of cDNA fragments with known sequences that can be studied and classified. Those fragments can be matched to a reference genome or reference transcripts or reassembled in order to identify the whole set of RNA molecules detected and to measure them [47]. Huber-Keener et al. studied gene expression in tamoxifen-resistant breast cancer cells using RNA-seq technology. They studied the genes involved in the development of tamoxifen resistance, indentifying the transcriptomes within tamoxifen-sensitive breast cancer cells, and compared them with the transcriptomes inside tamoxifen-resistant breast cancer cells. Through this comparison they found gene expression alterations of several mRNA and small RNA transcripts that are related to tamoxifen resistance mechanisms as estrogen receptor function, cell regulation, transcription regulation, and mitochondrial dysfunction. Therefore, the gene expression alterations found can be used for the diagnosis and treatment of tamoxifen resistance [48].

Proteomics

Proteomics involves the systematic study of proteins in order to provide a comprehensive view of their structure and function and their regulation of biological systems [49, 50]. Proteins are the working force of a cell—they catalyze enzymatic reactions, provide cell structure, serve as cellular signals, and provide many other cellular and intercellular functions. The complexity and variability of the proteome exceed that of the genome [7, 50].

Proteins can have multiple types of modifications, such as phosphorylation, acetylation, and ubiquitylation, which are called post-translational modifications (PTMs). Multiple proteomic techniques are used to separate, identify, quantify, and classify proteins existing in low levels inside breast cancer tissues. They include electrophoresis, mass spectrometry, and novel and combined high-throughput techniques such as surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS), two-dimensional difference in-gel electrophoresis (DIGE), and multidimensional protein identification technology.

Proteomics uses microarrays as well. The proteomic microarray technique uses a surface fixed with either a tissue or a panel of different proteins or antibodies, and these are matched with a corresponding protein/antibody sample. The identification and characterization of breast cancer expressed proteins provide future drug targets for more specific therapies and could find new biomarkers to detect breast cancer [44, 40, 51].

Two-dimensional electrophoresis (*2DE*) is a protein profiling technique that uses a base made of gel where proteins can be separated according to certain characteristics, such as the isoelectric point and molecular weight. Further methods are needed to identify the proteins previously separated as mass spectrometry (MS). MS is a basic proteomic technology that is capable of showing the different peptides inside a sample, ionizing their chemical compounds to generate charged molecules and measuring their mass and charge ratios. MS can be used to directly identify proteins previously separated on gels [44]. Using the combination of these two techniques, Rowell et al. studied the primary isoflavone component of soy (genistein) and its effect on breast tissue from rats. They found changes on the proteomics of the cells exposed to this isoflavone, suggesting that this exposure enhances cell proliferation, cell differentiation, and gland maturation therefore increasing the possibility of breast cancer [52]. This is an example of how proteomic studies can allow deep knowledge of how breast cancer

starts, and it could lead to find novel molecular markers and targets for breast cancer.

Surface-enhanced laser desorption/ionization mass spectrometry (*SELDI-MS*) is a high-throughput technique for proteomic analysis that permits the selection of proteins and peptides with specific properties using different chromatographic surfaces [44]. Hu et al. used proteomics with the help of SELDI-MS on breast cancer patients, patients with benign breast diseases, and healthy women and found four new biomarkers that are able to create a diagnostic model with significant sensitivity and specificity [53].

Two-dimensional difference in-gel electrophoresis (*DIGE*) is a technology that separates proteins from different samples inside the same gel, making it possible to detect and quantify the differences between two or three samples studied on the same 2D gel, enabling simultaneous visualization of relatively large portions of the proteome [54, 55]. Davalieva et al. made a proteomic analysis of breast cancer tissues and normal tissue samples from patients with infiltrating ductal carcinoma using DIGE. They found overexpressed proteins inside the tumor samples not previously associated with breast cancer and that are involved in cancer pathways [56].

Multidimensional protein identification technology (*MudPit*) uses two-dimensional liquid chromatography combined with mass spectrometry to identify several peptide sequences and their related proteins. It is a technology that improves the classification of proteins inside different samples. Sandhu et al. used this technology to profile protein expression of breast cancer cells. They found alterations associated with the malignant breast cancer phenotype, including differences in the apparent levels of key regulators of the cell cycle, signal transduction, apoptosis, transcriptional regulation, and cell metabolism [57].

Other Omics Techniques

Metabolomics

Metabolomics is the study of the whole set of small molecules that can be detected in organic fluids. These fluids represent the end products of cellular processes [7]. The new lab technologies enable profiling more kinds of cellular metabolites. The applications of a global metabolome analysis, or the detection of the whole set of molecules in organic fluids and tissues produced by cellular processes, include disease diagnosis and the identification of drugs or chemical exposures inside a cell. Using metabolomics technologies, it is possible to know the metabolic profile of an individual and predict toxicity of certain drugs, depending on the metabolizing capacities of the patient. The increasing availability of these novel methods with the capacity to detect, with high accuracy, the levels of the metabolome will lead to discovering the molecular response to a biological treatment or to the different metabolic characteristics of a tumoral cell [58].

Pharmacogenomics

Pharmacogenomics is an omics technology that studies how the response of a patient to different drugs is related to the patient's genetics. A combination between pharmacology and genomics, pharmacogenomics pursues the optimization of therapies using the genotype of the patient and the genetic characteristics of disease in order to obtain the best drug efficacy with the lowest adverse effects. Pharmacogenomics could classify and determine the relations between patient genetics and drug response. This is the basis of individualized medicine. The understanding of pharmacogenomics includes drug targets, drug metabolism, drug transport, disease susceptibility, and drug safety [59].

Interactomics

The network of all interactions between molecules and proteins from a cell is called interactome; therefore interactomics is the study of both the interactions and the consequences of those interactions [60]. This complete network of protein–protein interactions or molecule–protein interactions conform the signaling pathways, metabolic pathways, and cellular processes that are required for a cell survival. Therefore the

study of these interactions allows for a deep understanding of the pathogenesis of many diseases, including cancer [61, 62].

Conclusion and Future Perspective

Personalized cancer medicine is defined as "a medical model using molecular profiling technologies for tailoring the right therapeutic strategy for the right person at the right time, to determine the predisposition to disease at the population level, and to deliver timely and stratified prevention" [63].

The appearance of cancerous cells requires some alterations of their genetic material. Better treatment of cancer could be achieved if it is possible to decipher every single step that follows the creation of a tumor cell. There are many genetic alterations in a single tumor; maybe only a few of these alterations are responsible for the instauration of the disease, and we have to select the alterations that should be targeted in order to eradicate cancer [7, 64]. Every patient has different combinations of altered genes and should be treated according to their specific oncogenic characteristics, and perhaps each patient should receive more than one treatment with different oncogenic pathway targets [64].

Trastuzumab is a monoclonal antibody that interferes with the HER2 receptor. The HER2 receptors are proteins that turn genes on and off; therefore the HER2 proteins stimulate cell proliferation. As an example of personalized cancer medicine, if we were testing trastuzumab (Herceptin) in a population of breast cancer patients without knowing their HER2 receptor status, the results would be different from the reality of the trastuzumab (Herceptin) effectiveness, because as almost everyone knows, trastuzumab shows good results but only in patients with HER2-positive tumors [7, 64].

The use of a treatment previously studied and targeted for the right patient would improve healthcare services and may reduce cost, increase drug efficacy, and reduce toxicity. The research goal of omics technologies is to identify groups of at-risk patients with less-invasive diagnoses and the implementation of better cancer screening and prevention [7, 63, 64].

These high-throughput molecular profiling studies have provided great advances in the understanding of cancer biology. Researchers are hopeful to use the large data now stored and transform it into a new era of cancer management. Omics technologies are increasingly used and today are being regarded almost as standard research tools, notwithstanding that relatively only a small part of the information obtained via these novel techniques are currently being used on a clinical basis. The innovation of omics technologies applied in breast cancer research is focused to completely decode its pathogenesis, to endure the new classification using molecular subtypes with more specific characteristics, to accurately define its prognosis, and to find the best use of the actual therapy and novel drugs with the right targets.

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Omics of Hereditary Breast Cancer

2

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Abstract

Breast cancer is the leading cause of cancer-related deaths among women worldwide. Although advances in our understanding of this disease have been made in the last decade, the available treatments remain inadequate, particularly for the more intractable forms of breast cancer. Hereditary or familial breast cancer poses a particularly difficult challenge as only a few susceptibility genes with high penetrance have been identified, namely, BRCA1 and BRCA2. It is now suspected that the majority of hereditary and familial breast cancers are caused by various combinations of several moderate- and/or low-penetrance genes. Recent developments in research methodologies and conceptual frameworks within biology have revolutionized the study of cancer. This systems approach, which emphasizes a holistic understanding of biological systems, is referred to generally as "omics." A decade of omics research has led to the identification of many new therapeutic targets and biomarkers, allowing for more accurate and earlier diagnosis and treatment of the wide spectrum of diseases that are collectively referred to as breast cancer. Here we review the contributions of several omics fields to our understanding of hereditary and familial breast cancer, namely, genomics, transcriptomics, proteomics, and metabolomics.

Keywords

Hereditary breast cancer • Genomics • Transcriptomics • Proteomics • Metabolomics

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Introduction

With the sequencing of the human genome, the study of biological systems underwent a major transformation. Many researchers began to approach their work with a more global perspective. New fields of study have developed,

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Table 2.1 Publicly available Web-based databases containing microarray datasets

Database	Curator	Publication
Gene Expression Omnibus (GEO)	National Center for Biotechnology Information (NCBI)	[111]
Riken Expression Array Database (READ)	Riken	[112]
ArrayTrack	U.S. Food and Drug Administration (FDA)	[113]
ArrayExpress	European Molecular Biology Laboratory – European Bioinformatics Institute (EMBL-EBI)	[114]
BioGPS	Genomics Institute of the Novartis Research Institute	[115]
Microarray Retriever (MaRe)	Leiden University Medical Center (LUMC)	[116]

consisting of high-throughput data-rich methods aimed at understanding the intricacies of the biological systems around and within us. The development of automated high-throughput technologies able to carry out complicated experimental procedures and acquire detailed imaging and other data in a fraction of the time compared to previous labor-intensive methods has led to the generation of an almost unmanageable amount of data (see Table 2.1 for a list of publically available microarray dataset databases). Along with the development of these new technologies in the lab have come advancements in computer science that allow researchers with little or no background in computer software engineering to sift through these mountains of data in the hopes of mining relevant patterns [1]. These new and exciting fields of study have come to be collectively referred to as "omics" [2].

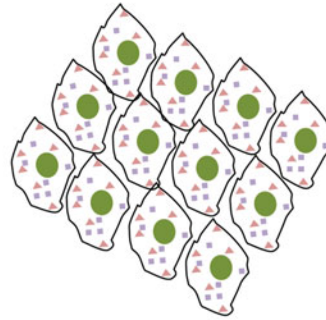
The power of the various omics fields lies in the vast and detailed information that can now be extracted relatively quickly and easily from a biological sample. The integration of data from several areas of omics (e.g., genomics, proteomics, and metabolomics) can offer a more informative, holistic view of the system under investigation, as shown in Fig. 2.1. This should lead to advancements in disease prevention (genome sequencing) detection (biomarkers), better and more individualized treatments for patients (pharmacogenomic profiling), as well as a more thorough and accurate picture of disease prognosis (biomolecular profiling).

Although we saw in the first decade of the twenty-first century an explosion in new methods that allow for more detailed and comprehensive exploration of biological systems, improvements in both detection and analysis of omics data are needed [1, 3, 4]. Ultimately, in the context of medicine, the goal of the omics revolution is for a better understanding of pathophysiological processes and better prevention, detection, and treatment/management of disease. This chapter aims to describe some of the main omics methods currently utilized in cancer research and how they have contributed to our current understanding of hereditary breast cancer.

A Growing Problem

Cancer places a heavy economic burden on health-care systems, making the need for early detection and more effective treatments not only a medical imperative but also an economic one as well [5–7]. Current treatment regimens, while improved and often more targeted, are still harmful to healthy cells and tissues. This harm to healthy cells is responsible for unpleasant side effects and carries the possibility of causing secondary cancers [8–10].

Cancer is the leading cause of death in high-income nations and the second leading cause of death in nations of low to moderate income. For women, breast cancer is the leading cause of cancer-related death. In 2008, breast cancer accounted for 23 % of newly diagnosed cancers and 14 % of cancer-related deaths in women. Fifty percent of breast cancers are diagnosed in economically developing countries, and 60 % of breast cancer-related deaths worldwide occur in these nations, suggesting an even more urgent need for better early detection and targeted, cost-effective treatments [11, 12]. The impact of breast cancer on a patient and their family is both physically and emotionally devastating, and in



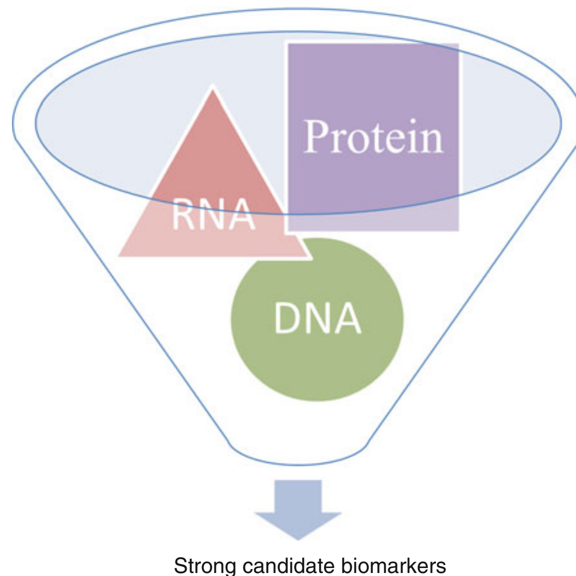


Fig. 2.1 Visual representation of the synthesis of high-throughput data for the identification of more robust predictive biomarkers

some nations, like the United States, which lack a comprehensive social health care system, it can also be economically crippling for a family. While great strides forward have been made in early detection and identification of new treatments, there still remains much work to be done. A concentrated effort is required to improve our understanding of the genetic, biochemical, and environmental factors that contribute to the development of breast cancer.

Heterogeneity

One of the major challenges in understanding any form of cancer is the heterogeneity inherent in the disease [12]. In fact, the word "cancer," while useful as a general descriptor, has led to a great deal of confusion and frustration among laypeople who may not be aware of the immense heterogeneity both between and within different cancer types. Owing to breakthroughs in understanding from the omics world, we have come to even better appreciate this aspect of cancer. Breast cancer is a particularly good example of why this term, while useful, is at the same time woefully inadequate [13]. Breast cancer is often broadly categorized as either hereditary or familial and sporadic. Tumors are also classified into subtypes based on various histological, genetic, and biomolecular characteristics. What has become increasingly clear in the past decade is that each tumor, while similar to others in many characteristics, is also unique. So while the search for new targets focuses on the similarities within subtypes, we must also remain aware of the unique nature of

each tumor, which may make it resistant to any number of available therapies. The promise of the omics revolution is that routine, inexpensive molecular profiling of individual tumors will lead to truly personalized treatment modalities.

Oncogenic Transformation

Oncogenic transformation is a complex, multistep process that differs widely between and even within cancer types. However different each cancer case may be, common to all are the characteristics of oncogene activation and mutations in tumor suppressors and other genes involved in a multitude of different signaling pathways that cumulatively produce the phenotype of a cancer cell [14]. Monitoring biological samples (e.g., blood, serum, or urine) taken from high-risk candidates over time using global transcription, metabolomic, and proteomic methods may help us to understand the early changes that occur during this transformation. Some recent studies have utilized breast cancer cell lines and/or patient-derived samples in order to examine the global changes that occur during the transformation to metastasis with the aim of identifying more specific and sensitive biomarkers. The hope is that early identification and treatment can prevent a cancer's advancement to metastasis [15]. Perhaps one day our understanding of the disease along with advancements in detection will even allow us to detect oncogenic transformation at a stage where its progression to cancer can even be blocked.

Four omics disciplines and their contributions to our understanding of hereditary breast cancer will be described in this chapter: genomics, transcriptomics, proteomics, and metabolomics. The order in which they are presented is meant to represent the flow of cellular information from genomics, the relatively fixed, molecular code of life; to transcriptomics, the first step in translating this code into "usable" parts; to proteomics, representing the workhorses of cellular activity; and finally ending with metabolomics, the downstream "end products" of the myriad cellular processes carried out by the aforementioned molecules.

Cancer Genomics

The discipline of genomics, as it is known today, started with the invention of DNA cloning in the 1970s and then the sequencing of the human genome [16]. "Classical" genomics is primarily concerned with the sequencing of genomes, the identification of all genes contained within a particular genome, and understanding gene structure and the complex interplay between genes and environment. There are now many subdisciplines within this field, such as structural and functional genomics, epigenomics, and pharmaco- and toxicogenomics. All aim to better understand the relationship between genetic sequences and biological processes or outcomes.

We are now living in the so-called "post-genomic" age. Gene mutations that increase a person's risk of developing various types of cancer have been identified. In high-risk breast cancer families, genetic screening can be carried out so that preventive measures can be taken, such as lifestyle changes, beginning mammograms at an earlier age, or prophylactic mastectomy [17–20].

Genomics of Hereditary Breast Cancer

Many attempts have been made to classify breast cancers into meaningful subgroups to aid in diagnosis, optimal treatment determination, and prognosis. Breast cancer tumor classification systems have evolved over time as our understanding of the heterogeneity of this disease has increased. Breast cancer tumors may be separated into four main types based on clinical and therapeutic characteristics. The luminal group is the most numerous and diverse subtype and is often subclassified into luminal A and luminal B, and several genomic tests are available to predict outcomes to endocrine therapy. The second group is the human epidermal growth receptor 2 (HER2 or ERBB2) amplified or HER-2 enriched group, which has responded very well to targeting of HER2 with monoclonal antibodies. The third group is referred to as normal breastlike. The fourth group is referred to as triple negative (or basal like) and is

so called because they lack estrogen receptor (ER), progesterone receptor (PR), and HER2 expression [21]. They have higher incidence in patients with germline BRCA1 mutations or who are of African ancestry and account for about 15 % of all breast cancer [22].

In 2009, Parker et al. reported subtype prediction by 50 genes using qRT-PCR and microarray technology, which came to be known as the Prediction Analysis of Microarray 50 (PAM50) and is commonly used to predict the best treatment modalities for individual cases [23]. In 2011, Ebbert et al. reported that the PAM50 system is generally accurate and that the assay is resistant to errors in the multivariate analyses (MVAs) used for classification. However, in the case of tumors that do not fit existing parameters very well, the system can lead to inaccurate conclusions [24]. In 2012, the IMPAKT task force compared the effectiveness of the PAM50 assay with a three-gene immunohistochemical (IHC) approach using antibodies against ER, HER2, and Ki67 and found that the former was "insufficiently robust" to make systemic treatment decisions. They recommend instead the combined use of ER and HER2 IHC.

In addition to the PAM50, there are germline genetic tests for BRCA1, BRCA2, and CYP2D6 and the Breast Cancer Index (BCI). OncotypeDX and MammaPrint assays are used in the United States and Europe for clinical decision-making [25]. Recently, more extensive and meaningful subgrouping has been made possible by genomic (as well as other omic) profiling of large sample groups [12, 26]. Such subtyping is essential for identifying and applying rational treatment combinations.

The first genes to be associated with hereditary breast cancer are also probably the best known. These breast cancer susceptibility genes, BRCA1 and BRCA2, are inherited in an autosomal dominant fashion and have high penetrance [27, 28]. Together, they account for about 30 % of familial cases of breast cancer [29]. Germline mutations in these genes result in what is called hereditary breast and ovarian cancer (HBOC) syndrome, which is associated with a lifetime risk of developing breast cancer of 50–80 % and of 30–50 % for ovarian cancer [30]. Interestingly, although primarily associated with breast cancer, the BRCA genes are more highly associated with ovarian cancer, with an overall mutation rate of about 12 % in women diagnosed with ovarian cancer [31]. After their identification, there was a great deal of excitement, with many hoping that more high-penetrance genes would be discovered. However, this has not been the case and this is one reason why many have great hope for advancements in understanding breast cancer via omics methodologies.

Although the two BRCA genes function in the same DNA repair pathway, homology-directed recombination repair (Fig. 2.2), the tumors that result from BRCA1 and BRCA2 mutation are remarkably different. BRCA2 tumors have characteristics similar to sporadic cases. BRCA1 tumors, on the other hand, are uniformly aggressive, difficult to treat, and are typically ER negative [30, 32]. In a recent review, Roy et al. propose several theories to explain why tumors arising from two genes involved in the same DNA repair pathway may vary so significantly, both genetically and clinically. It is possible that other gene mutations or polymorphisms are co-inherited with BRCA1; although, they note, there is no evidence currently available to support this. Another possibility they propose is that the role of BRCA1 in transcriptional co-activation or co-repression, which is not shared by BRCA2, may be able to modify expression of the ER biomarker. For this to be proven correct, the expression profiles of ER-negative BRCA1 and ER-negative sporadic tumors would need to be compared to identify a common mechanism. Finally, they suggest the possibility that BRCA1 and BRCA2 heterozygosity induce different mutational spectrums, perhaps resulting from their different roles in homologous recombination (HR) repair. Analyses using array-comparative genome hybridization (aCGH) show some similarities between BRCA1 and BRCA2 cancers, including large deletions and amplifications, but some differences are also seen [30].

In a response to this review, Simon A. Joosse puts forward an intriguing alternative to these explanations. He starts by noting that all mammary stem cells, from which all epithelial breast cells

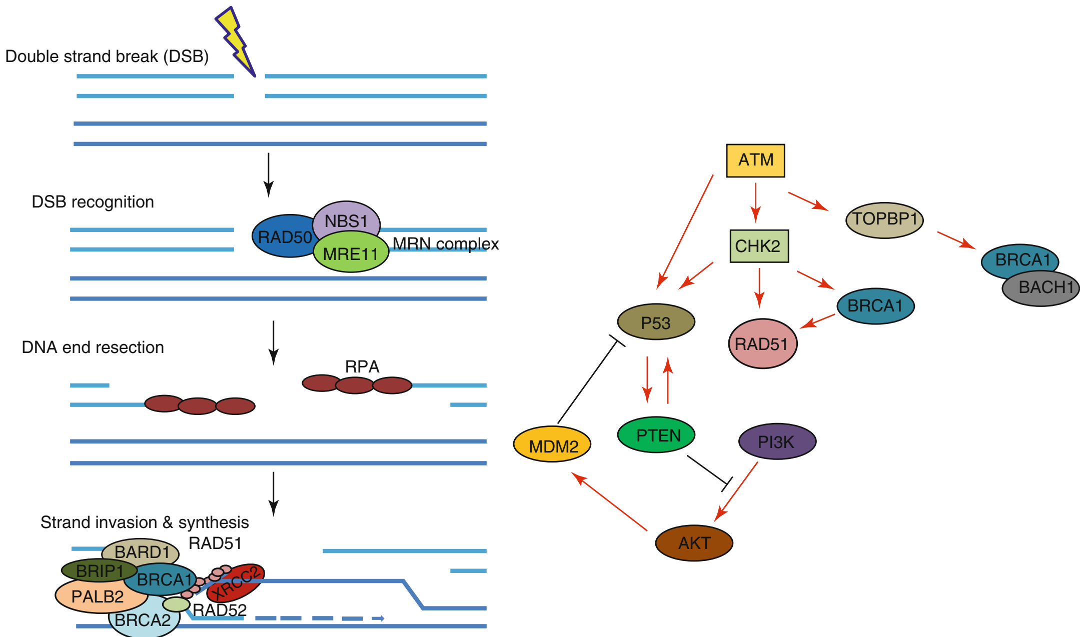


Fig. 2.2 Relationship between various genes involved in/implicated in the development and/or progression of breast cancer. Role of several proteins implicated in breast cancer susceptibility in homologous recombination directed repair of double strand breaks (DSBs) (*left*); Signaling between various proteins implicated in breast cancer susceptibility (*right*). Gene/protein abbreviations: *RAD50* RAD50 homolog (*S. cerevisiae*), *NBS1* Nijmegen breakage syndrome protein 1, *MRE11* MRE11 meiotic recombination 11 homolog (*S. cerevisiae*), *RPA* replication protein A, *BARD1* BRCA1 associated RING domain 1, *BRIP1* BRCA1 interacting protein C-terminal helicase 1, *PALB2* partner and localizer of BRCA2, *BRCA1* breast cancer 1, early onset, *BRCA2* breast cancer 2, early onset, *XRCC2* X-ray repair complementing defective in Chinese hamster cells 2, *RAD51* RAD51 homolog (*S. cerevisiae*), *RAD52* RAD52 homolog (*S. cerevisiae*), *ATM* ataxia telangiectasia mutated, *CHK2* checkpoint kinase 2, Topoisomerase (DNA) II binding protein 1, *P53* tumor protein p53, *PTEN* phosphatase and tensin homolog, *AKT* v-akt murine thymoma viral oncogene homolog 1, *MDM2* MDM2 oncogene, E3 ubiquitin protein kinase, *PI3K* phosphatidylinositol-4,5 bisphosphate 3-kinase, catalytic subunit alpha, *BACH1* BTB and CNC homology 1, basic leucine zipper transcription factor 1

originate, begin as ER negative and that BRCA1, but not BRCA2, is required for their maturation to ER-positive cells. Thus, BRCA1 deficiency may result in an accumulation of undifferentiated ER-negative stem cells with oncogenic potential. Following this line of thought, BRCA1 tumors would originate from a common cell lineage, explaining why they are so homogeneous as compared to tumors in BRCA2 mutation carriers. It could also explain why BRCA1 mutation carriers have a higher overall risk for developing breast cancer and why they are typically diagnosed at an earlier age than BRCA2 mutation carriers [32]. Further genomic and proteomic analyses will hopefully provide answers to these questions in the near future.

Apart from the BRCA genes, several others have been associated with familial cases of breast cancer. Among the high-penetrance genes are the tumor suppressors P53, PTEN, and STK11 [33–35]. Also implicated are genes of moderate penetrance, which include ATM, CHK2, RAD51D, and RAD51B [36–39]. Other moderate-penetrance genes that are part of the Fanconi anemia (FA) pathway are PALB2, BRIP1, RAD51C, and XRCC2 [38, 40–43]. A further 21 low-risk alleles have been identified [44–47]. Together these genes, including BRCA1 and BRCA2, account for approximately 35 % of all familial breast cancer worldwide. This leaves a gaping hole in our knowledge of what causes the majority of familial breast cancer; a hole that is slowly being filled with valuable omics data. (See Table 2.2 for a list of genes mentioned in this chapter, including brief descriptions.)

Gracia-Aznarez et al. analyzed seven BRCA1/BRCA2-negative families, each with six to ten

Table 2.2 Brief description of genes reviewed in this chapter: their basic function and role in various cancer types

Gene name	Function	Role in various cancer types
PPP2R2A (Protein phosphatase 2, regulatory subunit B, alpha)	Negative control of cell growth and cell division	Associated with complexes that directly dephosphorylate ATM at DSBs; loss inhibits homologous recombination directed DNA repair [143]; somatic deletion predicts prostate cancer [144]
MTAP (methylthioadenosine phosphorylase)	Polyamine metabolism, adenine and methionine scavenger	Loss is common in human cancer, addition of MTA to MTAP negative tumor cells increased sensitivity to 6TG and 5FU without affecting MTAP positive cells [145]
MAP2K4 (MKK4) (mitogen-activated protein kinase kinase 4)	Serine threonine protein kinase, phosphorylates and activates JNK	Metastasis repressor in prostate and ovarian cancers [146]
STK11 (LKB1) (serine/threonine kinase 11)	Serine threonine protein kinase, regulates cell polarity, tumor suppressor	Gastrointestinal polyposis-associated mutations [147]; inactivating mutations present in 20 % NSCLC [148]; pancreatic cysts resembling precancerous lesions in Lkb1 mutant allele knock-in mouse model [149]
DUSP4 (MKP2) (dual-specificity phosphatase 4)	Negatively regulates ERK, p38 and JNK	Low expression activates RAS-ERK signaling in residual breast cancer cells after neoadjuvant treatment [76]; frequently overexpressed in MSI-H colorectal cancer [150]
RUNX1 (AML1 or CBFA2) (runt-related transcription factor 1)	Transcription factor regulating differentiation of HSCs, ERα antagonist	Inactivation common in many hematopoietic and solid tumors, as expression decreases with increasing breast cancer aggression [151]; central to miRNA circuits involved in normal and malignant hematopoiesis [152]
AMBP (alpha-1-microglobulin/bikunin precursor)	Found in complexes with prothrombin, albumin, and immunoglobulin A (CD79a) in plasma	Differentially expressed in bladder cancer [153]
ABAT (4-aminobutyrate aminotransferase)	Catabolizes neurotransmitter GABA into succinic semialdehyde	Differentially expressed in ARMS
CDH1 (cadherin 1, type 1, E-cadherin (epithelial))	Suppresses re-accumulation of mitotic cyclins by recruiting them to APC for ubiquitination and subsequent proteolysis	Germline alterations associated with various gastric cancer syndromes [154]; normally considered tumor suppressor, but expression may cause progression of some ovarian and brain cancers [155]
RB1 (retinoblastoma 1)	Tumor suppressor, regulates cell growth, interacts with proteins involved in apoptosis and differentiation	Demonstrated role in various cancers [156]; possible role in EMT in TNBC [157]
HSP90 (Hsp90 chaperone)	Role in folding and activating proteins in various signal transduction pathways	HSP90 inhibitors used in treatment refractory HER2 BC and various other cancers [158, 159]
PIK3CA (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha)		Gene with highest frequency of gain-of-function mutations in breast cancer [160]
MLL3 (myeloid/lymphoid or mixed-lineage leukemia 3)	Histone methyltransferase, involved in circadian transcription	Germline mutations, i.e., by exome sequencing in colon cancer and AML [161]; loss of expression common in MSI-H gastric cancers [162]
GATA3 (GATA binding protein 3)	Transcription factor regulating luminal epithelial cell differentiation in the mammary gland, involved in regulation of T-cell development	Suppresses breast cancer metastasis by inducing miR-29b [163]; loss of expression in PTEN-deficient prostates accelerates tumor invasion [164]; may impact ESR1 enhancer accessibility [165]; mutant status correlated with suppression of proliferation upon aromatase inhibitor treatment [166]

(continued)

Table 2.2 (continued)

Gene name	Function	Role in various cancer types
MAP3K1 (mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase)	Serine threonine kinase	Correlated with breast cancer susceptibility in BRCA2 carriers [167]
CDKN1B (P27KIP1) (cyclin-dependent kinase inhibitor 1B)	Controls cell cycle progression at G1 by binding cyclin E-CDK2 or cyclin D-CDK4 complexes	HSP90 inhibitors alter its expression in melanoma cells [168]; its inhibition by Id3 may result in more aggressive prostate cancers [169]; induction by vitamin E δ-tocotrienol inhibits proliferation in PDCA cells [170]
TBX3 (T-box 3)	Transcription repressor	Overexpressed in HNSCC, represses PTEN [171]; BRAF/Tbx3/E-cadherin pathway may promote metastasis of BRAF-mutant melanomas [172]; methylation status of promoter identified glioblastoma patients with MGMT-methylated tumors [172]
CBFB (core-binding factor, beta subunit)	Beta subunit of a core-binding transcription factor complex involved in development and stem-cell homeostasis	CBFB-MYH11 fusion protein associated with AML [173]; decreased expression may be involved in malignant phenotype of some prostate and ovarian cancers [174]
NF1 (neurofibromin 1)	Negative regulator of several signal transduction pathway involved in proliferation, including Ras pathway	Mutations strongly associated with myeloid malignancies [175]; loss-of-function mutations cause neurofibromatosis 1, a tumor predisposition syndrome [176]; somatic mutations occur in OSCs [177]; allelic loss detected in CRC [178]
SF3B1 (splicing factor 3b, subunit 1, 155 kDa)	Subunit of U2 snRNP complex and minor U12-type spliceosome	Mutations common in B-CLL [179]; mutations at codon 625 in uveal melanoma [180]; mutated in PDCA [181]
CCND3 (cyclin D3)	Forms regulatory subunit of CDK4 and CDK6 which are required for G1/S cell cycle transition	Molecule targeting kinase function of cyclin D3:CDK4/6 inhibits cell cycle entry in human T-ALL [182]; expression upregulated in AC [183]; may be regulated by miR-138 which is often downregulated in HCC [184]; overexpressed in laryngeal squamous cell carcinoma [185]
PDZK1 (PDZ domain containing 1)	Scaffolding protein mediating localization of cell surface proteins and involved in cholesterol metabolism	Overexpression likely associated with drug resistance in multiple myeloma [186]; upregulated by 17beta-estradiol in some ovarian cancer cell lines [187]
PTX3 (pentraxin 3, long)	Involved in innate immunity and extracellular matrix formation	May be used as FGF2 antagonist in tumor cells resistant to anti-VEFG therapy [188]

5FU fluorouracil, *6TG* thioguanine, *AC* lung adenocarcinoma, *AML* acute myeloid leukemia, *APC* anaphase-promoting complex, *ARMS* alveolar pediatric rhabdomyosarcoma, *B-CLL* B-cell chronic lymphocytic leukemia, *CRC* colorectal cancer, *EMT* epithelial-to-mesenchymal transition, *GABA* gamma-aminobutyric acid, *HCC* hepatocellular carcinoma, *HNSCC* head and neck squamous cell carcinoma, *MSI-H* microsatellite instability high, *MTA* methylthioadenosine, *NSCLC* non-small cell lung cancer, *OSCs* ovarian serous carcinomas, *PDCA* pancreatic ductal cancer, *snRNP* small nuclear ribonucleoproteins, *T-ALL* T-cell acute lymphocytic leukemia

affected family members across generations who were diagnosed under the age of 60. A known moderate susceptibility indel variant, CHEK2 1100delC, was identified. CHEK2 (or CHK2) is a gene integral to cell cycle checkpoint regulation and is found within the same signaling pathway as ATM and p53 (see Fig. 2.2). Additionally, 11 rare variants were identified, although their association with breast cancer was not clear due to insufficient statistical power. Targeted re-sequencing of these gene candidates would need to be carried out in a larger cohort to determine whether or not an association actually exists [48].

In 2011, Rebbeck et al. published the results of a study in which they analyze a set of genes known to code for BRCA1 interacting proteins in

2,825 BRCA1 mutation carriers to try to identify breast cancer risk-modifying genes. The following genes were identified as potential modifiers: ATM, BRCC45, BRIP1, CTIP, MERIT40, NBS1, RAD50, and TOPBP1 [49]. ATM, BRIP1, NBS1, and RAD50 had previously been associated with hereditary breast cancer cases [50]. Mutation screenings of the MERIT40 and TOPBP1 gene had been previously carried out by the Winqvist group. In their work, MERIT40 was not found to be associated with disease in familial breast cancer cases. However, the sample size of the study was relatively small (125 families) and geographically limited (families originating in northern Finland); thus, their results may not be relevant to other populations [51]. The same group had performed a similar study in 2006 in which they examined TOPBP1 and identified several variants in familial breast cancer cases [52]. Two other studies examined the possible role of TOPBP1 in modifying breast cancer risk. The first found aberrant subcellular localization of the protein in breast carcinoma from an unselected consecutive cohort of 61 patients [53] and the other, specifically examining familial breast cancer cases in Poland, found that decreased mRNA levels and increased protein levels of TOPBP1 were associated with disease progression [54].

The power of genomic analysis is well illustrated in a study published by Banerji et al. in 2012 in which whole-exome sequences of 103 breast cancers from patients in Mexico and Vietnam were compared to matched normal DNA. They also performed whole-genome sequencing for 22 breast cancer/normal pairs. Results confirmed a number of previously identified somatic mutations as well as discovering some new mutations, including a recurrent MAGI3-AKT3 fusion enriched in triple-negative breast cancers. The fusion causes constitutive activation of AKT kinase. They found that treatment with an AKT small-molecule inhibitor was able to abolish AKT activation [55]. Although this work does not specifically assess cases of hereditary breast cancer, it illustrates the powerfully informative nature of high-throughput sequencing technologies that are now at many researchers' disposal.

DNA methylation is an epigenetic mechanism that is thought to contribute to the control of gene expression [56]. In a recent review the possibility of targeting epigenetic enzymes to specific DNA sequences to attain a more thorough understanding of epigenetic effects on gene expression was discussed [57]. With recent major advances in genome editing, it seems that epigenomic editing may be a reality in the near future [58, 59]. Recent research linking DNA methylation of particular genes with breast and other types of cancers suggests that the ability to edit epigenetic marks could be immensely useful both to basic and translational cancer research [60–63].

Swift-Scanlan et al. reported the use of quantitative multiplex-methylation specific PCR (QM-MSP) to examine the precise levels of methylation of genes known to be hypermethylated in breast cancer [64]. In a set of 99 formalin-fixed archival breast cancer tissue samples from patients with germline mutations in BRCA1 or BRCA2 and/or a family history of breast cancer, the authors were able to identify associations between levels of DNA methylation in several genes (APC, RASSF1A, TWIST, ERα, CDH1, and cyclin D2) and tumor stage, hormone receptor status, growth receptor status, and history of recurrent or metastatic disease. While not as high throughput as other methods, QM-MSP is very sensitive, allowing analysis of samples that are very limited in size (50–1,000 cells) [64]. Other studies investigating DNA methylation in breast cancer have found that GSTP1 and FOXC1 promoter methylation status could be used as a prognostic marker [65].

Another interesting and promising method that is increasingly being utilized is single-nucleus sequencing (SNS) from flow-sorted nuclei. This has clearly illustrated that tumors are composed of a number of distinct subpopulations, each with unique genetic characteristics but also shared genomic mutations. These various subpopulations may then travel to different parts of the body, forming genetically distinct metastases [15].

According to a number of different studies, the majority of mutations present in metastases

are also present in the primary tumor [66]. This is potentially good news in that the transformation to metastasis may be more easily inhibited than previously thought. Then again, cancer is a very "smart" disease with quickly evolving genetic characteristics allowing tumor cells, even if only a small subpopulation, to escape our attempts at its eradication.

Cancer Transcriptomics

With the development of microarray technologies and advanced bioinformatics analysis software, the focus of many researchers turned to such efforts as identifying patterns of differential gene expression in cells under various conditions. In 1999, Golub et al. showed that identification of tumor subtypes could be carried out using global gene expression data rather than histological and clinical observations [67, 68]. However, some recent reports suggest that a combination of various methods is currently the most effective way to make accurate diagnoses and prognoses [69].

Transcriptomics is the study of all the transcripts of a particular organism, including mRNAs, small RNAs (microRNA and siRNA), and noncoding RNAs. It also includes the characterization of transcriptional structure, splicing patterns, and other posttranscriptional modifications of genes. The characterization of differential gene expression between different types of cells or in the same cell type under variable conditions is an invaluable tool in cancer research. The gene expression profile of a cancer cell is strikingly different from surrounding noncancerous cells. It can also be used to define tumor subtypes for a variety of different cancers, including breast cancer [21, 70–73]. Examination of the changes in gene expression between cells of primary and metastatic tumors adds to our understanding of the mechanisms underlying metastatic transformation.

While traditional sequencing techniques have been modified for transcriptional profiling (serial analysis of gene expression (SAGE), cap analysis of gene expression (CAGE), massively parallel signature sequencing (MPSS)), these methods are low throughput, expensive, and imprecise. Microarray and gene chip technologies are currently the main tools of choice in this omics field. RNA-Seq (RNA sequencing), a relatively recent development, is a method that uses deep sequencing technology and has vastly improved precision in transcript measurement, does not rely on known genomic sequences, and can identify single nucleotide polymorphisms (SNPs) present in transcripts [74].

Transcriptomics of Hereditary Breast Cancer

The heterogeneity inherent in triple-negative breast cancer (TNBC), frequently associated with BRCA1 germline mutations, makes it an especially intractable disease. A recent study published by Cascione et al. examined miRNA and mRNA expression profiles of samples (formalin fixed and paraffin embedded) that had been obtained from women with TNBC between 1995 and 2005 (see Table 2.3 for a complete list of the miRNAs identified). Samples were obtained from tumor, adjacent non-tumor, and lymph node metastatic lesions from 173 patients. Due to low RNA yield, a somewhat limited array analysis was necessary. A human cancer-specific mRNA array and the human miRNA expression profiling v1 panel were used. Two miRNA signatures were linked to patient survival (miR-16, 155, 125b, 374a and miR-16, 125b, 374a, 374b, 421, 655 497) and miRNA/mRNA anticorrelations were used to identify four distinct molecular subclasses. One (subclass) group included seven mRNAs overexpressed in tumors compared to normal tissue (SPP1, MMP9, MYBL2, BIRC5, TOP2A, CDC2, and CDKN2A). The second group had 43 mRNAs downregulated in tumors with the top gene ontologies being enriched in NF-κB, PPAR, and PTEN signaling pathways. The third group had ten deregulated mRNAs and was enriched with gene ontologies associated with growth factors. Finally, the fourth group is composed of 64 mRNAs with NF-κB signaling pathway as the most enriched gene ontology [22].

Adjuvant and neoadjuvant treatments are often coupled with primary therapeutic modalities

Table 2.3 Deregulation of miRNAs identified by Cascione et al. (in TNBC expression signatures)

miRNA	Dereguation of identified miRNAs in other types of cancer cells
miR-16	Prostate [117]
	Myeloma [118]
	Breast [119]
	Colon [120]
	Oral [121]
	Lung [122]
	Liver [123]
	Brain [124]
miR-155	Colon, cervix, pancreas, lung, thyroid, lymphoma, leukemia [125]
	Pancreas [126]
miR-374a	Lung [127]
	Breast [128]
	Colon [129]
miR-421	Head and neck [130]
	Stomach [131]
	Liver [132]
	Pancreas [133]
	Prostate [134]
	Breast [135]
miR-497	Cervix [136]
	Breast [137]
	Skin [138]
	Colon [139]
	Brain [140]
	Head and neck [141]
	Stomach, lung [142]

with the aim of improving the effectiveness of the primary therapy. For tumors that do not respond well to treatment, there is a good chance of disease recurrence and/or progression. Some theorize that this is due to the difficulty of eradicating tumor cells especially resistant to cancer therapy, what are commonly referred to as cancer stem cells [75]. To identify genes associated with drug resistance in TNBC (also referred to in the paper as basal-like breast cancer, BLBC), transcriptional profiling was performed on 49 archival samples that had been surgically resected following neoadjuvant treatment. To estimate long-term clinical outcome, IHC staining with Ki67 (a commonly used marker of proliferation) was performed on all samples. Ki67 staining highly correlated with tumor subtype, both clinically and molecularly, with the highest positive staining observed in BLBC cases. Expression profiling data from BLBC samples with high Ki67 staining, when compared with the Molecular Signatures Database, indicated activation of the Ras-ERK pathway.

To rule out KRAS mutation, which is infrequent in breast cancer, DNA sequencing was performed and no mutations were found. However, expression of DUSP4, a negative regulator of the Ras-ERK pathway, was significantly downregulated in these samples. Low DUSP4 expression had previously been correlated with shorter DFS in a cohort of 286 patients who had not received adjuvant therapy. To further verify the significance of DUSP4, the authors measured its expression in another cohort composed of samples obtained from 89 TNBC patients after neoadjuvant treatment and found a similar pattern of high Ki67 staining together with low DUSP4 expression. Experiments conducted in BLBC cell lines with siRNA knockdown of DUSP4 resulted in decreased apoptosis, increased mitogen-activated protein kinase (MEK)-dependent proliferation, and an increased half-maximal inhibitory concentration (IC_{50}) of docetaxel, an antimitotic drug used in the clinic.

After restoring DUSP4 expression in three BLBC cell lines, phosphorylation of ERK was inhibited and viability in two of the three cell lines was reduced. The addition of MEK inhibitors was found to increase sensitivity to docetaxel in 17 BLBC cell lines. In cell lines with loss of PTEN expression, the PI3K pathway is activated resulting in what is likely MEK-independent proliferation and evasion of apoptosis. Thus, the authors conclude DUSP4 expression coupled with PTEN status may effectively predict efficacy of MEK inhibitors in patients with BLBC tumors [76].

Taking advantage of the vast number of tumor samples available in tissue banks, Curtis et al. carried out an analysis of copy number variation and its effects on the transcriptome using a discovery set of 997 fresh-frozen primary breast tumor samples with accompanying clinical information. Another set of 995 tumors was then used as a test set to verify the predictive ability of data gleaned

from the discovery set. The aim was to identify the underlying genetic mechanisms that translate into observed variance between and among breast cancer subgroups. Patients were clinically homogenous with most ER-positive/lymph node (LN)-negative patients having not received treatment while ER-negative/LN-positive patients had received treatment. A number of putative cancer genes were identified including PPP2R2A, MTAP, and MAP2K4. The patients also stratified into a high-risk, ER-positive 11q13/14 *cis*-acting subgroup and a subgroup without any copy number aberrations, which corresponded to favorable prognosis, providing a new method for identifying breast cancer subgroups [77].

Cancer Proteomics

Proteomics is the study of the entire complement of proteins expressed by a particular biological system. As with genomics, subspecialties of proteomics have developed. The four major subfields are expression proteomics, functional proteomics, structural proteomics, and the proteomics of posttranslational modifications [78]. The aim of proteomics is not only to identify all proteins in a particular system, but also to understand the regulation of their expression, the interactions that occur between them, and their effects on cellular function. An example of one of the many programs available for visualizing protein-protein interactions is presented in Fig. 2.3.

The primary method in the proteomics toolbox is mass spectrometry (MS), a technology that measures the mass-to-charge ratio of ions in the gas phase. Throughout the twentieth century, MS technologies developed, but it was not until the late 1980s that its widespread use in biological research became feasible. This was made possible by the development of electron spray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [78, 79].

A recent review outlines the main challenges facing the field of proteomics. According to the authors, the primary bottleneck in proteomics development is in data analysis [3]. For a review of available methods for MS data analysis and protein identification via database search, we refer the reader to Brusniak et al. and Eng et al., respectively [80, 81]. For a basic look at how to analyze protein-protein interaction networks and regulatory networks, we recommend Koh et al. and Poultney et al. [82, 83]. Finally, for integrated analysis of omics data from multiple platforms, the reader is referred to Chavan et al. [84].

Proteomics of Hereditary Breast Cancer

Cohen et al. examined plasma from 76 breast cancer patients and were able to identify a signature consisting of four proteins previously found to be associated with breast cancer tissue: fibronectin, clusterin, gelsolin, and α-1-microglobulin/inter-α-trypsin inhibitor light chain precursor (AMBP). The plasma levels of these proteins differed between the two tumor types such that they were able to distinguish between infiltrating ductal and invasive mammary breast carcinomas [85].

A powerful methodology for identifying putative breast cancer biomarkers was used in a recent study by Pavlou et al. First, proteomic data was collected from the secretome (the full complement of proteins secreted by a cell) of eight different breast cancer cell lines, representing the three major breast cancer tumor subtypes. Out of 5,200 nonredundant proteins identified by MS, 23 were unique to basal breast cancer cells, 4 were unique to HER2-neu-amplified, and another 4 were unique to luminal breast cancer cells. These results were then compared with four publicly available breast cancer mRNA microarray data sets queried from the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO). In total, 24 out of the 30 candidate proteins had microarray expression patterns similar to those identified in the proteomic approach.

They next tested the clinical applicability of this data by performing MS on cytosol collected from eight ER-positive and eight ER-negative breast cancer tissue samples. Eighteen out of the 30 subtype-specific proteins were identified and three proteins in particular (ABAT, PDZK1, and

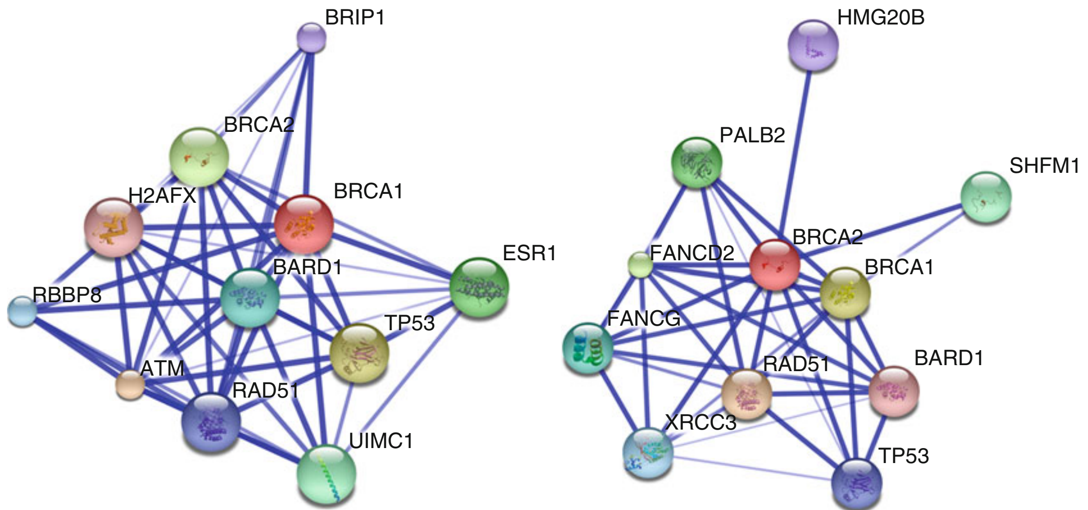


Fig. 2.3 Protein interaction networks for BRCA1 (*left*) and BRCA2 (*right*) generated using Search Tool for the Retrieval of Interacting Proteins/Genes (STRING) 9.05. The *thickness of lines* represents strength of association, with *thicker lines* indicating stronger associations

PTX3) had significantly different expression in the different subtypes. Finally, they examined the 2-year and 5-year disease-free survival (DFS) data that accompanied the four gene expression array data sets. ABAT was the most robust candidate of the three potential biomarkers. Expression levels of ABAT were, on average, 2.3 times higher for patients with DFS of more than 2 years. ABAT expression remained significantly different in all four datasets at the 5-year DFS mark.

They also queried the Gene Expression-Based Outcome for Breast Cancer Online Database and found that patients with higher ABAT expression had slightly longer disease-free survival than those with low expression. Patients with ER-positive disease and high ABAT expression as well as tamoxifen-treated patients with high ABAT expression had better prognosis than those with low expression [86]. This work demonstrates that in vitro proteomic analysis of breast cancer cell lines combined with publicly available transcriptomic data from patients can be used to successfully identify new candidate biomarkers that are breast cancer subtype specific.

Lee et al. carried out protein expression profiling of 38 sample pairs from lymph node metastases of varying grades (classified according to the TNM staging system, which includes physical examination, biopsy, and imaging) alongside adjacent normal tissue collected from patients with infiltrating ductal carcinoma (IDC). Using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and high-performance liquid chromatography and tandem mass spectrometry (LC-MS/MS), they found a number of proteins upregulated specifically in metastatic tissue and also identified possible markers to distinguish between the various metastatic stages. Calreticulin was significantly upregulated in metastases of all three stages with a rate of 77 % in stage N0, 92 % in stage N1, and 83 % in stage N2. Tropomyosin alpha-3 chain was also upregulated in all three stages, albeit at lower overall incidence (N0 and N1 69 %, N2 75 %). They suggest that HSP70 is a possible marker for stage N0 metastases, 80 k protein H precursor and PDI may serve as biomarkers for N1 stage metastases, and immunoglobulin heavy chain binding protein (BIP) is a potential identifier of stage N2 metastases [87].

Another study aimed at stratifying tumors into subgroups based on protein expression profiles found increased expression of STAT1 and CD74 to be associated with metastatic potential in TNBC, both in patient samples and in MDA-MB-231 cells. The authors suggest that the

mechanism by which this increased capability occurs is likely the CD74/CD44/ERK, MIF receptor pathway with a positive feedback loop between CD74 and STAT1 [88].

Cancer Metabolomics

Metabolomics is another piece of the omics puzzle that will improve our understanding of cancer cells and their transformation to the metastatic state. Metabolomics may be defined as "the comprehensive analysis of the low-molecular-weight molecules, or metabolites, that are the intermediates and products of metabolism" [89]. The Human Metabolome Database (www.hmdb.ca) is a publicly available collection of detailed information about the 40,250 small-molecule metabolites that have been thus far identified in human cells. "The large number of different metabolites, differences in their relative concentrations and variability in their physicochemical properties (polarity, hydrophobicity, molecular mass or chemical stability) require the application of different technologies and a huge range of experimental conditions" [90]. The most common techniques used in metabolomic profiling are nuclear magnetic resonance (NMR) and mass spectroscopy (MS) [89].

While metabolomics, like the other omics disciplines, offers great hope, it also comes with many challenges. The complete human metabolome is very large, almost twice that of the human proteome, and they exist in a constant dynamic flux. While collection of samples for metabolomic analysis is relatively easy and noninvasive (typically serum, plasma, or urine), because the molecules of interest are so easily modified during the process of sample transport and preparation, this presents potential variability that may be very difficult to control for. For translation to use in medicine, standard protocols and conditions for collection, storage, and processing must be designed and strictly adhered to.

One of the distinguishing characteristics of cancer cells is their unique "reprogramming" of metabolic pathways, in which they acquire changes that affect the metabolism of the four major types of macromolecules (carbohydrates, lipids, proteins, and nucleic acids) [14, 91, 92]. This phenomenon was first formally described by Otto Warburg in the 1920s and refers specifically to a cancer cell's "preference" for performing glycolysis even in the presence of oxygen (aerobic glycolysis) [14, 93, 94]. Known as the Warburg effect, this characteristic of "glucose addiction" is exploited in the clinic for the identification of cancerous lesions. Positron emission tomography (PET) is used to detect radioactively labeled glucose (2-deoxy-2-[18F] fluoro-D-glucose, FDG), which accumulates more in tumor cells relative to other cells due to their heavy reliance on glycolysis [93]. This has helped thrust cancer metabolism back into the spotlight in recent years. Evidence that activated oncogenes and mutant tumor suppressors can impact metabolism has also helped feed this interest. *Nature* and *Nature Reviews Cancer* published a "Web focus" on cancer metabolism where they highlight some recent developments in the field [27]. One review notes that the oxygen and nutrient-rich environment in which cancer cell lines are typically maintained and studied is markedly different from the in vivo tumor microenvironment [91]. Cocultures of breast cancer cells with fibroblasts may more accurately reflect the conditions in which tumors grow and can add to our understanding of how cancer cells evade death during treatment.

An example of how metabolomics, integrated with the other omics fields, can enrich our understanding of biological systems comes from a study published in 2011 in which a panel of 59 cell lines from different cancer types is used to identify links between genetic and metabolic profiles. They examined these cell lines before and after treatment with a variety of chemotherapy agents, including a variety of platinum-containing drugs. Their approach required an integrated analysis of two very different sets of data. Using the common method of overrepresentation (OR) analysis to first analyze the two sets of data individually and then develop a method to integrate analysis of both data sets together, they illustrate the potential power of inter-omics analysis [95].

Metabolomics of Hereditary Breast Cancer

A flurry of papers specifically investigating breast cancer metabolism were published by the Lisanti group in 2009 and 2010. As a result of their findings, they proposed that it is not only the cancer cells that have altered metabolic pathways but that cancer-associated fibroblasts (CAFs) present in the tumor microenvironment also have major alterations in metabolism [25, 96, 97]. Known as the "reverse Warburg effect" or "stromal-epithelial metabolic coupling," this theory posits that epithelial cancer cells actually induce the Warburg effect in stromal fibroblasts located in the tumor microenvironment so that they produce and secrete additional pyruvate and lactate that can then be utilized by cancer cells as inputs to the mitochondrial TCA cycle, oxidative phosphorylation, and ATP production [98]. They proposed that CAFs, which are thought to be derived from mesenchymal stem cells of the bone marrow, are essentially like TGFβ-activated fibroblasts (myofibroblasts) that cannot be "turned off" [99]. They, and others, have shown that CAFs exhibit a loss of caveolin-1 (Cav1), an inhibitor of TGFβ signaling.

In another paper, the Lisanti group shows that Cav1 was dramatically downregulated in breast cancer CAFs as compared with normal fibroblasts taken from the same patients. Other studies have shown that Cav1 is an effective predictor of breast cancer tumor recurrence, lymph node metastasis, tamoxifen resistance, and poor clinical outcome even independent of ER, PR, and HER2 tumor status [100–102].

Jerby et al. designed and validated an in silico metabolic phenotypic analysis (MPA) to measure whole metabolomic flux. These phenotypes were inferred by integration of transcriptomic and proteomic data and this technique was applied to conduct the first genome-scale study of breast cancer metabolism. This method differs from previous models in that it does not require an optimal fit to the data. The model used in MPA allows the data to deviate somewhat from the optimal fit so that one can estimate the cell or system's adaptive potential. Predictions were made based on data from nearly 400 clinical samples and subsequently studied in vitro in metastatic and nonmetastatic breast cancer cell lines. Consistent with results from previous studies examining other types of cancers, they found that metastatic breast cancer cells had a proliferative capacity similar to that of cells in the primary tumor; that is, metastatic cells did not have higher proliferative activity than primary tumor cells. There was also an apparent trade-off between proliferation and detoxification of reactive oxygen species. Production of lipids is necessary for cell proliferation but hinders a cell's ability to detoxify oxidative molecules. The authors identified metabolic differences between ER-positive and ER-negative tumor cells, which suggested that the latter have a lower capacity for producing lactate from glucose. The authors suggest that their findings may apply broadly to many different types of cancer, but also note that their work was in cell lines and thus in vivo experiments are necessary to explore the applicability of their results to disease in the organismal context [103].

In 2011, Possemato et al. presented a new tool for target identification with an in vivo RNA interference-based loss-of-function screen of metabolic genes associated with aggressive breast cancer and stemness in a human breast cancer xenograft model. Increased expression of phosphoglycerate dehydrogenase (PHGDH) was found to be required for increased serine pathway flux in some breast cancers. Inhibition of PHGDH in cell lines with elevated PHGDH resulted in decreased proliferation and a reduction in serine synthesis. While overall cellular serine levels were not affected, a reduction in α-ketogluterate, another output of the serine biosynthesis pathway, was observed [104].

Another study published in 2011 found that basal, but not luminal, breast cancer cells are highly glutamine dependent and that the glutamine independence of luminal breast cancers is associated with cell lineage-specific expression of glutamine synthetase (GS). Glutamine synthetase is induced by GATA3 and glutaminase expression is repressed by GATA3 [105]. This is yet another aspect of tumor biology that must be considered when formulating treatment plans for individual patients.

For a review of breast cancer metabolism, we refer the reader to a piece by Davison and Schafer, published in 2010 [106]. A review from 2011 that looks specifically at the role of the PI3 kinase pathway in breast cancer is also recommended [107]. For a more recent review, we refer the reader to a piece by Deblois and Giguere about estrogen-related receptors and their role in breast cancer cell growth and metabolism [108].

Pharmacogenomics

Perhaps equally important to an understanding of the genetic and metabolic profiles of cancer cells is knowledge of the status of the genes involved in the body's response to cancer therapeutics. In terms of chemotherapy, the status of genes involved in drug metabolism and transporters may be used to determine the suitability of a particular treatment for a patient. Likewise, the status of genes involved in the body's response to radiation therapy (e.g., DNA repair and radiation-induced fibrosis) can also aid in determining the best treatment options for each individual [109]. This will certainly be a key component in the "personalized medicine" revolution.

The Power of Omics Integration

The Cancer Genome Atlas Research Network published a paper in 2012 presenting results from a comprehensive molecular analysis of tumors and germline DNA samples from 825 breast cancer patients. Samples were analyzed by genomic DNA copy number arrays, DNA methylation, exome sequencing, messenger RNA arrays, microRNA sequencing, and reverse-phase protein arrays. Data from the six different platforms were analyzed both individually and in an integrated manner, which resulted in identification of four basic breast cancer classes, each with a distinct molecular signature. Almost all genes that had been previously identified in breast cancer were confirmed in this study, including PIK3CA, PTEN, AKT1, TP53, GATA3, CDH1, RB1, MLL3, MAP3K1, and CDKN1B. Novel genes were also identified and include TBX3, RUNX1, CBFB, AFF2, PIK3R1, PTPRD, NF1, SF3B1, and CCND3 [26].

Conclusion and Future Perspective

The *Oxford English Dictionary* defines the suffix *–ome* in cellular and molecular biology applications as "forming nouns with the sense 'all of the specified constituents of a cell, considered collectively or in total'" [110]. This definition very neatly captures the broad vision shared by the many omics fields. Each field has its own unique methods and instruments designed by engineers, biologists, chemists, and physicists working together. And each has its own set of molecules of interest. However, combined they all share the vision of a more complete and nuanced understanding of biological systems.

The task of sifting through the data produced by high-throughput omics methodologies is a daunting task. However, after about a decade of work in the various omics fields, the value of doing so has become evident. Here we reviewed some of the recent literature relevant to our understanding of breast cancer as seen through the prism of various omics fields. It is expected that these powerful methods will continue to provide a more holistic understanding of cancer and other diseases and contribute to the quest for truly personalized medical treatment.

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3 Oncogenes and Tumor Suppressor Genes as a Biomarker in Breast Cancer

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Abstract

Breast cancer is the most common cause of cancer in women in the United States and the Western world. The important question is what can be done to limit the human suffering associated with cancer and to reduce the burden on society? One solution is early detection. Early diagnosis of breast cancer before symptoms emerge is the most effective prevention of breast cancer.

Currently, mammography is the gold standard for breast cancer screening. The procedure is suggested and often reimbursed for women between the ages of 50 and 75. Yet it is presumed that between 15 and 25 % of women with early-stage breast cancers are presently missed by commonly used diagnostic procedures such as mammography. Since breast cancer is also diagnosed in an increasing number of younger women, the screening strategy should be modified. Hence, oncogenes and tumor suppressor genes could be used as biomarkers for early detection of breast cancer. Eventually, researchers aim to use the molecular data collected from an individual tumor for prognostication and personalized therapy for each patient. Genetic profiles of tumors are now providing information about clinical outcome, and some prognostic and predictive indicators have appeared based on this research. In the near future, prospective tissue collection for molecular analysis may become routine in order to classify patients for alternative treatment options and to optimize treatment strategies based on molecular structure of the cancer.

Keywords

Breast Cancer • Early Diagnosis • Oncogenes • Tumor Suppressor Genes • Biomarker

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Introduction

Breast cancer is the second most common cause of cancer death in women in the United States and the Western world. Recently, an increase in incidence has occurred in many of the countries that have screening programs. Significantly, such an increase is especially observed in younger women in Europe and Asia [1].

Breast cancer causes disability, psychological trauma, and economic burden. The burden of cancer affects not only the patients and their loved ones, who suffer physically, emotionally, and financially, but society in general. The loss of human potential due to cancer-related morbidity and mortality is very hard to estimate, particularly when the effect of the disease on the patient's family and friends is also counted in. As the prevalence of cancer is highest among older people [2] and the population is getting older, the overall costs to society will most likely increase in the next years. The important question is that what can be done to limit the human suffering associated with cancer and to reduce the burden on society. One solution is early detection.

Breast cancer morbidity increases meaningfully when it is not diagnosed early in its progression. Early diagnosis of breast cancer before symptoms emerge is the most effective prevention of breast cancer. When the cancer is diagnosed early, patients live longer and need less extensive treatment [3]. Early detection of breast cancer decreases the suffering and cost to society associated with the disease. Hence, research leading to an understanding of the mechanisms that lead to breast cancer and ways to avoid it should be increased.

Currently, mammography is the gold standard for breast cancer screening. It is presumed that between 15 and 25 % of women with early-stage breast cancers are presently missed by commonly used diagnostic procedures such as mammography [4]. Yet this screening is suggested and often reimbursed for women between the ages of 50 and 75 [5]. Since breast cancer is also diagnosed in a progressing number of younger women, the screening strategy should be modified.

Molecular Predictors of Response to Therapy for Breast Cancer

Breast cancer is a clinically heterogeneous disease; patients with the same stage of disease and similar pathological diagnoses can present very different clinical outcomes [6]. This clinical heterogeneity is due to the genetic variability of patients and tumors. Eventually, researchers aim to use the molecular data collected from an individual tumor for prognostication and personalized therapy for each patient.

The US NIH Consensus Conference explained that a clinically useful prognostic biomarker must be a proven independent, significant factor that is easy to determine and interpret and that has therapeutic consequences.

As yet, measurements from tumor tissue biomarkers have been used to diagnose breast cancer patients who may profit from specific therapies. Estrogen receptor testing has been routinely executed since the 1980s on breast carcinoma samples in order to decide whether hormonal therapy is indicated. Recently, estrogen receptor, progesterone receptor, and human epidermal growth factor receptor type 2 testing to direct treatment decisions are standard of care. Nowadays, multigene assays have been introduced to estimate breast tumor behavior. Specifically, the Oncotype Dx and MammaPrint assays have been used in North America and Europe to direct clinical assessments. Others, including the Breast Cancer Index (BCI; bioTheranostics) and PAM50 (Expression Analysis, Inc.), are gaining acceptance as validated assays with related clinical results. Also, some germ line genetic tests are now reported to estimate response to specific treatments (e.g., BRCA1, BRCA2, CYP2D6) [7].

Important Oncogenes and Tumor Suppressor Genes for Breast Cancer on Chromosome 17

Abnormalities of chromosome 17 are significant molecular genetic changes in human breast cancers. Some very well-known oncogenes (HER2,

Table 3.1 Breast cancer predisposition genes on chromosome 17 and their basic functions

Gene ID	Symbol	Function of gene
2064	ERBB2/HER2	Epidermal growth factor (EGF) receptor family of receptor tyrosine kinases. Amplification and/or overexpression has been reported in numerous cancers
7153	TOP2A	DNA topoisomerase controls and alters the topologic states of DNA during transcription. It is associated with the development of drug resistance
7157	P53	P53 responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, and DNA repair. It is accumulated in a variety of transformed cells
672	BRCA1	BRCA1 plays a role in maintaining genomic stability. It acts as a tumor suppressor. BRCA1 combines with other tumor suppressors, to form a BRCA1-associated genome surveillance complex (BASC). Mutations in this gene are responsible for approximately 40 % of inherited breast cancers and more than 80 % of inherited breast and ovarian cancers
3090	HIC-1	Hypermethylated in cancer 1, a candidate tumor suppressor gene which undergoes allelic loss in breast and other human cancers. The human HIC-1 gene is a target gene of p53
4137	TAU	Microtubule-associated protein TAU (MAPT) functions to keep cell shape, microvesicle transportation, and spindle formation. Interfering spindle microtubule dynamics will cause cell cycle arrest and apoptosis. TAU detection helps to identify those patients who are most likely to benefit from taxane treatment and resistant to paclitaxel treatment

This table has been modified by utilizing [60]

TOP2A, and TAU) and tumor suppressor genes (p53, BRCA1, and HIC-1) are located on chromosome 17, as shown in Table 3.1.

HER2

HER2 amplification is widely utilized as molecular markers for trastuzumab target treatment. Hence, analysis of HER2/*neu* expression on breast tissue is used as the standard of care now [8]. The HER2 gene encodes a transmembrane tyrosine kinase receptor (the human epidermal growth factor receptor). It is located on 17q21.1, which encodes two oncogenes. Most recently, a series of HER2-targeting agents were developed, including trastuzumab, pertuzumab, ertumaxomab, and lapatinib. HER2 gene amplification or protein overexpression is demonstrated in approximately 25 % of newly diagnosed breast cancers. HER2/*neu* gene amplification is associated with a worse prognosis in patients with node-positive breast cancer due to increased proliferation and angiogenesis and inhibition of apoptosis [9, 10].

Even though the incidence and importance of HER2 amplification in lobular carcinoma are less than that in ductal carcinoma, the HER2 amplification is still an important adverse prognostic factor in lobular breast cancer [11]. It is suggested that tumor-initiating cells (side population fraction by cytometry, SP) of breast cancer present important HER2 expression—the SP fraction was reduced by HER2 inhibitors [12]. A meta-analysis for cohort randomized trials on women with HER2-positive early breast cancer explored that trastuzumab-based adjuvant chemotherapy derived profit in disease-free survival, overall survival, and recurrence to adjuvant chemotherapy [13]. The candidate gene is regarded as amplification when demonstrating more than six HER2 copies per nucleus or with a ratio of HER2 to centromere 17 greater than 2.2. The HER2 amplification and overexpression have been associated with negative responses to conventional chemotherapy and poor prognosis, but better overall survival rate for trastuzumab in breast cancer [14, 15].

TOP2A

DNA topoisomerase II alpha (TOP2A) is a new marker of cell cycle turnover. TOP2A gene is located on chromosome 17q21-q22. TOP2A is a

major target of anthracycline activity [16]. In breast cancer, TOP2A expression has been associated with HER2 protein overexpression and cell proliferation.

The TOP2A gene showed an increase in responsiveness to anthracycline-containing chemotherapy modalities relative to non-anthracycline regimens [17]. Because the gene locus of TOP2A is fairly close to the HER2 gene, the amplification of TOP2A is often cooperated with HER2 gene amplification. Twenty-three patients with T2–T4 ER-negative and HER2-overexpressed breast cancers treated with anthracycline-based chemotherapy were evaluated by Orlando et al. TOP2A was amplified in five (22 %) of the tumors. In all patients with TOP2A amplification, HER2 gene amplification was determined as well. It was reported that the pathological complete remission ratio in TOP2A amplified tumors is higher than in tumors without TOP2A amplification (respectively, 60 % and 15 %). It is postulated that in endocrine-unresponsive/HER2 overexpression cases, TOP2A amplification or the polysomy of chromosome 17 is associated with meaningfully high remission after anthracycline-based chemotherapy [18]. TOP2A is used as a molecular target of anthracycline drug and is pretty helpful as a predictive marker of response to anthracycline therapy [19].

TAU

TAU is one of the microtubule-associated proteins (MAPs) and is located on chromosome 17q21.1. Tubulin-targeting agents alter the microtubule function to disrupt cell shape, spindle formation, and microvesicle transportation. Detection of TAU expression may direct doctors to choose the patients who are likely to benefit more from taxane treatment. The decreased expression of TAU is accompanied with high responsiveness to paclitaxel in vitro. TAU enhances microtubule assembly and stabilizes microtubules and it is likely that TAU competes with taxanes for microtubule binding [20, 21]. It was shown that high TAU mRNA expression in ER-positive breast cancer presented as endocrine-sensitive but chemotherapy-resistant. On the contrary, low TAU expression in ER-positive cancers presented a poor prognosis with tamoxifen alone, but may profit from taxane-containing chemotherapy [22]. Patients with TAU-negative expression showed better response to paclitaxel administration compared to patients with TAU-positive expression (respectively, 60 % and 15 %) [23]. Amplified TOP2A and TAU genes are correlated to an important response to anthracycline-based chemotherapy, taxane, or cisplatin, respectively.

BRCA1

The breast cancer predisposition gene BRCA1 is located on chromosome 17q12-21. The breast cancer genes BRCA1 and BRCA2 are involved in tumor suppressor pathways, such as DNA repair and cell cycle control, but are not directly interacting [24], and the respective protein products do not show any homology [25]. However, it is interesting that breast cancers related to mutations of either BRCA1 or BRCA2 result in tumors with a similar phenotype of high genomic instability [26]. The BRCA1 protein presents its function via its ubiquitin ligase activity, which implicate in DNA repair pathways in response to DNA damage, cell cycle checkpoints, and mitosis by targeting proteins for degradation [27, 28].

BRCA2 acts with BRCA1 and RAD51 in genotoxic stress response [29]. BRCA2 also functions in exit of mitosis and is essential for formation of the contractile ring and appropriate abscission [30]. This function is in accordance with the observed genetic instability in cells lacking BRCA2. Loss of heterozygosity for BRCA1 and BRCA2 is determined in nearly all BRCA1- and BRCA2-associated carcinomas, correspondingly [31]. Hereditary breast cancer, appearing in carriers of mutations in the BRCA1 and BRCA2 genes, differs from sporadic breast cancer and from non-BRCA1/2 familial breast carcinomas, which imply defects in specific pathways [32]. Particularly, BRCA1 carcinomas have the basal-like phenotype and are high-grade, highly proliferating, estrogen receptor-negative, and

HER2-negative breast carcinomas, characterized by the expression of basal markers such as basal keratins, P-cadherin, and epidermal growth factor receptor.

Inherited mutations in breast cancer predisposition genes, mainly BRCA1 and BRCA2, account for approximately 5–10 % of breast cancer cases [33]. Most mutations in BRCA1 and BRCA2 result in unstable protein products; however, how this led to cancer predisposition is not obvious, and as yet, no treatment methods have been developed to target BRCA1 functions and mutations [34]. However, it is possible that mutations that are related with increased cancer risk might produce gene products that interfere with tumor suppressor pathways or support oncogenic pathways.

Germ line mutation of BRCA1 increases the risk of having breast cancer. Genetic factors comprise about 5 % of all breast cancer cases. However, somatic BRCA1 mutations are seldom determined in sporadic breast tumors. BRCA1 methylation has been described to take place in sporadic breast tumors and to be accompanied with decreased gene expression. Hence, epigenetic modification and deletion of the BRCA1 gene might act as Knudson's two "hits" in sporadic breast tumorigenesis [35, 36].

Retrospective studies explored a high level of responsiveness to platin derivatives in BRCA-associated tumors [37] and first clinical trials demonstrate good efficacy and tolerability for PARPs, or poly ADP (adenosine diphosphate)-ribose polymerase inhibitors, in mutation carriers with advanced breast and ovarian cancers [38]. But, the advantages of risk-reducing prophylactic surgery in mutation carriers have been verified [39].

P53

The tumor suppressor gene p53 is located on 17p13.1. P53 somatic change is determined in approximately 50 % of all human cancers [40]. MDM2 can inactivate the p53 through binding to the transactivation domain of p53. High expression level of this gene can cause excessive inactivation of p53 tumor suppressor protein. MDM2 targets p53 for proteasomal degradation through E3 ubiquitin ligase activity. Regulating p53 activity with MDM2 inhibitor is a striking approach for treatment of cancer [41]. A small-molecule MDM2 antagonist, nutlin-3, has been developed at last. Cancer cells with MDM2 gene amplification are most susceptible to nutlin-3 in vitro and in vivo. It is suggested that patients with wild-type p53 tumors may profit from antagonists of the p53-MDM2 interaction [42].

HIC-1

Hypermethylated in cancer 1 (HIC1, also named ZBTB29 or ZNF901) is located at 17p13.3. It is a candidate tumor suppressor gene, which generally undergoes allelic loss in breast and other human cancers. High HIC1 protein expression has been accompanied with better outputs in breast cancers. Lastly, it is demonstrated that HIC1 can hold down the ephrin-A1 transcription and it involves in the pathogenesis of epithelial cancers. Restoration of HIC1 in breast cancer cells causes a growth arrest in vivo [43]. It is shown that HIC1 controls breast cancer cell responses to endocrine therapies. A demethylating drug 5-aza-2′-deoxycytidine can restore the HIC1 expression in MDAMB231 cells. Hence, restoration of HIC1 function by demethylation may suggest a therapeutic opportunity in breast cancer [44].

Hereditary Breast Cancer

Monogenic Inheritance of BRCA1 and BRCA2 Mutations

Hereditary breast and ovarian cancers are brought about by an autosomal dominant inheritance with incomplete penetrance. Population-based studies put its reentrance for breast cancer at 45–65 % [37, 38]. This emphasizes the effect of modifying factors and lifestyle. Lastly, approximately 50 % of the monogenically determined breast and ovary cancers are through a mutation in one or the other of the highly penetrate BRCA genes.

Table 3.2 Breast cancer-related genes and their effect on risk

Risk genes	Increase in risk	Genes/syndromes
Highly penetrant genes	5- to 20-fold	BRCA1/BRCA2/RAD51C: hereditary breast and ovarian cancer syndrome TP53: Li-Fraumeni syndrome STK11/LKB1: Peutz-Jeghers syndrome PTEN: Cowden syndrome
Moderately penetrant genes	1.5- to 5-fold	CHEK2, PALB2, BRIP1, ATM
Mildly penetrant genes	0.7- to 1.5-fold	FGFR2, TOX3, MAP3K1, CAMK1D, SNRPB, FAM84B/c-MYC, COX11, LSP1, CASP8, ESR1, ANKLE1, MERIT40, etc.

This table adapted from [61]

Women carrying a mutated gene have an 80–90 % risk of breast cancer and a 20–50 % risk of ovarian cancer [45].

Monogenic Inheritance in Mutations of the Gene RAD51C and as Yet Unidentified, Highly Penetrant Genes

The third highly penetrant gene for breast and ovarian cancer, RAD51C, was determined in the summer of 2010 [46]. It is mutated in almost 1.5–4 % of all families predisposed toward breast and ovarian cancer with high or moderate penetrance. Like BRCA1 and BRCA2, it has an important role in DNA repair as a tumor suppressor gene. First studies in other populations verify that mutation in RAD51C is a predisposing factor for development of breast cancer. But, as it is rarely mutated and the data available on its penetrance are as yet inadequate, it is most recently not offered as part of routine diagnostics.

Moderately and Mildly Penetrant Gene Variants

A significant proportion of BRCA1/2- negative high-risk families possibly may have mutations in highly penetrant genes, which have not been identified yet. Therefore, it is postulated that the total effect of moderately and mildly penetrant gene variants is probably the cause for the majority of carcinomas [47]. This may be correct for 50 % of cases of hereditary breast cancer and 20 % of all cases of breast cancer (see Table 3.2). For instance, ATM, CHEK2, BRIP1, and PALB2 have been classified in moderate-risk gene groups with low heterozygote frequency [47]. Some low-risk variants located within the intron or regulatory areas were identified in the following genes: FGFR2, MAP3K1, TNRC9, and LSP1 (2q35, 6q22.33, 8q24) [48, 49]. The risks inherent in these variants are very low, with relative risks (RRs) of just about 1.1–1.3; however, their heterozygote frequencies are high.

Other Predisposition Genes Associated with Breast Cancer

G-Protein-Coupled Receptor-Associated Sorting Protein 1 (GASP-1)

Zheng et al. identified a specific fragment of G-protein-coupled receptor-associated sorting protein 1 (GASP-1) which was found in the sera of patients with early-stage disease but absent in sera of normal patients. They immunohistochemically determined overexpression of GASP-1 in all 107 cases of archived ductal breast carcinoma tumor samples, although normal adjacent breast tissue from 12 cases of ductal carcinoma presented little or no staining. Moreover, all 10 cases of metastatic breast carcinoma present in lymph nodes were positive. These studies point to GASP-1 as a potential new serum and tumor biomarker for breast cancer and suggest that GASP-1 may be a novel target for the development of new breast cancer therapeutics [4].

Estrogen-Related Receptor-α (ERR-α)

Jarzabek et al. showed that breast cancer tissues showed a slightly higher expression level of estrogen-related receptor-α (ERR-α) mRNA compared to the normal breast tissue (mean, 57.7 ± SD 58.7, 46.2 ± SD 42.0, respectively). But, ERR-α mRNA levels in breast cancer tissues demonstrated greater diversity than in normal tissues. It is probable that ERR-α could play a significant role in the alternative pathway to classical estrogen receptor-dependent pathway in cell signaling. The development and use of ERR modulators in the near future could help to design new well-tolerated and individualized therapeutic agents [50].

Survivin

Petrarca et al. showed that survivin overexpression in the primary tumor may be used as a promising predictive biomarker of complete pathological response (pCR) to neoadjuvant chemotherapy in patients with stage II and stage III breast cancer [51].

BCL2

Callagi et al. showed that the meta-analysis strongly supports the prognostic role of BCL2 in breast cancer and this effect is independent of lymph node status, tumor size, and tumor grade as well as a range of other biological variables on multi-variety analysis. Further large prospective studies are now essential to establish the clinical utility of BCL2 as an independent prognostic marker [52].

STAT3 and STAT5

Also in recent years, recognition of the intrinsic subtypes of breast cancer has enabled the disease to be categorized into different types, with the use of adjuvant therapies targeted to the biological profile of each type [3, 53]. STAT3 has been suggested as a prognostic factor in node-negative breast cancer patients and STAT5 has a role in estimating response to endocrine therapy [54, 55]. In a retrospective study of 346 node-negative tumors, nuclear expression of STAT3 was determined in 23.1 % of cases and phospho-STAT3 (Tyr705) in 43.5 %; both were accompanied with a reduced risk of recurrence and longer survival. It is demonstrated by multivariate analysis that phosphorylation of STAT3 is an independent prognostic factor for survival in this group, with no relation to hormone-receptor expression, proliferation index, or HER-2 amplification [56].

Visfatin

Mean serum visfatin was significantly greater in cases than in controls and patients with benign breast lesions (BBL) BBL ($p < 0.001$). In cases, visfatin was significantly correlated with CA 15-3 ($p = 0.03$), hormone-receptor status ($p < 0.001$), and lymph node invasion ($p = 0.06$) but not with metabolic and anthropometric variables ($p > 0.05$). Multivariable regression analysis explored that absence of estrogen and progesterone receptors (ER−PR−) was the strongest determinant of serum visfatin level ($p < 0.001$) in cases adjusting for demographic, metabolic, and clinicopathological features [57].

TGF-β-Signaling Pathway in Breast Cancer

The transforming growth factor-β (TGF-β) pathway has dual effects on tumor growth. Discordant results have been apparently demonstrated on the association between TGF-β-signaling markers and prognosis in breast cancer. It is shown that tumors with high expression of TβRII (TGF-β receptors II), TβRI (TGF-β receptors I), and TβRII and p-Smad2 ($p = 0.018$, 0.005, and 0.022, respectively) and low expression of Smad4 ($p = 0.005$) had a poor prognosis concerning progression-free survival. Low Smad4 expression combined with high p-Smad2 expression or low expression of Smad4 combined with high

expression of both TGF-β receptors showed an increased hazard ratio of 3.04 (95 % confidence interval (CI) 1.390–6.658) and 2.20 (95 % CI 1.464–3.307), respectively, for disease relapse.

As a result, combining TGF-β biomarkers give us prognostic information for patients with stages I through III breast cancer. This can determine patients at increased risk for disease recurrence, so they might be candidates for additional treatment [58].

PTEN or PIK3CA

MK-2206 was shown to repress Akt signaling and cell cycle progression and increased apoptosis in a dose-dependent manner in breast cancer cell lines. Cell lines with PTEN or PIK3CA mutations were meaningfully more sensitive to MK-2206; however, several lines with PTEN/PIK3CA mutations were MK-2206 resistant. siRNA knockdown of PTEN in breast cancer cells arose Akt phosphorylation consistent with increased MK-2206 sensitivity. Stable transfection of PIK3CA, E545K, or H1047R mutant plasmids into normal-like MCF10A breast cells increased MK-2206 sensitivity. Cell lines that were less sensitive to MK-2206 had lower ratios of Akt1/Akt2 and had reduced growth inhibition with Akt siRNA knockdown. In PTEN-mutant ZR75-1 breast cancer xenografts, MK-2206 treatment repressed Akt signaling, cell proliferation, and tumor growth. In vitro, MK-2206 demonstrated a synergistic interaction with paclitaxel in MK-2206-sensitive cell lines, and this combination had significantly further antitumor efficacy than either agent alone in vivo.

As a result, MK-2206 has antitumor activity alone and in combination with chemotherapy. This effect may be greater in tumors with PTEN loss or PIK3CA mutation [59].

Conclusion and Future Perspective

In conclusion, genetic profiles of tumors are now being informative about clinical outcome, and some prognostic and predictive indicators have appeared based on this research. While progress of therapeutics in this field is rapid and laudable, many hindrances must be overcome for these molecule-based therapies to become a reality for use in common cancers.

Pharmacological difficulties include developing safe, effective, and site-specific delivery mechanisms for these molecule-directed therapies. Despite these challenges, the remarkable potential of miRNAs as cancer biomarkers and therapeutics cannot be undervalued. If the current studies in these molecular targets can be sustained, it will bring a new dimension to the field of diagnostics and therapeutics for breast cancer. Hence, studies with the molecules mentioned in this chapter have the potential to transform current practice to the ideal of individualized care for breast cancer patients. Additionally, prospective tissue collection for molecular analysis may become routine in the near future in order to classify patients for alternative treatment options and to optimize treatment strategies based on molecular structure of the cancer.

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4 Breast Cancer Genomics

Birendra Kumar

Abstract

Breast cancer is a complex disease caused by the progressive accumulation of multiple gene mutations combined with epigenetic dysregulation of critical genes and protein pathways. There is substantial interindividual variability in both the age at diagnosis and phenotypic expression of the disease. With an estimated 1,152,161 new breast cancer cases diagnosed worldwide per year, cancer-control efforts in the post-genome era should be focused at both population and individual levels to develop novel risk assessment and treatment strategies that will further reduce the morbidity and mortality associated with the disease.

The discovery that mutations in the BRCA1 and BRCA2 genes increase the risk of breast and ovarian cancers has radically transformed our understanding of the genetic basis of breast cancer, leading to improved management of high-risk women. A better understanding of tumor host biology has led to improvements in the multidisciplinary management of breast cancer, and traditional pathological evaluation is being complemented by more sophisticated genomic approaches. A number of genomic biomarkers have been developed for clinical use, and increasingly, pharmacogenetic end points are being incorporated into the clinical trial design.

For women diagnosed with breast cancer, prognostic or predictive information is most useful when coupled with targeted therapeutic approaches, very few of which exist for women with triple-negative breast cancer or those with tumors resistant to chemotherapy. The immediate challenge is to learn how to use the molecular characteristics of an individual and their tumor to improve detection and treatment and, ultimately, to prevent the development of breast cancer.

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Introduction

Breast cancer is the most common malignancy affecting women, with more than one million cases occurring worldwide annually. There were 412,000 deaths attributed to breast cancer for women in the world, representing 1.6 % of all female deaths [1]. Breast cancer is mainly a disease of the genome, with cancers occurring and progressing through accumulation of abnormalities that amend the genome—by altering DNA sequence, copy number, and construction—in ways that supply to sundry aspects of tumor pathophysiology. Classic examples of genomic events that contribute to breast cancer pathophysiology include inherited mutations in BRCA1, BRCA2, TP53, and CHK2 that contribute to the initiation of breast cancer; amplification of ERBB2 (formerly HER2) and mutations of elements of the PI3-kinase pathway that activate aspects of epidermal growth factor receptor (EGFR) signaling; and deletion of CDKN2A/B that contributes to cell cycle deregulation and genome instability. It is now apparent that accumulation of these aberrations is a time-dependent process that accelerates with age [2].

Although American women living to an age of 85 have a 1 in 8 chance of developing breast cancer, the incidence of cancer in women younger than 30 years is uncommon. This is consistent with a multistep cancer progression model whereby mutation and selection drive the tumor's development, analogous to traditional Darwinian evolution [3, 4]. In the case of cancer, the driving events are changes in sequence, copy number, and structure of DNA and alterations in chromatin structure or other epigenetic marks. Our understanding of the genetic, genomic, and epigenomic events that manipulate the development and progression of breast cancer is increasing at a remarkable rate through application of powerful analysis tools that enable genome-wide analysis of DNA sequence and structure, copy number, allelic loss, and epigenomic modification. Application of these techniques to elucidation of the nature and timing of these events is enriching our understanding of mechanisms that increase breast cancer susceptibility, enable tumor initiation and progression to metastatic disease, and determine therapeutic response or resistance. These studies also reveal the molecular differences between cancer and normal that may be exploited to therapeutic benefit or that provide targets for molecular assays that may enable early cancer detection and predict individual disease progression or response to treatment.

In this chapter, I assess present and prospective instructions in genome analysis and précis studies that provide insights into breast cancer pathophysiology or that propose policy to improve management of breast cancer.

Primary Prevention of Breast Cancer Through a Genomic Approach

High-Penetrance Breast Cancer Genes

A developing body of evidence credentials the benefits of preventive measures with minimal possibility to women with identifiable highly penetrant mutations in the BRCA1 and BRCA2 genes. While other genes, such as PTEN in Cowden syndrome and TP53 in Li–Fraumeni syndrome, also contribute to a small fraction of hereditary breast cancer, alterations in these genes are rare and account for a relatively

small percentage of inherited breast cancer possibility [5].

About 100 genes for hereditary diseases showing Mendelian patterns of inheritance in families are known [6]. These are consistently rare genes and associated with high relative risks. Most of the genes have been identified through linkage analysis of carefully selected families, followed by positional cloning. Within this class are the breast cancer BRCA1 and BRCA2 genes, which contain over 1,000 alterations. Genetic screening for the spectrum of significant mutations in these genes in high-risk families is well established. The BRCA1 "breast cancer 1 early-onset" gene [7] is involved in susceptibility to breast and ovarian cancer at a young age, and tumors can arise through somatic or germ line mutations. Impaired or lost BRCA1 function underlies substantial genome instability, including augments in the number of mutations, DNA breakage and chromatid exchanges, increased sensitivity to DNA damage, and defects in cell cycle checkpoint functions.

The role of BRCA1 in the DNA damage response is that of "caretaker" or "master regulator" in the genome [8–10]. Jensen et al. isolated the large protein encoded by the BRCA2 gene and showed it to be a key mediator of homologous recombination [11]. It is a crucial element in the DNA repair process, which, if impaired through mutation, can lead to chromosome instability and cancer. It is known to mediate recombinational DNA repair by promoting assembly of RAD51 onto single-stranded DNA. This has a key role in catalyzing the invasion and exchange of homologous DNA sequences. Mutations in the BRCA2 gene may disrupt this mechanism and impair repair of DNA breaks, using homologous sequences from an intact homolog or sister chromatid, leading to errors in the repair process and chromosome instability.

BRCA1 and BRCA2 are likely to be the only major high-penetrance genes underlying breast cancer. Germ line mutations in the TP53 gene cause Li–Fraumeni syndrome, a phenotype that includes early-onset breast cancer [12], but these mutations are far rarer. Both BRCA1 and BRCA2 genes were identified using linkage mapping in families, a method that has been successful in identifying many Mendelian disease genes. However, this strategy has contributed little to the study of more common or "complex" forms of disease, mediated by genetic variants with reduced penetrance which may interact with environmental and other genetic factors. The complexity of this pattern of inheritance greatly reduces the power to detect genes through family-based studies.

Rare Cancer Syndromes and Rare Moderate-Penetrance Breast Cancer Genes

Garcia-Closas and Chanock have provided a comprehensive review of recent work from large consortial studies that have led to the discovery of additional breast cancer susceptibility loci through candidate gene or whole-genome approaches [13]. These studies suggest that much of the genetic component of breast cancer risk remains uncharacterized and probably arises from combinations of low-penetrance variants that, individually, might be quite common in the population. There are a number of syndromes that include breast cancer as a component of the disease phenotype. Rare to uncommon mutations in the PTEN [14] and STK11 [15] genes reason Cowden and Peutz–Jeghers syndromes, respectively, and both are associated with considerably increased breast cancer risk [16]. The E-cadherin gene (CDH1) encodes a cellular adhesion protein and is an influential tumor suppressor of breast cancer [17]. It is particularly implicated in invasive lobular breast carcinomas. RAD51C is another gene involved in the recombinational repair of double-stranded DNA breaks. Rare germ line mutations have been shown to confer increased risks of breast and ovarian cancer [18]. Segregation in families follows Mendelian patterns, and the disease phenotype resembles that of BRCA1 and BRCA2 mutation carriers.

There are also a number of gene mutations associated with more moderate risks of breast cancer, which show marked departures from Mendelian patterns of inheritance. As a result,

segregation of disease with the mutation may be unhelpful to confirm relationship with disease. Genes in this class include germ line mutations in the ataxia-telangiectasia (A-T) gene, which are associated with increased risk (~2.2-fold) of breast cancer in carriers of heterozygous mutations, with apparently higher risks below the age of 50 years [19]. Other rare moderate-penetrance genes include heterozygous mutations in BRIP1 (encoding a BRCA1-interacting protein) that confers elevated risks of breast cancer and Fanconi anemia subtype FA-J for bi-allelic mutations. The partner and localizer of BRCA2 (PALB2) gene interacts with BRCA2, and mono-allelic mutations are involved in familial breast cancer, conferring a 2.3-fold risk. Mutations in BRCA2 are also known to underlie Fanconi anemia (subtype FA-D1), and bi-allelic mutations of PALB2 underlie the very similar Fanconi anemia subtype FA-N [20]. Rare variants in the cell cycle checkpoint kinase 2 (CHEK2) genes are known to underlie an approximately twofold increase in risk of breast cancer. Products of this gene are involved in DNA damage repair, and mutations are found in 1–2 % of unselected women with breast cancer [21].

Common Low-Penetrance Breast Cancer Genes

In recent years the research of low-penetrance allelic variants was conducted mainly through genome-wide association studies (GWAS). These studies use a large number of common genetic single-nucleotide polymorphisms (SNPs) to identify associations with disease that rely upon patterns of linkage disequilibrium (LD) in the human genome [22]. The power of GWAS is to evaluate the association of genetic variants at different loci on different chromosomes (LD) in a large series of cases versus controls, analyzing a panel of 100,000 SNPs simultaneously, to identify new alleles of susceptibility to breast cancer [23].

In the human genome, it has been estimated that there are seven million common SNPs that have a minor allele frequency (m.a.f.), 45 %, and because recombination occurs in different hot spots, the nascent polymorphisms are often strongly correlated. GWAS have identified more than 100 such low-penetrance loci involved in cancer, including at least 17 related to breast cancer (Table 4.1). These variants have allele frequencies in the range 0.05–0.5, but they confer only small increases in disease risk [24]. Because of the greatly reduced penetrance and strongly non-Mendelian patterns of inheritance, there is often considerable uncertainty about the exact underlying genetic mutation. Not only are the most strongly associated SNPs unlikely to be the causal sites, but there also may be uncertainty about the gene involved. It has also been suggested that multiple rare variants create “synthetic association” signals in a GWAS if they occur more often in association with a common tag SNP. This implies that causal variants could be many mega bases away from variants detected in GWAS [25], although this scenario appears to be rare [26].

Perhaps one of the unexpected findings from these studies is a greater-than-anticipated role for noncoding variants in common diseases [27]. From the analysis of population sequences [28], 30 % of common variants associated with disease are annotated as, or in linkage disequilibrium with, non-synonymous (coding) variation. This supports the view that many of the common disease variants have gene regulatory roles. Among the set of well-established common susceptibility genes are variants in intron 2 of the FGFR2 gene [29], which, among the common variants, are likely to make one of the larger contributions to relative risk, at least for postmenopausal disease. Easton et al. [30] found that the rs2981582 SNP (allele frequency 0.38) contributes odds ratios of 1.23 and 1.63 for heterozygote and homozygote genotypes, respectively. The FGFR2 gene encodes a fibroblast growth factor (FGF) receptor. FGFs and their corresponding receptors are involved in regulation of the proliferation, survival, migration, and differentiation of cells. The considerable importance of FGF signaling in a range of tumor types is now becoming recognized [31].

SNPs within intron 2 are involved in FGFR2 upregulation, and aberrant signaling activation induces proliferation and survival of tumor cells

Table 4.1 Recognized breast tumor vulnerability genes and region

Recognized gene/region	Position	Mapped by	Allele incidence	Recognized/feasible role
BRCA1	17q21	Linkage	Rare	DNA repair/genome stability
BRCA2	13q13.1	Linkage	Rare	Recombinational repair
TP53	17p13.1	Linkage	Rare	Li–Fraumeni syndrome, apoptosis
ATM	11q22.3	CS	Rare	DNA repair
BRIP1	17q23.2	CS	Rare	DNA repair, associated with BRCA1
CHEK2	22q12.1	CS	Rare	DNA repair/cell cycle
PALB2	16p12.2	CS	Rare	Associated with BRCA2
RAD51C	17q22	CS	Rare	Homologous recombination repair
PTEN	10q23.3	Linkage	Rare	Cowden disease, cell signaling
STK1 (LKB1)	19p13.3	Linkage	Rare	Peutz–Jeghers syndrome, cell cycle arrest
CDHå1	16q22.1	Linkage	Rare	Intercellular adhesion: lobular BC
FGFR2	10q26	GWAS	Common	Fibroblast growth factor receptor
TOX3(TNRC9)//RBL2	16q12	GWAS	Common	Chromatin structure/cell cycle
MAP3K1	5q11.2	GWAS	Common	Cellular response to growth factors
LSP1	11p15.5	GWAS	Common	Neutrophil motility
8q24	8q24	GWAS	Common	Intergenic, enhancer of MYC proto-oncogene?
2q35	2q35	GWAS	Common	–
CASP8	2q33	GWAS	Common	Apoptosis
SLC4A7/NEK10?	3p24.1	GWAS	Common	Cell cycle control?
COX11/STXBP4?	17q22	GWAS	Common	Transport?
MRPS30?	5p12	GWAS	Common	Apoptosis?
NOTCH2/FCGR1B?	1p11.2	GWAS	Common	Signaling/immune response?
RAD51L1	14q24.1	GWAS	Common	Homologous recombination repair?
CDKN2A/CDKN2B?	9p21	GWAS	Common	Cyclin-dependent kinase inhibitors?
MYEOV/CCNDL?	11q13	GWAS	Common	Cell cycle control/fibroblast growth factors?
ZNF365?	10q21.2	GWAS	Common	Zinc finger protein gene
ANKRD16/FBXO18?	10p15.1	GWAS	Common	Helicase?
ZMIZ1?	10q22.3	GWAS	Common	Regulates transcription factors?

Notes: ? refers to "possible" gene or function in the breast cancer context. There is uncertainty about the exact genes and their useful roles in breast cancer
Abbreviation: *CS* candidate resequencing, *GWAS* genome-wide association studies

[32]. The identification of this gene, which was unanticipated as a cancer gene, has prompted research into related genes and their potential roles in cancer. Other FGFs (e.g., FGF-8) appear to be involved in breast cancer cell growth through stimulation of cell cycle and prevention of cell death [33].

Other low-penetrance variants that have been identified through GWAS include CASP8 (caspase 8), which encodes an apoptotic enzyme [34]. The variant rs1045485 is protective, contributing odds ratios of 0.89 and 0.74 for heterozygotes and rare homozygotes, respectively. Recently, variants in CASP8 have been shown to alter risks (in a protective direction) in individuals with a family history of breast cancer [35].

Breast tumors are classified according to whether they have receptor proteins that bind to estrogen and progesterone. Such cells are termed ER+and PR+and require estrogen and progesterone to

grow. Conversely, ER−and PR−tumors lack the protein that allows the hormones to bind. Tumor classifications manipulate the choice of treatment regimes for the patient. A further classification arises through tumors that overexpress the human epidermal growth factor receptor 2 (HER2) genes, which are termed HER2+ (conversely, HER2−). The triple-negative subtypes are ER−, PR−, and HER2− and are characterized by aggressive tumors and reduced range of effective treatment options. Several common gene variants are more strongly associated with specific cancer subtypes. These include the TOX3 gene, formerly called TNRC9 in which variant rs3803662 contributes a 1.64-fold homozygote risk, specifically in ER+cancer [36]. This gene encodes a high-mobility group chromatin-associated protein, and increased expression is implicated in bone metastasis [37].

Fine mapping has shown that hypothesized susceptibility variants lie in an intergenic region consistent with a gene regulatory function [38]. These authors note there remains uncertainty as to whether the causal variant is actually involved in the regulation of the nearby retinoblastoma-like gene 2 (RBL2) gene, which is involved in cell cycle regulation, given gene expression evidence.

The mitogen-activate protein kinase (MAP3K1) breast cancer gene [30] is a member of the Ras/Raf/MEK/ERK signaling pathway (as is FGFR2) and is involved in regulating transcription of a number of cancer genes. MAP3K1 has been found to be more strongly associated with ER+and PR+tumors than ER−/PR−subtypes. There is also a stronger association with HER2+ tumors [39]. The LSP1 gene was identified as breast cancer susceptibility locus by Easton et al. [30] who identified a SNP within the intron as the most strongly associated. LSP1 encodes lymphocyte-specific protein 1, which is an F-actin binding cytoskeletal protein. The same study also identified a breast cancer variant in the 8q24 region containing no known genes. This region is also associated with prostate cancer [40]. Stacey et al. identified a SNP on 2q35, a region with no known genes, as associated with breast cancer in Icelandic patients with ER+breast cancer [36]. Milne et al. also found an association with ER−disease, although there was a stronger signal for ER+ [41]. Other breast cancer associations include signals on 3p24, potentially relating to the genes SLC4A7 or NEK10, and on 17q22, perhaps related to COX11. These SNPs contribute odds ratios of 1.11 and 0.97 for heterozygote and homozygote genotypes, respectively [42]. Additionally, a common variant close to MRPS30 on 5p12 was found to confer higher risk of ER+disease [43]. Turnbull et al. described five new associations on chromosomes 9, 10 (three regions), and 11 [44]. Two further signals reported by Thomas et al. [45] include a SNP in the pericentromeric part of chromosome 1, within a region containing NOTCH2 and FCGR1B, and a signal associated with another double-strand break repair gene (RAD51L1) on 14q24.1. There is evidence that the chromosome 1 locus is more strongly associated with ER+disease.

Considerable additional follow-up investigation will be required to establish the relationships between many of the SNPs and the actual causal variant(s) and to further elucidate the role in disease for many of these common genes.

Genome-Wide Association Studies

In modern years, the research of low-penetrance allelic variants was conducted mainly through GWAS. These studies use a large number of common genetic SNPs to identify associations with disease that rely upon patterns of linkage disequilibrium in the human genome [22]. The power of GWAS is to evaluate the association of genetic variants at different loci on different chromosomes in large series of cases versus controls, analyzing a panel of 100,000 SNPs simultaneously, to identify new alleles of susceptibility to BC [23]. In the human genome, it has been estimated that there are 7 million common SNPs that have a minor allele frequency (m.a.f.), 45 %, and because recombination occurs in different hot spots, the nascent polymorphisms are often strongly correlated. These studies therefore supply a powerful tool to recognize novel markers for susceptibility and prognosis of disease [46–48].

In the GWA studies, the accumulation of a large number of data is crucial. Houlston and

Peto have estimated the number of cases required to identify low-penetrance alleles conferring a relative risk of two both in an unselected population and in families with first-degree relatives affected [47]. In an unselected population, the identification of a susceptibility allele with a frequency of 5 % requires over 800 cases. In the same population, the identification of a susceptibility allele with a frequency of 1 % requires over 3,700 unselected cases, whereas about 700 would be enough if three affected families are selected. Therefore, the power of association studies can be significantly increased using selected cases with a family history of cancer because fewer cases are required to demonstrate the association with the disease [47].

The potential of the association studies of cases with a family history to identify low-penetrance alleles conferring a relative risk of 2 has been demonstrated by the mutation CHEK2 1100delC in patients with BC. This variant carried by 1 % of the population confers an increased risk of 1.7-fold. The frequency was not significantly increased in unselected cases (1.4 %), but it was strongly increased in familial cases without BRCA1 and BRCA2 mutations (5.1 %) [49].

In the past several years, several novel risk alleles for BC were identified by four recent GWA studies: Breast Cancer Association Consortium, Cancer Genetic Markers of Susceptibility, DeCode Islanda, and Memorial Sloan–Kettering Cancer Center [29, 30, 43, 50]. In each of them, the association study was shared in three phases: the first phase identifies the common SNPs in cases and controls, the second phase evaluates how many of the above SNPs are common to a greater number of cases and controls, and, finally, the third phase aims to identify new alleles of susceptibility to BC. Easton et al., in their study, identified five independent loci associated with increased susceptibility to BC (Po10_7) [30]. This multistage study involved in the first stage 390 BC cases with a strong family history and 364 controls and 3,990 cases and 3,916 controls in the second stage. To define the risk associated with the 30 most significant SNPs, a third stage of the study was conducted involving 21,860 cases and 22,578 controls from 22 additional studies in the Breast Cancer Association Consortium. These combined analyses allowed the observation that the SNPs showing a stronger statistical evidence of association with an increased familial risk were rs2981582 in intron 2 of FGFR2, rs12443621 and rs8051542 within TNRC9, rs889312 in a region that contains the MAP3K1 gene, rs3817198 in intron 10 of lymphocyte-specific protein 1 (LSP1), and rs2107425 within the H19 gene.

In brief, GWAS analyze thousands of cases and controls (huge numbers are necessary to reach sufficient statistical power) in order to compare SNPs, which makes it possible to identify genetic variants associated with disease risk. More than 100 GWAS have related a slight increase in cancer risk [51]. In breast cancer, at least 18 variants have been identified, with a 1.1- to 1.5-fold increase in risk. Interestingly, these SNPs might not only provide useful information on the risk of breast cancer, but they could also be linked to specific molecular subtype of breast cancer, such as FGFR2-RS2981582 and TNRC9-RS3803662 with positive estrogen receptor [52] and rs8170 in chromosome 19p13 with negative estrogen receptors [53]. However, the clinical impact of these approaches is not clear, and this could illustrate how techniques are developing faster than knowledge. For example, a 1.2-fold risk with one of these variants is comparable to that of delaying the age of the first pregnancy to more than 35 years [51]. In addition, these approaches give no information about other complex factors that can affect risk, and there are no data on the possible effects of combining two or more variants. Consequently, large research consortia are essential if a high number of cases and controls are to be attained. Finally, direct access to some GWAS via the Internet can generate anxiety and dangerous misinterpretation of genetic risk.

Breast Cancer Genomics Based on Biobanks

Endeavors to discover genes' contributions to intricate diseases, such as cancer, require new study designs that incorporate an efficient use of

population resources and modern genotyping technologies. There are two approaches used for the study of breast cancer, both of which incorporate the use of biobanks. One uses a cancer registry as a source of case information, which is then linked to a biobank on blood DNA. The biobank also provides samples from matched controls. After genotyping, clinical data are retrieved from hospital records, and the results can be presented for genotype-specific cancer risks or similarly for genotype-specific clinical and survival parameters. The second approach uses registered data on cancer in families or among twins. With defined groups of patients, paraffin tissue is collected by contacting the pathology departments of the hospitals where the patients were diagnosed. Tumor and healthy tissue are prepared and used for mutation, the loss of heterozygosity, or copy number analysis. In the era of whole-genome genotyping technologies, the importance of well-characterized sample sets cannot be overemphasized. Samples, rather than technologies, limit the rate of gene discovery in complex diseases [54].

Linking Genotype to Phenotype in Breast Cancer

Women with germ line BRCA1 or BRCA2 mutations are estimated to have a 45–70 % risk of breast cancer by age 70 years [55–58]. The identification of BRCA1 and BRCA2 mutations was a major step in personalizing breast cancer risk assessment, screening, and risk reduction strategies. Studies are ongoing to determine whether or not certain subgroups of BRCA mutation carriers may be at a higher risk for breast cancer. It has been proposed that certain BRCA mutations may confer a differential risk of future breast cancer development, suggesting an important genotype–phenotype linking [59, 60].

In a recent kin-cohort study in Ontario, Risch et al. observed a trend of increasing breast cancer risk associated with increasing downstream location of BRCA1 mutation with a continuous linear trend and a 32 % increase in risk associated with each additional 10 % or 559 nucleotides of downstream distance [60]. Over the past few years, a considerable effort has been made to characterize genetic abnormalities in cancer, the general idea being that tumor genotyping would be valuable in defining cancer phenotypes. In a previous study, we showed that it was possible to delineate subsets of breast tumors according to specific combinations of DNA amplifications [61]. The present work allowed us to extend the phenotypic description to prognostic significance. We show here that some of the markers tested presented prognostic significance in specific subsets of patients. This was particularly evident for MDM2 amplification and p53 mutations, which showed a strong prognostic value in the N2 subset of patients, or for the amplification of CCND1, EMS1, and FGFR1 in N1 patients. During the course of this study, we also made some observations that suggest the existence of correlation clustering in other patient subsets, such as MYC in patients under 50 years or MDM2 in ER1 patients (data not shown).

Our data constitute an attempt to delineate tumor subsets according to their genotypic specificity. Knowing the complexity of the genetic rearrangements in breast cancer, the nine events studied here probably correspond to a small portion of the genes involved in tumorigenesis. Genotyping of breast tumors will involve the analysis of an ever-larger number of parameters and sorting of the significance of complex combinations. Because different combinations of genes or genetic anomalies may bear a meaning in different populations of patients, the analysis of specific phenotypic subsets will be necessary, thus leading to an increase of the number of comparisons. This will require the analysis of very large cohorts of patients (several thousand) and consequently the use of high-throughput analytical methods [62] in association with statistical tools, especially devised for multiple-comparison analyses.

Genomics Landscape of Breast Cancer and Comprehensive Atlas of Breast Cancer Genomes for Various Applications

A pilot genome-wide sequencing exertion on breast cancers identified a total of 1,137 somatically mutated genes from 11 breast cancers, with an average of 52 non-synonymous mutations per

sample. Using gene mutation incidence as the principal criterion, 140 genes were identified as candidate cancer genes that require further assessment to confirm their functions as causal contributors to tumorigenesis [63, 64]. These studies also portrayed the genomic landscape of human breast cancer that consists of a few repeatedly mutated gene “mountains” and a huge number of rarely mutated (usually <5 %) gene “hills” [64].

Besides the detection of novel candidate genes, more recent studies have delineated new aspects of breast cancer exomes. Interrogating luminal-type breast cancer genomes with clinical data revealed that somatic mutations in TP53 signaling pathway, DNA replication, and mismatch repair are associated with aromatase inhibitor resistance [65]. Determination of clonal frequencies by deep sequencing provided new insights into the initiating events of TNBCs [66]. Massively parallel paired-end sequencing technologies enable whole-genome detection of gene rearrangements at the DNA sequence level [67]. An analysis on 24 breast cancers revealed more than 2,000 gene rearrangements, enriched with tandem duplications [68]. Analysis of breast cancers across a variety of subtypes revealed that luminal B and HER2-enriched breast tumors harbor many more structural rearrangements when compared to the luminal A subtype. However, no repeatedly recurrent rearrangements have been discovered in breast cancer by earlier studies except for the MAGI3–AKT3 gene fusion detected in 4 % (9 out of 257) of breast cancers [69].

Like all cancer types, breast cancer progression is thought to be a dynamic multistep Darwinian evolution process. Independent mutations arise in a stepwise fashion, of which those conferring selective advantages promote cell proliferation and clonal expansion [70]. Through deep whole-genome sequencing of 21 breast cancers and analysis of subclonal genetic alterations, Nik-Zainal et al. proposed a model for clonal evolution that many molecular aberrations accumulate in dormant cell lineages before final expansion of the most recent common ancestor, which triggers diagnosis [71]. Integrative breast cancer studies aim at developing new definition of breast cancer subtypes with better prognostic and predictive values. A cluster analysis integrating copy number and gene expression profiles of ~2,000 breast cancers suggested a novel classification system [72]. A recent multiplatform study on hundreds of breast cancers revealed subtype-specific pattern in numerous tumor characteristics including gene mutations, microRNA expression, DNA methylation, copy number changes, and protein expression. Moreover, in whole-exome sequencing of more than 500 tumors, this study also revealed almost all repeatedly altered pathways (PI3K/AKT, TP53, RB) in breast cancer [73].

The quickly evolving sequencing technologies generate massive genomic data at an increasing rate with reduced cost. In recent years in particular, large-scale analyses of cancer genomes have produced a prosperity of information, which greatly expanded our knowledge on human breast cancer, summarized in Table 4.2. The launch of comprehensive cancer genome projects including the Cancer Genome Project (CGP) [78], the Cancer Genome Atlas (TCGA) [79], and the International Cancer Genome Consortium (ICGC) [80] facilitates the compilation of an encyclopedic catalogue of the genomic changes involved in cancer. Whole-genome sequencing studies enable observation of genetic alterations earlier undetectable by protein-coding sequence screens, including mutations in noncoding regions and large rearrangements. Primary breast cancers were reported to harbor ~7,000–10,000 somatic point mutations per genome [65, 69, 81] in which tens to hundreds reside in the protein-coding regions [63, 64, 69, 77], as well as up to hundreds (average 20–50) of somatic structural variants [65, 68, 69, 75, 81]. The minimum number of mutations necessary for tumorigenesis has been estimated to be around 5–6, according to incidence modeling of solid tumors such as breast and colorectal cancers, and this number would be smaller in leukemia and childhood cancers [82]. However, recent systematic mutational screens of cancer genomes suggested a higher number of causal gene mutations in each tumor (range 10–20 genes) [63, 83].

Distinguishing the driver mutations from passengers cannot be accomplished by analyzing genetic data alone but requires functional validation of the cancer-relevant activities. Since most

Table 4.2 Summary of genome sequencing studies of breast cancer

Study	Cancer type	Sequencing target	T/N pairs[a]	Findings
Stephens et al. [74]	Breast	Protein kinome (518 genes)	25	Identified diverse patterns of somatic mutations in breast cancer
Wood et al. [64]	Breast, Colorectal	All RefSeqGene (18,191 genes)	11 + 24 per tumor type	*The first sequencing effort of all coding regions in cancer genomes*. Identified 280 candidate genes and revealed the mutation landscape of breast and colorectal cancer genomes
Shah et al. [70]	Breast (metastasis)	Whole genome whole transcriptome	1[b]	Demonstrated single-nucleotide mutational heterogeneity and mutational evolution in breast tumor progression
Stephens et al. [68]	Breast	Whole genome	24	*The first genomic screen for somatic rearrangements in tumor samples*. Revealed the genome landscape of somatic rearrangements in breast cancer
Ding et al. [75]	Breast	Whole genome	1[c]	Indicated that metastasis may arise from a minority of cells within the primary tumor
Edgren et al. [76]	Breast	Whole transcriptome	4	Discovered novel fusion genes (e.g., *VAPB-IKZF3*) with potential functional role in breast cancer
Nik-Zainal et al. [71]	Breast	Whole genome	21	Identified distinct nucleotide substitution signatures, observed localized hypermutation, and constructed a model of breast cancer evolution
Ellis et al. [65]	Breast	Whole genome ($n = 46$), whole exome ($n = 31$)	77 + 240	Identified novel significantly mutated genes (e.g., *GATA3*, *TBX3*, *ATR*, *RUNX1*, *LDRAP1*, *STMN2*, *AGTR2*, *SF3B1*) in luminal breast cancer and revealed pathways (e.g., TP53, DNA replication, MMR) associated with aromatase inhibitor response
Shah et al. [66]	Breast	Whole genome ($n = 15$), whole exome ($n = 54$)	65	Revealed mutations and structural alterations with clonal frequency and suggested involvement of cytoskeletal gene mutations in breast cancer
Stephen et al. [77]	Breast	Whole exome	100 + 250	Revealed multiple mutation signatures of breast cancers and identified novel driver mutations (e.g., *AKT2*)
Banerji et al. [69]	Breast	Whole genome ($n = 22$), whole exome ($n = 130$)	108 + 235	Identified novel recurrent mutations in *CBFB* and a recurrent fusion gene *MAGI3–AKT3*
TCGA [73]	Breast	Whole exome	507	Revealed molecular subtype-specific patterns of mutations and identified novel candidate genes

[a]T/N pairs, patient-matched tumor/normal pairs investigated. In some cases, numbers of T/N pairs in discovery screen and validation screen are indicated before and after the plus sign
[b]DNA from primary tumor and metastasis was analyzed in this study
[c]DNA from blood, primary tumor, metastasis, and xenograft was analyzed in this study

functional assays are relatively labor- and time-intensive, prioritization of the genes for functional studies presents a great challenge in cancer genomic data interpretation. Several measurements have been adopted to identify the most promising driver mutations. First, analyzing the ratio of non-synonymous mutations to synonymous mutations of a given gene would indicate whether the mutations have been under positive selection during tumor development, thus a higher than expected ratio always suggests driver mutation [84, 85]. Second, assessment of the mutation prevalence in genes also identifies drivers that contribute to cancer if they are highly unlikely to be mutated by chance [63]. Third, several tools have been employed to predict the effect of non-synonymous single nucleotide variants on protein function based on phylogenetic conservation and physical considerations (e.g., Sorting Intolerant From Tolerant (SIFT) [86], Polymorphism Phenotyping (PolyPhen) [87], Panther [88], MutationTaster [89], etc.). Last but not least, as the number of pathways involved in cancer is much smaller than that of cancer genes and a variety of mutations in multiple cancer genes from the same pathway would likely to have similar pathological effects [90], evaluation of the combined prevalence of somatic alterations at the pathway level provides strategies for identification of cancer-associated processes [91, 92].

In the past few years, comprehensive mutation interpretation implementing most, if not all, of these measurements has been introduced into cancer genome analyses. For example, Carter and her colleagues developed a computational pipeline for cancer-specific high-throughput annotation of somatic mutations (CHASM), which takes a total of 49 predictive features into account for driver identification [93]. Another example is a package for determination of mutational significance in cancer (MuSiC), designed by Dees et al. MuSiC is the first software suite that integrates clinical data with coverage data and database references to identify drivers from large mutational discovery sets [94].

Although many tools can help to prioritize the candidates of interest for downstream analyses, only the evidence from functional assays and biological studies can fully credential a candidate gene as a bonafide cancer gene. It is clear from cancer genome resequencing efforts that not all cancer genes are mutated at high prevalence. On the contrary, despite conferring selective advantage, the vast majorities of cancer genes are not frequently mutated and are therefore difficult to identify through sequencing of a limited number of samples. In order to discover these infrequent driver mutations, systematic screens of large cohorts of patients are required. For example, it was estimated that 500 tumor samples of a particular tumor type are needed in whole-exome sequencing studies to get an ~80 % detection power of genes with ~3 % true mutation frequency [80].

Classification of Breast Cancer Based on Genome Profile

Gene expression profiling has proven to be a useful and reliable tool for classifying breast cancers into subgroups that reflect different histopathological characteristics as well as differential prognostic outcome. It has been suggested that estrogen receptor-negative and estrogen receptor-positive breast cancers can be subdivided into Her-2 positive basal-epithelial-like, normal breast-like, and luminal-like [95]. The potentially different origins of the tumor cells may signify distinct pathways of tumorigenesis and differences in the clinical course of the disease. Germ line mutations in the BRCA1 and BRCA2 genes together account for a significant portion of hereditary breast cancers. They have been shown to leave a characteristic imprint on the panel of genes expressed by the tumors [96], with BRCA1-dependent tumors exhibiting a transcriptional profile similar to the basal subtype of tumors [97]. These findings suggest that the cellular origin of BRCA1- and BRCA2-mutation-positive tumors may differ or that these tumors traverse down separate pathways in their progression toward malignancy [96]. Furthermore, the molecular subclassification of non-BRCA1/2 familial breast cancers into homogeneous subgroups underscores the potential differences in

cellular origin and/or disease progression due to the presence of multiple diverse underlying genetic alterations, which is reflected in the phenotype of the tumors [98].

The diversity of breast cancer has been acknowledged for decades, but recent technological advances in molecular biology have given detailed knowledge on how extensive this heterogeneity really is. Traditional classification based on morphology has given limited clinical value, mostly because the majority of breast carcinomas are classified as invasive ductal carcinomas, which show a highly variable response to therapy and outcome [99]. The first molecular subclassification with a major impact on breast cancer research was proposed by Perou and colleagues where the tumors were subdivided according to their pattern of gene expression [95, 100]. Five groups were identified and named luminal A, luminal B, basal-like, normal-like, and HER-2-enriched subgroups. These intrinsic subgroups have been shown to be different in terms of biology, survival, and recurrence rate [95, 97]. The molecular subgroups have been extended to also include a sixth subgroup, which has been named the claudin-low group, based on its low expression level of tight junction genes (the claudin genes) [101].

Different methods for the assignment of individual tumors to its molecular subgroup are proposed, each based on the expression levels of different sets of genes [97, 102, 103]. The agreement between methods on how to classify individual tumors are not optimal, and how to establish more robust single sample predictors is actively debated [104–107].

Aneuploidy is the presence of an abnormal number of parts of or whole chromosomes and is one feature that clearly separates cancer cells from normal cells. This was proposed as being important in cancer nearly a century ago by Theodor Boveri. With array-based comparative genomic hybridization (aCGH), a genome-wide profile of the copy number alterations in the tumor can be obtained. These patterns are related to the molecular subtypes with distinct differences in the number of alterations between the subtypes [108–111]. These copy number alterations (CNAs) alter the dosage of genes and highly influence the level of expression [112, 113]. This frequently affects the activity in oncogenes and tumor suppressor genes, and in this way CNAs are important for the carcinogenic process.

CNAs in tumors are a result of deregulated cell cycle control and of DNA maintenance and repair [114]. Different patterns of copy number alterations have been identified with distinct differences; simplex profiles are characterized by few alterations and complex genomic profiles have extensive changes [115]. Complex genomic rearrangements are areas with high-level amplifications and have prognostic value in breast cancer even when they do not harbor known oncogenes, suggesting that the phenotype of defect DNA repair may be associated with more aggressive disease [115, 116]. Alterations in the expression pattern are caused by changes at the genomic level, and a robust classification of breast cancer for clinical use should probably take these more into account. Changes at the genomic level include point mutations, changes in copy number, and epigenetic events. These are characteristics that enable and drive carcinogenesis together with tumor-promoted inflammation [117].

Risk Assessment and Prognosis by Genetic Test: Market Players in Genomics-Based Personalized Diagnosis, Prognosis, and Therapy

Risk Assessment

Hereditary alterations have been associated with 10–15 % of all breast cancer cases; however, disease etiology in the majority of women appears to be sporadic, lacking a momentous family history. Because sporadic breast cancer may be influenced by a number of lifestyle and environmental factors as well as common low-risk variant in a number of genes, numerous models have been developed in an endeavor to quantify individualized breast cancer risk:

- The Gail model measures risk based on patient age, age at menarche, number of prior breast biopsies, age at first live birth, and number of first-degree relatives affected by breast cancer [118].

- The Claus model estimates risk based on the number of affected relatives and their respective age at diagnosis [119].
- The BRCAPRO model calculates risk of developing breast cancer based on the probability of carrying a BRCA1 or BRCA2 mutation [120].

These models have been widely used to predict risk and direct patient care, but each model has limitations, because no model accounts for the spectrum of risk factors influencing breast cancer. For example, the Gail model considers only first-degree relatives without regard to age at diagnosis or presence of ovarian cancer, thus potentially underestimating genetic risk. The Claus and BRCAPRO models only consider family history, potentially underestimating risk in women with other risk factors [121]. In addition, these models were developed 10–20 years ago, when incidence of breast cancer in the general population was lower than it is today, and use of lower baseline risk estimates may contribute to an underestimation of recent risk [122]. More recent models, such as the Tyrer–Cuzick model, utilize family history, endogenous estrogen exposure, and presence of benign disease to model breast cancer risk [123], but contributions from other factors such as mammographic breast density, weight gain, steroid hormone levels, and susceptibility genes have not been incorporated [124].

The discovery of the BRCA1 and BRCA2 genes advanced risk assessment in families affected by hereditary breast and ovarian cancer, but identification of molecular markers associated with increased breast cancer risk in patients without a family history of breast cancer has remained far more challenging. Without a strong family history, linkage approaches involving large pedigrees such as those used to identify BRCA1 and BRCA2 are not applicable. Sporadic breast cancer is not usually associated with other cancers, such as ovarian or male breast cancer, and unlike BRCA1-positive carcinomas, which exhibit specific histological characteristics, sporadic breast cancer cases comprise a vast array of phenotypes. Early approaches to identify sporadic breast cancer susceptibility genes compared the frequency of DNA variants in genes from molecular pathways believed to be involved in breast cancer development between cases with disease and healthy matched controls. An association study using candidate genes recently identified caspase 8 (CASP8) as a low-risk susceptibility gene where the major (H) allele of the D302H polymorphism had a protective effect on the development of breast cancer [125]. Despite success in identifying CASP8, candidate gene approaches have not been widely successful in identifying additional breast cancer susceptibility genes [126].

Whole-Genome Approaches

Candidate gene approaches are rapidly giving way to genome-wide association studies (GWAS), which evaluate a dense array of genetic markers representing common variation throughout the genome. Completion of the human genome sequence and subsequent identification of single-nucleotide polymorphisms (SNPs) now permits millions of informative SNPs across the genome to be assayed simultaneously. GWAS are useful for mapping genes of interest to small, localized regions of the genome and for detecting the effects of common (>5 % minor allele frequency) alleles on disease risk [127]. Moreover, GWAS are performed without a priori knowledge of the underlying genetic defect(s), which may be advantageous since many genes identified through whole genome approaches were not previously suspected to influence the disease under investigation [128].

Recent GWAS have identified a number of loci that appear to be associated with breast cancer susceptibility. For example, the fibroblast growth factor receptor 2 (FGFR2), mitogen-activated protein kinase kinase kinase 1 (MAP3K1), lymphocyte-specific protein 1 (LSP1), and trinucleotide repeat-containing 9 (TNRC9/LOC643714) genes, along with a 110-kb region of chromosome 8q24, have been associated with breast cancer in large studies involving thousands of subjects [29, 30]. Associations with other chromosomal regions—2q35, 5p12, 6q22, and 16q12—also have been

Table 4.3 Leading direct-to-consumer genetic testing companies

Company	Headquarters	Website	Price (USD)	Genetic counseling	Breast tumor susceptibility variants
23andMe	Mountain View, CA	www.23andme.com	$399	No	2 SNPS
deCODEme	Reykjavik, Iceland	www.decodeme.com	$985[a]	Yes	11 variants[b]
Knome	Cambridge, MA	www.knome.com	Custom[c]	Yes	DNA sequence
Navigenics	Foster City, CA	www.navigenics.com	$999[d]	Yes	Unknown

[a]Complete scan
[b]For women of European descent
[c]KnomeSELECT™ is $24,500 for complete sequence of 20,000 genes; KnomeCOMPLETE™ is $99,500 for complete genome sequence
[d]Option for ongoing subscription ($199 per year) for updates

reported [36, 43, 50]. Further analysis has shown that allelic variation at FGFR2, TNRC9, 8q24, 2q35, and 5p12 is associated with physiological characteristics of breast tumors, such as ER status [36, 43, 129], and specific FGFR2, MAP3K1, and TNRC9 variants may interact with BRCA1 and BRCA2 mutations to increase breast cancer risk [130].

Despite recent success in identifying genetic determinants of breast cancer, susceptibility alleles identified through GWAS are believed to account for only ~5 % of breast cancer risk [131]. If future studies are to be successful in identifying additional low-risk susceptibility alleles and low-frequency, highly penetrant variants [132], interactions between genes and environmental exposures must be assessed [133], and methods must be developed to evaluate mechanisms by which DNA variants in intronic or intergenic regions contribute to disease. As risk associated with susceptibility alleles may vary between racial/ethnic populations due to differences in frequency, patterns of disequilibrium, and interactions with environmental factors [5, 30, 36], sufficiently powered genetic studies in women from various ethnic groups are needed to improve risk reduction strategies for all women.

Direct-to-Consumer Testing

New susceptibility variants identified by GWAS have not yet been incorporated into genetic tests with beneficial clinical utility for breast cancer patients. However, genetic analysis and risk assessment are available commercially through direct-to-consumer (DTC) testing. A number of for-profit companies offer personal genetic information based on DTC tests—the largest and most recognized companies include 23andMe, deCODEme, Navigenics®, and Knome®, Inc. (Table 4.3). For a fee of $99 to $99,500 consumers provide a blood, buccal, or saliva sample for targeted SNP analysis or whole-genome sequencing. Genetic information provided to the consumer varies greatly among companies, from trivial facts such as earwax type and ancestry information to information on risk for disease [134, 135]. Although DTC tests epitomize "personalized genomics" by providing consumers with individual genotypes, critics note that the clinical utility of such tests is limited and often incongruent with marketing claims. Because information on family history and environmental exposures is usually not accounted for, DTC risk estimates may not be sufficiently accurate to enable consumers to make appropriate medical decisions [136, 137].

The majority of genetic risk assessments developed thus far focus on DNA variants; however, a new RNA-based signature has been developed for noninvasive breast cancer screening using peripheral blood samples. Although based on a small number of cases ($n = 24$) and controls ($n = 32$), a subset of 37 genes in the assay correctly classified 82 % of patients [138]. Despite a relatively high misclassification rate, DiaGenic (www.diagenic.no) has since developed this gene

expression signature into a clinical screening tool, currently available only in India as BCtect™ India.

Breast Cancer Personalized Prognostics

Pathological Characterization of Breast Cancer

Human breast carcinomas exhibit diverse pathological characteristics that are associated with different clinical outcomes and thus are routinely used to guide treatment options. Accordingly, an accurate definition of prognosis is dependent on the ability to detect and quantify differences in tumor attributes, such as rates of proliferation and propensity to metastasize. Routine tumor evaluation currently includes (1) histopathological classification; (2) grade determination; and (3) quantification of tumor size, surgical margin status, and lymph node involvement.

Histopathological characterization, based on microscopic cellular morphology, classifies breast carcinomas into common subtypes (ductal or lobular carcinoma), which tend to have similar prognoses [139]; or less common forms such as mucinous, tubular, and papillary (favorable prognosis) [140]; or inflammatory breast cancer (poor prognosis) [141]. Increasing tumor size has long been associated with poor prognosis [142], but improved mammographic detection of smaller tumors has decreased the prognostic utility of tumor size [143]. Presence of positive surgical margins has been associated with local recurrence, but only 27 % of patients with extensively positive margins will have recurrent disease [144, 145].

Likewise, the Nottingham Histological Score, widely used for assessing histological grade, is clinically useful for stratifying patients into low-risk (low-grade disease, 95 % 5-year survival) and high-risk (high-grade disease, 50 % 5-year survival) groups [146, 147], but the reliability of breast tumor grade in predicting survival is hampered by subjectivity associated with its assessment [148]. Axillary lymph node status is the most reliable predictor of survival, differentiating women who are likely to have >90 % 5-year survival (patients with negative nodes) from those who are likely to have <70 % survival (women with nodal metastasis) [149]. Although these clinical attributes are currently the standard of care for breast cancer patients, many are imprecise in their ability to accurately predict outcomes.

Immunohistochemistry

Molecular markers have the prospective to provide additional prognostic information to supplement traditional pathological assessments for disease management in breast cancer patients. As mentioned above, traditional immunohistochemistry (IHC) markers routinely used in the classification of breast cancer include ER, PR, and HER2. Tumors positive for ER and PR expression frequently have low cellular proliferation rates, tend to exhibit lower histological grade, and are associated with more favorable prognosis [150]. ER and PR expression also is useful for identifying patients who will likely benefit from hormonal therapy, as women with ER- and PR-negative breast cancer do not gain a survival benefit from antiestrogen tamoxifen [151].

The HER2 gene is a member of the epidermal growth factor receptor family with tyrosine kinase activity and is amplified at the DNA level and/or overexpressed in 15–25 % of breast cancers. Carcinomas with amplified/overexpressed HER2 exhibit high histological grade and usually have a poor prognosis [152, 153]. Some patients with positive HER2 status (15–20 %) are eligible to receive trastuzumab, a monoclonal antibody targeting HER2, in combination with standard chemotherapy [154].

Rigorous clinical studies have shown that evaluating ER, PR, and HER2 status provides additional prognostic information beyond that normally achieved by histological assessment alone. For example, breast carcinomas that are ER negative and PR negative and do not have HER2 overexpressed (triple negative) are marked by aggressive behavior, but because women with triple-negative disease are not eligible for tamoxifen or trastuzumab treatment, they usually have relatively low long-term survival [155]. Other markers such as nuclear antigen Ki67 are not routinely used to guide treatment selection, but hold

Table 4.4 Selected molecular diagnostic tests for breast cancer

Test	Company	Assay type#	Number of genes/proteins	Classification	Study
Breast Bioclassifier™	University genomics	qRT-PCR	55	Tumor subtype Therapeutic guidance	Perou et al. [100]
MammaPrint™	Agendia	Microarray	70	Prognostic Therapeutic guidance	van't Veer et al. [160] van de Vijver et al. [161]
MammoStrat®	Applied Genomics	IHC	5	Prognostic	Ring et al. [162]
Map*Quant* DX™	Ipsogen	Microarray	97	Tumor grade	Sotiriou et al. [163] Loi et al. [164]
Onco*type* DX™	Genomic Health	qRT-PCR	21	Prognostic Therapeutic guidance	Paik et al. [165] Paik et al. [166]
Rotterdam signature	Veridex	Microarray	76	Prognostic	Wang et al. [167]

#*qRT-PCR* quantitative real-time PCR, *IHC* immunohistochemistry

great promise for monitoring the effectiveness of neoadjuvant chemotherapy and predicting recurrence-free survival [156–158].

Individual estimates of outcome using clinical and pathological characteristics of breast tumors, including age, menopausal status, comorbid conditions, tumor size, number of positive lymph nodes, and ER status, have been incorporated into a computer program, Adjuvant! Online (www.adjuvantonline.com/index.jsp), which is available over the Internet as a decision aid for patients and their physicians [159]. The program estimates the efficacy of endocrine therapy and chemotherapy as well as overall and disease-free survival in a user-friendly format that effectively brings patients into the decision-making process regarding personalized treatments.

Although IHC analysis of ER, PR, and HER2 is widely used in the pathological evaluation of breast tumors, additional molecular signatures involving multiple genes and/or proteins are desperately needed to more accurately classify tumors and guide treatment selection. Recently, a multigene IHC-based test known as MammoStrat® (Applied Genomics, Huntsville, AL; www.applied-genomics.com/mammostrat.html) was developed to classify breast cancer patients into low-, moderate-, or high-risk categories for disease recurrence (Table 4.4) [162]. MammoStrat® uses conventional paraffin-embedded tissue to assay five markers by IHC:

- Tumor protein p53 (TP53)—known to play a central role in cell cycle regulation
- *Hpa*II tiny fragments locus 9C (HTF9C)—involved in DNA replication and cell cycle control
- Carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5)—aberrantly expressed in some cancers
- N-myc downstream-regulated gene 1 (NDRG1)—may function as a signaling protein in growth arrest and cellular differentiation
- Solute carrier family 7 (cationic amino acid transporter, y + system), member 5 (SLC7A5)—mediates amino acid transport

The MammosStrat® test may have utility for predicting patient outcomes but currently requires five separate slides (one slide per antibody), which has the potential to show variability in staining intensity and scoring between patients.

Gene Expression Signatures and Disease Risk

Molecular profiles are now being used more frequently as clinical tools to determine treatment for certain groups of patients by categorizing them into low-risk and high-risk groups. The MammaPrint™ assay (Agendia, Amsterdam,

The Netherlands; www.agendia.com) is a 70-gene signature developed using tumor tissue from young women (<55 years of age) with node-negative disease, who either developed distant metastasis or remained disease-free after 5 years [160]. Overall 10-year survival for the "poor-prognosis" signature is ~55 %, while 10-year survival in women with the "good-prognosis" signature is 95 %. The probability of being free from distant metastasis after 10 years is 51 % for the poor prognosis and 85 % for the good prognosis profile [161].

A second group of researchers subsequently developed a 76-gene profile (Rotterdam signature) that could identify breast cancer patients at high risk for distant recurrence. The signature could identify patients who developed distant metastases within 5 years when traditional prognostic factors were considered (hazard ratio 5.55, 95 % CI 2.46–12.5) and could predict metastasis in both premenopausal and postmenopausal patients [167].

The gene expression signatures outlined above were refined from global expression profiling experiments involving thousands of genes and flash-frozen tumor specimens. An alternative approach relied on an extensive literature search to identify candidate genes ($n = 250$) believed to be involved in disease development based on known function. Gene expression levels were assayed in 447 patients with ER-positive, node-negative breast cancer to identify a small subset of 16 genes (plus five reference genes) amenable to analysis by real-time PCR (RT-PCR) on RNA isolated from formalin-fixed, paraffin-embedded (FFPE) specimens. The resulting 21-gene signature, known as Onco*type* DX® (Genomic Health, Redwood, CA; www.genomichealth.com/), provides a probability of recurrence score for women with early-stage (stage I or II), ER-positive, node-negative breast cancer and categorizes patients as low, intermediate, or high risk.

In validation studies using patients from the National Surgical Adjuvant Breast and Bowel Project (NSABP) clinical trial B-14 who received tamoxifen, the probability of distant recurrence at 10 years for the three risk categories was low risk, 6.8 % (95 % CI 4.0–9.6); intermediate risk, 14.3 % (95 % CI 8.3–20.3); and high risk, 30.5 % (95 % CI 23.6–37.4). Recurrence scores also correlated significantly with relapse-free interval and overall survival [165]. In a subsequent study, Onco*type* DX™ was used to assess the benefit of adjuvant chemotherapy in ER-positive, node-negative patients. Because the highest benefit was observed in patients with high-risk scores, while women with low-risk recurrence scores did not benefit from chemotherapy [166], Onco*type* DX™ may be useful in guiding treatment options in ER-positive, node-negative patients.

Clinical trials of the MammaPrint™ and Onco*type* DX™ assays are currently in progress. In the Microarray In Node negative Disease may Avoid ChemoTherapy (MINDACT) trial, 6,000 node-negative women will be assigned to treatment groups based on risk stratification by traditional clinical–pathological factors (Adjuvant! Online) and the MammaPrint™ molecular signature [168]. Patients classified as low risk by both methods will not receive chemotherapy, while those considered high risk for relapse by both methods will be given the opportunity to receive adjuvant chemotherapy. Patients of primary interest, those with discordant results, will be randomized to treatment based on either Adjuvant! Online or MammaPrint™ to determine which test is more effective in defining treatment in node-negative patients.

The Trial Assigning Individualized Options for Treatment (TAILORx) is examining whether hormone receptor-positive patients with an intermediate Onco*type* DX™ risk recurrence score benefit from chemotherapy. The trial is recruiting 10,000 hormone receptor-positive patients with HER2-negative and lymph-node-negative disease. Treatment will be based on the risk recurrence score as follows: <10, hormone therapy alone; >26, hormone and chemotherapy; and intermediate scores, randomization to either hormone therapy alone or to hormone therapy and chemotherapy. The goal is to integrate Onco*type* DX™ into the clinical decision-making process and refine the utility of the assay in clinical practice [169].

Molecular signatures have improved the ability to predict outcome and identify breast cancer

Table 4.5 Selected genetic polymorphisms affecting response to therapy in breast cancer patients

Treatment	Gene	Variant	Functional change	Response to treatment	Study
Chemotherapy					
Doxorubicin	CBR3	11G>A	Decreased enzyme activity	Hematological toxicity	Fan et al. [174]
Anthracyclines	MnSOD	Ala[16]	Higher levels of reactive oxygen species	Decreased mortality	Ambrosone et al. [176]
	MPO	—463GG	Higher levels of reactive oxygen species	Decreased mortality	Ambrosone et al. [176]
	GSTP1	313A>G	Altered drug transport	Hematological toxicity	Zárate et al. [177]
	MTHFR	1298A>C	Altered drug metabolism	Non-hematological toxicity	Zárate et al. [177]
Endocrine therapy					
Tamoxifen	CYP2D6	*3, *4, *5, *10, *41	Reduced function/ nonfunctional enzyme	Poor clinical outcome	Schroth et al. [178] Goetz et al. [179]
Aromatase inhibitors	CYP19A1	Cys264, Thr364	Decreased enzyme activity	Reduced benefit	Ma et al. [180]
Radiotherapy					
	TP53	Arg72Pro, PIN3	Decreased apoptosis	Risk of telangiectasia	Chang-Claude et al. [181].
Targeted therapy					
Trastuzumab	HER2	Heterodimer	Prevents disruption by trastuzumab	Poor response to treatment	Lee-Hoeflich et al. [182]

*The different variant alleles of CYP2D6, in this case tamoxifen treatment, is related to variants 3, 4, 5, 10, and 41 of CYP2D6

patients who would most likely benefit from systemic therapy, thus providing an additional layer of personalized medicine. However, no current molecular signature is 100 % accurate, and 5–10 % of patients now classified as low risk are likely to relapse. Furthermore, current classification systems were developed to predict only short-term (<5 years) outcomes; thus, there is a need to develop signatures that identify patients with protracted disease progression who may benefit from prolonged therapy [170]. Although outcome prediction tends to be similar between gene expression signatures, overlap among genes comprising the signatures is relatively low, suggesting that these profiles assess common biological pathways but have not identified the actual genes driving tumor behavior and outcome [171].

Finally, some multigene predictor assays are being adopted and marketed before they have been properly validated and proven to be clinically informative; thus, the degree to which expression-based tests will alter the course of patient treatment remains unclear [172, 173].

Pharmacogenomics of Breast Cancer

Pharmacogenomics in breast cancer assesses the effect of inherited genomic variation on patient response or resistance to treatment. Genetic variability is commonly measured at the DNA level in the form of chromosomal alterations or DNA sequence variants (Table 4.5). Conversely, somatic genomic changes (DNA variants and gene expression profiles) in breast tumors can influence rates of apoptosis, cell proliferation, and DNA damage repair, which may have direct effects on response to treatment and survival.

To be most effective, personalized medicine must incorporate information from innate genetic variation as well as somatic mutations in diseased tissue [183].

Endocrine Therapy

Estrogens play an important role in the etiology of breast cancer by stimulating growth and proliferation of ductal epithelial cells in the breast; thus, the status of the estrogen receptor in breast carcinomas provided one of the earliest avenues for personalized medicine. Fortunately, hormone receptor-positive tumors usually are responsive to agents such as tamoxifen that block the function of estrogen. Tamoxifen is a potent antagonist of the ER with inhibitory effects on tumor growth that has become the gold standard for endocrine treatment of estrogen receptor-positive breast cancer in premenopausal and postmenopausal women [184]. Tamoxifen is associated with side effects such as blood clots, stroke, and increased risk of endometrial and uterine cancer, but 5-year use of tamoxifen has been shown to reduce risk of cancer recurrence by ~50 % [185]. For most patients, the benefit of using tamoxifen for hormone receptor-positive disease outweighs the risk of serious side effects; however, a small subgroup of hormone receptor-positive patients who carry specific variants in the cytochrome P450 2D6 (CYP2D6) gene do not benefit from tamoxifen. The CYP2D6 gene is a key enzyme in the metabolism of tamoxifen to its active metabolite endoxifen. Several DNA variants in CYP2D6 result in poor metabolism of tamoxifen and lower levels of endoxifen [186]. Patients who carry reduced-function or nonfunctional CYP2D6 alleles have been found to derive inferior therapeutic benefit from tamoxifen and thus are at increased risk of breast cancer recurrence [178] or have significantly shorter disease-free survival than noncarriers [179]. Studies are underway to determine the utility of CYP2D6 genotyping for making clinical decisions about tamoxifen and the potential to optimize breast cancer therapy [187, 188].

Alternate forms of directed antiestrogen therapies do exist for patients with hormone receptor-positive breast cancer, including aromatase inhibitors that block the production of estrogen and compounds such as fulvestrant (Faslodex®) that downregulate and degrade the ER protein. Aromatase inhibitors such as anastrozole (Arimidex®), letrozole (Femara®), and exemestane (Aromasin®) target cytochrome P450 19 (CYP19A1 or aromatase), an enzyme involved in estrogen synthesis in peripheral organs. Premenopausal women with functional ovaries do not receive aromatase inhibitor therapy because first- and second-generation aromatase inhibitors did not effectively suppress estrogen levels and because decreased estrogen levels in peripheral tissues could be counteracted by increased estrogen synthesis in the ovaries [189]. In postmenopausal women, aromatase inhibitors are well tolerated and improve both disease-free and recurrence-free survival [190–192]. Similar to CYP2D6, the Cys264 and Thr364 variants in aromatase are associated with decreased activity and lower levels of immunoreactive protein, which may contribute to variation among patients in response to aromatase inhibitor therapy [180]. Although directed endocrine therapies provide treatments specific for patients with hormone receptor-positive breast cancer, factors such as menopausal status and innate genetic variability may alter the effectiveness of treatment.

Treatment for HER2-Positive Breast Cancer

Therapies directed at the HER2 protein provide a second avenue of targeted treatment for some patients with breast cancer. Trastuzumab (Herceptin®, Genentech, South San Francisco, CA; www.gene.com/) is a humanized monoclonal antibody that binds to the extracellular domain of the HER2 protein, blocking tumor cell growth. Trastuzumab is the current standard of care in adjuvant therapy for HER2-positive breast cancer, effective as a single agent or in combination with chemotherapeutics for the 20–25 % of patients with HER2-positive cancer [193]. However, many patients with HER2-positive disease do not derive tangible benefit from trastuzumab. Given that the cost per patient for trastuzumab ranges from $20,000 to $80,000 per year with the potential for significant adverse

side effects [194], a more precise classification of HER2-positive patients who will derive benefit from trastuzumab and improved understanding of how amplification and/or overexpression of HER2 contribute to aggressive tumor biology are critical to improving patient treatment. The major oncogenic unit in HER2-positive breast cancer appears to be a heterodimer between the HER2 and epidermal growth factor receptor-3 (HER3) proteins, where HER3 functions as a necessary dimerization partner for HER2 to achieve full oncogenic signaling potential [195].

Recent studies have shown that HER2/HER3 heterodimers promote cellular proliferation in both in vitro and in vivo models, suggesting that HER3 may be an important therapeutic target in HER2-positive patients [182]. Pertuzumab has been shown to bind to the dimerization arm of HER2, blocking HER2/HER3 heterodimerization and attenuating growth of solid tumors in model systems [196]. Thus, combining pertuzumab with trastuzumab may augment therapeutic benefit by blocking HER2/HER3 signaling. Monogram Biosciences (South San Francisco, CA; www.monogrambio.com/) has developed the commercially available HERmark™ test to measure total HER2 levels and HER2 homodimers in FFPE tissue and is developing a *VeraTag*™ assay to quantify levels of HER2/HER3 heterodimers. These assays may allow patients with HER2-positive breast cancer to receive the most efficacious combination of new drugs targeting HER2.

Chemotherapeutics

Chemotherapy involves use of chemical agents as part of a systemic treatment targeting proliferative cancer cells. Adjuvant chemotherapy is used to reduce risk of recurrence after primary therapy in women with localized breast cancer and to provide palliative care in patients with advanced (metastatic) disease. In contrast, neoadjuvant chemotherapy is normally used to shrink moderate- to large-sized breast carcinomas prior to surgical resection, which permits use of less aggressive surgical options, including breast conservation, and may be useful in guiding longer-term treatment based on tumor response to specific drug combinations [197]. Obviously, the ability to predict which patients will benefit from adjuvant therapy and identify who will respond favorably to neoadjuvant regimens would provide an additional level of personalized care.

Gene Expression and Chemotherapeutic Agents

Gene expression profiling has been used to study the biological responses of human breast carcinomas to optimize chemotherapeutic treatments. Cell lines derived from luminal and basal epithelium have been observed to respond differently to agents commonly used in chemotherapy, such as doxorubicin (DOX) and 5-fluorouracil (5FU). In culture, luminal cell lines show low levels of expression for genes regulating cellular proliferation and the cell cycle, while basal cell lines tend to repress genes involved in cellular differentiation when exposed to DOX and 5FU [198]. Similarly, different molecular subtypes of breast cancer defined by gene expression profiling respond differently to preoperative chemotherapy, with basal-like and HER2-positive subtypes being more sensitive to paclitaxel and doxorubicin than luminal and normal-like cancers [199].

Expression signatures also have been used to predict clinical response of breast cancer patients receiving either cyclophosphamide–adriamycin or epirubicin–5FU as part of their adjuvant chemotherapy regimen [200] and to distinguish primary breast tumors that are responsive or resistant to docetaxel chemotherapy [201]. These observations further highlight the vast amount of molecular variability among breast carcinomas and emphasize the need for additional molecular signatures to more effectively guide treatment.

DNA Variation and Chemotherapeutic Agents

Clinical responses in breast cancer patients to commonly used chemotherapeutic agents vary considerably, from optimum therapeutic response to partial (beneficial) response to severe adverse events. Variation at the DNA level in an increasing number of genes is now known to affect the pharmacokinetics and pharmacodynamics of many chemotherapeutic drugs [202, 203], thus

influencing toxicity and patient response. To improve the safety and efficacy of current treatments, therapies could be tailored to individual patients based on their genetic makeup [204]. For example, the carbonyl reductase 3 (CBR3) gene contributes to the reduction of DOX to doxorubicinol, a less potent metabolite, and the extent of metabolism is believed to be a source of variability in doxorubicin chemotherapy. The 11G > A variant (rs8133052) in CBR3 has been shown to influence tumor tissue expression of CBR3 and is associated with interindividual variability in clinical outcomes. Women with the 11GG genotype experience greater leukocyte toxicity and are less likely to show a reduction in tumor size than women carrying 11AA [174].

A number of chemotherapeutics generate reactive oxygen species that function by damaging DNA and triggering the apoptotic cascade. Women carrying variants in genes associated with oxidative stress, such as manganese superoxide dismutase (MnSOD), catalase (CAT), and myeloperoxidase (MPO) that result in higher levels of reactive oxygen species, tend to have better overall survival than women with genotypes associated with lower levels of reactive oxygen species when treated with chemotherapy [176]. Due to the large number of drug-metabolizing enzymes and drug transporters containing polymorphisms that affect chemotherapy-related toxicity and treatment outcomes in breast cancer patients, improved pharmacogenetic information is needed to identify individuals at risk for toxicity and poor response.

Genomics in Clinical Practice

Recent developments in the clinical arena are indicative of the emerging importance of personal genomics in the prevention, surveillance, and treatment of breast cancer. Professional organizations such as the American Society of Clinical Oncology (ASCO) have issued recommendations on the use of molecular markers for guiding therapy and determining prognosis in breast cancer patients [175].

- CA 15-3 and CA 27.29 (assays to detect circulating MUC-1 antigen in peripheral blood)—contribute to decisions regarding therapy for metastatic breast cancer in conjunction with diagnostic imaging, history, and physical examination
- Carcinoembryonic antigen (CEA)—contributes to decisions regarding therapy for metastatic breast cancer in conjunction with diagnostic imaging, history, and physical examination
- ER/PR—should be measured on every primary invasive breast cancer to identify patients most likely to benefit from endocrine therapy
- HER2—should be measured on every primary invasive breast cancer at diagnosis or recurrence to guide trastuzumab therapy
- Urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1)—measured by ELISA on fresh or frozen tissue for determining prognosis in newly diagnosed, node-negative breast cancer patients
- Onco*type* DX®—in newly diagnosed patients with node-negative, ER-positive breast cancer, can be used to predict risk of recurrence in women treated with tamoxifen

Large cancer centers such as Massachusetts General Hospital and Memorial Sloan-Kettering Cancer Center are now embracing the importance of genomics in clinical practice, recently implementing policies to routinely assay a number of breast cancer-related genes: vakt murine thymoma viral oncogene homolog 1 (AKT1) and HER2 at Memorial Sloan-Kettering, phosphatase and tensin homolog (PTEN) and TP53 at Mass General, and phosphatidylinositol 3-kinase, catalytic, alpha (PIK3CA) at both institutions [205]. As genomic medicine becomes an integrated part of health-care delivery, use of personalized genomics in the clinical treatment of breast cancer will increase.

Genomic Studies of Breast Cancer Initiation, Progression, and Metastasis

Breast cancers progress through multiple genomic and epigenomic steps. However, the evolutionary process is unlikely to be the result of

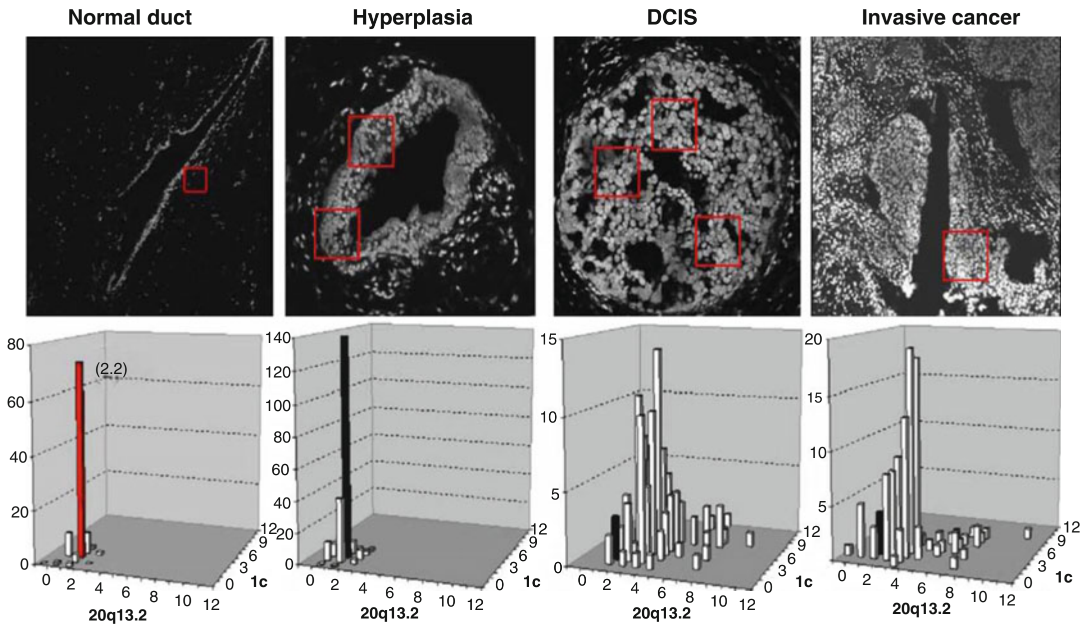

Fig. 4.1 Genome instability measured during breast tumor progression using FISH [206]. Histological sections depicting stages of evolution are illustrated above. Bivariate measures of genome copy number at the centromere of chromosome 1 and at chromosome 20q13. The onset of instability at the DCIS stage of evolution is apparent (Reprinted from Korkola and Gray [211]. With permission from Elsevier)

a linear and more or less constant rate of successive genomic and epigenomic aberration accretion. It seems more likely that cancer progression varies between individuals and over time within an individual. The breast progenitor cell in which the tumor arises likely determines the spectrum of aberrations needed to enable progression. Evidence for this comes from studies showing that the spectrum of genomic aberrations accumulated is strongly influenced by the normal progenitor cell type in which the cancer arises [108, 206]. In general, the events associated with early aspects of breast tumor appear to be epigenomic in nature, wherein stepwise DNA methylation changes enable escape of telomerase-negative epithelial cells from proliferation barriers [207]. This leads to proliferation in the absence of telomerase and culminates in a period of high genome instability owing to checkpoint deregulation [208, 209] and/or entry into telomere crisis when telomeres become critically short [206, 210]. This barrier is highly effective but not perfectly so. As a consequence, most cells become genomically unstable and die. The increase in genome instability during breast tumor progression is illustrated in Fig. 4.1. Rarely, a single cell accumulates genomic or epigenomic alterations that reactivate telomerase and confer a proliferative advantage. This cell might be considered the tumor initiation cell and will have multiple characteristics that appear "stem cell like." The extent to which this is related to normal stem cells remains unclear. However, it is likely that the genomic characteristics of this cell—both transcriptional and genomic—will be reflected in subsequent progeny. This may explain why tumor genomes appear to evolve relatively slowly after telomere crisis and why metastases that develop years after immortalization usually retain the genomic characteristics of the primary tumor from which they were derived [212].

That said, not all breast tumors progress this way. A recent analysis of cancer progression termed Sector-Ploidy-Profiling (SPP) demonstrates that breast tumor evolution may evolve as a single major clonal subpopulation or as multiple clonal subpopulations [213]. The latter model

may explain why a small percentage of metastatic cancers do not resemble the primary tumor from which they were derived. Figure 4.1 also shows that the cells that survive telomere crisis remain genomically unstable. Thus, tumors are likely to consist of a large number of cells that are not faithful genomic representations of the tumor-initiating cell and are likely to be biologically compromised. These cells are likely to be much more sensitive to treatment than the tumor-initiating cells from which they were derived. This may partially explain why breast tumors initially respond well to treatment but fail to exhibit a durable response.

Various Genomic Signatures

Unsupervised molecular classification identified three major and robust groups of breast cancers that differ in the expression of several hundred to a few thousand genes. These include basal-like breast cancers, which are negative for ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2); low histological grade ER-positive breast cancers (also called luminal A); and high-grade, highly proliferative ER-positive cancers (luminal B). Several smaller and less stable molecular subsets (such as normal-like, HER-2-positive, and claudin-low) have also been proposed but are less consistently seen and are distinguished by substantially smaller molecular differences [95, 104]. Importantly, among the various molecular subsets, one group, the luminal A class that includes low-grade ER-positive cancers, stands out with a very favorable prognosis with or without adjuvant endocrine therapy. The other groups have worse but rather similar prognosis [95, 102].

If one understands these close associations between clinical phenotype, molecular class, and prognosis, it is no longer surprising that comparing gene expression profiles of breast cancers that recurred (mostly the ER-negative and the high-grade, ER-positive cancers) and those that did not (low-grade, ER-positive cancers) in the absence of any systemic therapy (or after antiestrogen therapy alone in the case of ER-positive cancers) yields a very large number of differentially expressed genes. The relative position of individual genes in a rank-ordered gene list varies greatly, but the consistency of the gene list membership is fairly high across various datasets [214].

Functional annotation indicates that the majority of these prognostic genes are proliferation-related genes, and the remainder are mostly ER-associated and, to a lesser extent, immune-related genes [215–217]. Because these genes function together in a coordinated manner in the regulation and execution of complex biological processes, such as cell proliferation, or originate from a particular cell type, such as immune cell infiltrate, many of these prognostic genes are also highly coexpressed with one another. It is therefore expected that a large number of nominally different prognostic signatures can be constructed that will all perform equally well. For example, a particular gene may be highly significantly discriminating in two datasets but it is ranked 5th among the most discriminating genes in one dataset (based on *P*-value or fold difference) but only 35th in another dataset (which is still very high, considering the thousands of comparisons!).

In multivariate prediction model building, the top few informative features are usually combined, and genes are added incrementally to increase the predictive performance. However, because many of the genes are highly correlated with each other, adding genes lower on the list yields less and less improvement in the model as a result of lack of independence. Therefore, the gene in question will be included in a predictor developed from the first dataset (because it is ranked as 5th) and will work well on validation in the second dataset; but if a new predictor were to be developed from the second dataset, this gene may not be included in the predictor (because it is ranked 35th).

These three features of the breast cancer prognostic gene space—the large number of individually prognostic features, the unstable rankings, and the highly correlated expression of informative genes—explain why it is easy to construct many different prognostic predictors that perform equally well even if they rely on nominally different genes in the model. However, this does not

mean that all published prognostic gene signatures are equally ready for clinical use. Before adoption in the clinic, a molecular diagnostic assay has to be standardized, the reproducibility within and between laboratories and stability of results over time have to be demonstrated, and its predictive accuracy has to be validated in the right clinical context, preferably in multiple independent cohorts of patients. Most importantly, clinical utility implies that the assay improves clinical decision making and complements or replaces older standard methods, which in turn leads to better patient outcomes. Few published prognostic predictors have met these criteria [175, 218].

The predictive performance of a multivariate model largely depends on the number of independent informative genes included in the model, the magnitude of differential expression of the informative genes, and the complexity of the background. Different clinical prediction problems show different degrees of difficulty. From the discussion above, it should be apparent that prediction of ER status, histological grade of breast cancer, or better or worse prognosis associated with these clinical phenotypes should be relatively easy when considering all breast cancers together and that such prediction can therefore yield predictors with good overall accuracy. Indeed, prognostic gene signatures developed for breast cancer in general or for ER-positive cancers tend to have good performance characteristics [161, 165, 167, 217]. However, the first-generation prognostic signatures share some limitations. Because these were invariably developed by analyzing all subtypes of breast cancers together, they tend to assign high risk category to almost all ER-negative cancers (which are almost always high grade), even though a substantial majority of these cancers have good prognosis [219, 220]. Similarly, the good- and poor-prognosis ER-positive cancers, as assigned by gene profiling, tend to correspond to the clinically low-grade/low-proliferation versus high-grade/high-proliferation subsets, respectively. This strong correlation between prognostic risk as predicted by gene signatures and routine clinical variables, such as histological grade, proliferation rate, and ER status, limits the practical value of these tests.

Efforts are under way to develop simple multivariate prognostic models that use routine pathological variables (such as ER, histologic grade, and HER2 status), and these could eventually rival the performance of the first-generation prognostic gene signatures [221, 222]. However, standardization of the pathological assessment of breast cancer and reducing the interobserver variability remain an important challenge. Predicting clinical outcome, such as prognosis or response to chemotherapy, within clinically and molecularly more homogeneous subsets (such as triple-negative breast cancers or high-grade, ER-positive cancers) would be highly desirable. Unfortunately, these prediction problems seem to be more difficult [223, 224]. It seems that fewer genes are associated with outcome in homogeneous disease subsets and the magnitude of association is modest when currently available datasets are analyzed. This leads to predictors that are specific for a particular dataset from which they were developed. These prediction models are fitted to the dataset and rely on features that have no or limited generalizability. This means that they fail to validate when applied to independent data or may demonstrate only nominally significant predictive value (i.e., they may predict outcome slightly better than chance). Also, the discriminating value may not be substantial enough to be clinically useful [225, 226]. For example, if the good-prognosis group has a recurrence rate of 30 % compared with 50 % in the poor-risk group, these may be significantly different, but the risk of recurrence in the good-risk group is still too high to safely forego adjuvant chemotherapy.

Genomic Mechanism of Breast Cancer Dormancy and Recurrence

Factors that determine the length of the dormancy period remain unclear [227]. Current data have led to various experimental models that address the phenomenon of tumor dormancy. It appears that dormant cancer cells can persist either by completely withdrawing from the cell

cycle (mitotic arrest) or by continuing to proliferate at a slow rate that is counterbalanced by cell death [228, 229]. These two types of dormancy are not mutually exclusive; both forms of latency could coexist in the entire population of disseminated tumor cells (DTC) of a particular cancer patient.

Single-Cell dormancy

In the single-cell dormancy model, isolated tumor cells detached from the primary tumor arrive at the future metastatic organ and enter a prolonged state of mitotic arrest. This model of arrested apoptosis contrasts with the micrometastatic dormancy model, in which proliferation in micrometastatic foci is counterbalanced by cell death. Speculations about the metabolic status of minimal residual disease (MRD) during dormancy are not yet sufficiently investigated. Cell cycle regulation mechanisms are highly complex, with an assortment of stimuli interacting at numerous cell cycle checkpoints to determine the proliferative status. The presence of tumor cell dormancy due to a growth arrest of cancer cells is supported by evidence that within tissues where primary tumors are developing or tissues that harbor disseminated cells and have a functional vasculature, tumor cells are found to be in a non-proliferative mode [230–232]. In numerous studies, dormant cancer cells have been demonstrated to be in a G0–1 arrest; this was linked to negative staining for proliferation markers (e.g., Ki67, PCNA) [233, 234]. Dormant tumor cells may gain a survival advantage by blocking the receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which would result in arrested apoptosis. Two exemplary mechanisms of TRAIL-receptor blocking have been described and may be of relevance to dormant tumor cells. TRAIL receptors in cancer cells can be blocked by osteoprotegerin, an important member of the tumor necrosis factor receptor superfamily [235, 236]. Interestingly, bone marrow stromal cells from breast cancer patients secrete enough osteoprotegerin to inhibit apoptosis in vitro [237]. More recently, c-Src (a tyrosine-specific kinase involved in breast cancer progression) was demonstrated to support cancer cell survival in the bone marrow microenvironment by conferring resistance to TRAIL [238].

Micrometastatic Dormancy

In contrast to dormancy due to mitotic arrest, dormancy of a micrometastasis seems to be caused by a balance of cell proliferation and apoptosis, such that the tumor does not increase in size. This constant balance is regulated by proangiogenic proteins and angiogenic inhibitors produced by tumor and stromal cells, as well as immunologic, hormonal, or other microenvironmental switches [239]. According to Naumov et al., a failure to activate the angiogenic switch can maintain a group of cancer cells in a dormant state [240]. Indraccolo et al. reported that a short-term perturbation in the tumor microenvironment, in the form of a transient angiogenic burst, could suffice to interrupt tumor dormancy [239]. Genetic data support the interpretation that minimal residual cancer might be divided into “active” and “dormant” groups, in which an advantageous mutation is acquired shortly before a highly aggressive metastatic clone appears [241]. At present, there is no definite answer to the question of which model best represents tumor dormancy in breast cancer. Hussein and Komarova hypothesized that indolent breast cancers might fit into the single-cell dormancy model, while more aggressive diseases are linked to the micrometastatic dormancy model [229]. Indeed, in a series of experiments, Barkan et al. demonstrated that more aggressive basal-type cell lines, such as MDA-MB-231, proliferated readily, while estrogen receptor (ER)-positive MCF7 remained in a state of mitotic arrest, potentially linking the dormancy type associated with arrested growth with the less aggressive disease phenotype [242].

Genes driving recurrence may be encoded in the gene expression profile of primary tumors. Transcriptomic analysis of a variety of primary tumors by Ramaswamy and colleagues identified a 17-gene signature present in a wide range of human primary tumors that predicted metastasis and poor clinical outcome [243]. Although the genomes of clinically similar breast cancers are remarkably different, some regions of the genome are recurrently aberrant. The number of genes

mapping to regions of significant abnormality is remarkable. In fact, correlative analyses of genome copy number and gene expression indicate that 10–15 % of the entire genome is deregulated by recurrent genome copy number abnormalities [108, 109]. Analysis of CGH data suggests the existence of at least three general classes—those with "simple" genomes displaying relatively few genome aberrations, those with "complex" genomes displaying many aberrations, and those displaying high-level amplification [109, 244]. It is likely that these subtypes may be caused by differences in DNA repair defects. For example, complex genomes typically carry p53 mutations while simple genomes do not.

CGH profiles for tumors from BRCA1 carriers also display high genome complexity, although the spectrum of recurrent abnormalities differs between sporadic and heritable breast cancers. In particular, tumors from BRCA1 mutation carriers typically display recurrent aberrations at chromosomes 3 and 5 that sporadic tumors do not have [245]. Relatively few recurrent mutations have been found in breast cancers. High-frequency somatic mutations (i.e., present in >3 % of reported cases) include PIK3CA, TP53, CDH1, CDKN2A, and AKT1 (http://www.sanger.ac.uk/genetics/CGP/cosmic). However, almost 200 others have been reported as recurrently mutated but at lower frequency [63]. Prevalent germ line mutations that contribute to breast cancer genesis involve BRCA1, BRCA2, CHEK1, and TP53. The mechanisms by which low-level copy number abnormalities contribute to cancer pathophysiology have not yet been determined definitively. However, we have suggested that these deregulate transcription of large numbers of genes that collectively increase metabolic activity [109]. The contributions of regions of amplification at 8p11–12, 8q24, 11q13, 12q13, 17q11–12, 17q21–24, and 20q13 as somewhat better understood since each encodes a known oncogene (e.g., FGFR1, MYC, CCND1, MDM2, ERBB2, PS6K, and ZNF217, respectively).

However, substantial evidence is now emerging that demonstrates that multiple genes in each region of amplification contribute to the pathophysiology of the disease. Functionally important coamplified genes demonstrated so far include LSM1, BAG4, and C8orf4 at 8p11–12 [246]; MYC and PVT1 at 8q24 [247]; CCND1 and EMSY at 11q13 [248]; and ERBB2 and GRB7 at 17q21 [249]. Recent studies also suggest that amplifications of regions that are well separated in the normal genome are not independent events. For example, coamplification of regions at 8p11–8p12 and 11q12–11q14 [250] or 8q24 and 17q21 [251] have been reported to contribute collectively to breast cancer pathophysiology. These interactions may be just the tip of the iceberg since dozens of genes have been identified as overexpressed as a result of recurrent, high-level amplification and most have not been functionally assessed [109]. Recurrent mutations, although relatively infrequent in breast cancer, provide particularly strong evidence of functional importance. Bringing all of these observations together, recent integrative analyses of homozygous deletions, high-level amplification, and recurrent mutations suggest that genomic deregulation of pathways involving ERBB2, EGFR, and PI3K signaling and DNA topology is particularly important in breast cancer genesis and progression [252].

Epigenomic alterations also contribute to breast cancer pathophysiology [253, 254]. Targeted and genome-wide analyses of promoter region methylation in breast cancer have shown that genes with known or suspected tumor suppressor function including BRCA1, CCND2, CDKN2A, RARβ, and RASSF1A [255, 256] and members of the WNT signaling pathway [257] are recurrently downregulated by hypermethylation. Consistent with this, genes found to be recurrently mutated in large-scale genome analysis studies are frequently targets of hypermethylation, and hypermethylation is usually mutually exclusive from genetic changes [258]. At the chromatin level, modification via acetylation and/or phosphorylation directly modifies estrogen receptor alpha and other steroid hormone receptors superfamily in breast cancer, thereby modifying ligand sensitivity and hormone antagonist responses [259]. Chromatin remodeling also has been implicated in growth and metastasis. In particular, SATB1 has been

reported to be a genome organizer that tethers multiple genomic loci and recruits chromatin-remodeling enzymes to regulate chromatin structure and gene expression. Overexpression of this "master chromatin regulator" appears to drive breast cancer progression by upregulating metastasis-associated genes and downregulating tumor-suppressor genes [260].

Various Genomic Approaches and Their Outcome in Breast Cancer Research

To facilitate understanding of the scope of epigenetic modifications that occur in normal and cancer cells, a range of gene-specific or genome-wide technological approaches have been developed. We present an overview of recent technological developments and discuss the merits and the limitations of these approaches with respect to studies on cancer cells.

DNA Methylation

DNA methylation is the first identified epigenetic mechanism of gene regulation. The initial methods of detection of methylation were restricted to the quantitation of total 5-methylcytosine content by high-performance liquid chromatography (HPLC) or high-performance capillary electrophoresis (HPCE) [261] and the study of DNA methylation of selected sequences using restriction enzymes that can distinguish between methylated and unmethylated recognition sites in genes of interest [262]. For restriction-enzyme-based methods, incomplete restriction-enzyme digestion and the limitation of enzyme-recognition sites restricted their extensive application. These technical limitations caused delay in advances in the cancer epigenetic field in contrast to the rapid development in cancer genetics field. The development of a bisulfite-conversion technique that reproducibly changes unmethylated cytosines to uracil but leaves methylated cytosines unchanged [263] was a key development that drastically sped up progress in the field.

Several sensitive DNA methylation detection techniques (summarized in Table 4.6) were developed upon the basis of bisulfite conversion, including bisulfite sequencing, methylation-specific PCR (MSP), combined bisulfate restriction analysis (COBRA), and so on [263–265]. Among these technologies, MSP is the most popular and powerful method for DNA methylation detection, which needs limited amounts of DNA material [264]. Since MSP is a gel-based assay, it cannot provide quantitative information and is subjective. Several real-time methylation-specific PCR methods, such as bisulfite treatment in combination with MethyLight™ [266], quantitative multiplex MSP (QM-MSP) [267, 268], or pyrosequencing [269], have been developed and used in DNA methylation studies, which have improved features to detect minimal amounts of aberrant DNA methylation in a quantitative manner.

In addition to the gene-locus specific DNA methylation detection methods mentioned above, several recently developed genome-wide techniques have been applied to the study of global DNA methylation profiles in normal and cancer cells (summarized in Table 4.6). Restriction landmark genomic scanning (RLGS) is one of the earliest methods to be applied to genome-wide methylation analysis [270]. RLGS has an ability to globally analyze the methylation status of approximately 1,000 unselected CpG islands. Other important techniques for analyzing altered DNA methylation patterns across the genome have relied on an arbitrarily primed PCR technique (e.g., AIMS) [271, 285]. Further advances in this field are derived from the application of the DNA microarray technology. A widely used example is differential methylation hybridization (DMH) that uses CpG island and promoter sequence microarrays, which enables the simultaneous analysis of the methylation levels of a large number of CpG islands in the genome [272].

A recently developed, related technique called HpaII tiny fragment enrichment by ligation-mediated PCR assay (HELP) uses a modified approach to globally analyze DNA methylation patterns [273]. Methods (e.g., methylated

Table 4.6 Technologies for assessing and profiling DNA methylation and histone modifications

Technology	Description	Study
Gene-specific detection of DNA methylation		
Bisulfite sequencing	Bisulfite-converted DNA is PCR-amplified to enrich the target templates. The purified DNA templates are subjected to sequencing analysis directly or after cloning into plasmid	Fraga and Esteller [261]
Methylation-specific PCR (MSP)	This technique takes advantage of the altered sequence of bisulfite-converted unmethyated and methylated DNA for designing primers, which can amplify DNA in a methylation-state-specific manner	Herman et al. [264]
Combined bisulfite restriction analysis (COBRA)	The qualitative and quantitative detection of methylated alleles is achieved by restriction enzyme-mediated digestion of PCR amplified target amplicons from bisulfite-modified DNA	Xiong and Laird [265]
MethyLight™	By including the fluorescent probe technology (TaqMan®), this method is able to quantitatively and sensitively detect methylated alleles	Eads et al. [266]
Quantitative multiplex MSP (QM-MSP)	This technology is a modified version of fluorogenic probe-based quantitative MSP assay. This method includes the multiplex PCR step that allows amplification of multiple target alleles. The diluted multiple PCR products are subjected to quantitative MSP assay for multiple gene detection	Fackler et al. [267] Swift-Scanlan et al. [268]
Pyrosequencing	This method is based on sequencing-by-synthesis technology to quantitative detect methylation levels of individual CpG site by monitoring the real-time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a bioluminometric signal	Tost and Gut [269]
Genome-wide profiling of DNA methylation		
Restriction landmark genomic scanning (RLGS)	In the RLGS technique, restriction-enzyme digestion, radioactive labeling, and two-dimensional electrophoresis combine to quantitatively display DNA methylation levels of thousands of CpG islands	Costello et al. [270]
Amplification of intermethylated sites (AIMS)	The AIMS method combines methylation-sensitive restriction enzyme digestion with the display of methylation fingerprint of PCR-amplified DNA fragments, which can then be isolated and characterized individually by sequencing	Frigola et al. [271]

(continued)

Table 4.6 (continued)

Technology	Description	Study
Differential methylation hybridization (DMH)	DMH is a promoter-sequence-microarray- basis method that combines methylation-sensitive restriction-enzyme digestion with linker-basis PCR labeling to serve as probes for array hybridization, capable of globally displaying methylated CpG islands	Huang et al. [272]
HpaII tiny fragment enrichment by ligation-mediated PCR assay (HELP)	The HELP method adopts differential digestion with a methylation-sensitive restriction enzyme or its methylation-insensitive isoschizomer and ligation mediate PCR amplification of digested templates for cohybridization to a genomic microarray, enabling the display of genome-wide methylated CpG islands	Khulan et al. [273]
Methylated DNA immunoprecipitation (methyl-DIP)	The methyl-DIP technique uses antibodies against methyl- CpG-binding domain proteins (MBDs) to immunoprecipitate sheared genomic DNA for isolation of methylated DNA fragments. Methyl-DIP has been combined with tiling microarrays or with high-density promoter arrays to map the human methylome	Keshet et al. [274] Weber et al. [275] Weber et al. [276]
Microarray-based gene expression profiling	Gene expression microarray has been applied to display expression-profile changes in cells treated with epigenetic inhibitors for identification of methylation-targeted genes	Suzuki et al. [277] Yamashita et al. [278] Hoque et al. [279]
Methylation-specific digital karyotyping (MSDK)	The MSDK method is a modified serial analysis of gene expression (SAGE) assay that combines a methylation-sensitive restriction enzyme and a fragmenting restriction enzyme to generate short sequence tags for providing information on gene loci and their methylation levels	Hu et al. [280]
Genome-wide profiling of histone modifications		
Chromatin immunoprecipitation (ChIP)-on-chip	This method combines ChIP technology with high-density microarrays for measuring and mapping histone-binding genomic loci	Bernstein et al. [281]
ChIP-SAGE	This method adopts a combination of ChIP and SAGE technologies to globally quantify and map genomic binding sites for specifically modified histones	Roh et al. [282]
ChIP-Seq	This new method employs a high-throughput sequencing technique to analyze ChIP DNA for genome-wide mapping of histone-DNA binding patterns	Barski et al. [283] Mikkelsen et al. [284]

DNA immunoprecipitation [methyl-DIP]) based on chromatin immunoprecipitation (ChIP) using the ChIP-on-chip technology are other seminal recent advances in the epigenomic profiling of cancer cells [274–276]. Finally, gene expression profiling using microarrays is another powerful and widely used technique for assessing genome-wide DNA methylation patterns. This approach is used to compare gene expression levels from cancer cell lines before and after treatment with a demethylating drug, an HDAC inhibitor, or a combination of both drugs [277–279]. As with all microarray-based technologies, the identified candidate genes are further verified, in this case, by quantitative RT-PCR and promoter methylation analyses.

In addition to microarray-based techniques, a serial analysis of gene expression (SAGE)-technology-based method, known as methylation-specific digital karyotyping (MSDK), has been developed recently for the genome-wide analysis of methylation profiles [280]. The advantage of this new technique is that there is no need of prior sequence information for analysis and the obtained data can be used to map the methylated gene loci and to determine their methylation levels.

Histone Modifications

Characterization of posttranslational histone modifications is a greater challenge than analysis of DNA methylation and needs special technology. Currently, the gold standard for accurately assessing global levels of histone modifications is mass spectrometry [286]. Since antibodies specifically recognizing the amino acid modifications of histone proteins are available, a simple Western blot analysis is also used for detecting histone modifications. In addition to determining the types and relative levels of histone modification in cells, characterization of distribution of each type of histone modification on chromosomes also provides very important information.

The current techniques for genome-wide analysis all adopt the ChIP technology with antibodies against specifically modified histones (summarized in Table 4.6). The first developed and widely used technique is ChIP-on-chip [281]. In contrast to the ChIP-on-chip method, another new technique for profiling histone modifications at a genomic scale is ChIP-SAGE, which combines ChIP experiments with the SAGE technology [282]. The merit of ChIP-SAGE is that unlike ChIP-on-chip that requires sequence information of preselected genomic regions to manufacture genomic microarrays, no prior genomic sequence information is required in this assay. However, the cost for ChIP-SAGE is higher than ChIP-on-chip due to the use of the more expensive traditional sequencing methods. Besides, the fact that not every region of chromosomes contains restriction-enzyme recognition sites used to cleave the ChIP DNA limits the capacity of ChIP-SAGE to study the entire genome.

More recently, a new technique, chromatin immunoprecipitation combined with high-throughput sequencing techniques (ChIP-Seq), has been developed for analyzing ChIP DNA using a high-throughput massively parallel signature sequencing-like technique developed by Solexa [283, 284]. This technique is more powerful and cost-effective than the ChIP-SAGE technique. There are several advantages in this new technique, including the use of less PCR amplification of ChIP DNA after ligation to adaptors and a highly efficient sequencing procedure (sequencing by synthesis). In contrast to ChIP-on-chip, more quantitative data of histone modification levels at different chromosomal regions can be obtained in ChIP-Seq experiments. In addition to mapping the genome-wide histone-DNA binding patterns, it can be envisioned that this new technique has great potential for globally defining the methylome of a particular cell type. With rapid and striking technological advancements, global analysis of DNA methylation and histone modification mapping on chromosomes has become eminently practical.

Treatment of Breast Cancer Through a Genomic Approach

Treatment is complicated by the complexity of cancer genomes and the large and growing numbers of candidate anticancer agents that should be

considered for use in the treatment. In fact, a 2009 survey by the pharmaceutical industry indicates that more than 800 experimental therapies are now being evaluated clinically, including 106 in breast cancer (http://www.pharma.org/files/09-046PhRMACancer09_0331.pdf). Identification of genomic and epigenomic features that predict response to these agents is challenging in the context of clinical trials because of the difficulty of obtaining tumor samples during trials [211].

Schneider et al. discuss triple-negative breast cancer, a distinct molecular subtype of breast cancer that has emerged from DNA microarray gene expression studies [287]. Human mammary glands contain two distinct subtypes of epithelial cells, basal (myoepithelial) and luminal, which can be easily distinguished by the pattern of expression of certain cytokeratins. Basal cells lie closest to the basement membrane and stain positive for cytokeratin 5/6. Luminal cells compose the upper, more differentiated, layer of the mammary gland epithelium and express cytokeratin 8/18. The cytokeratin pattern is largely conserved after transformation of epithelial cells, allowing determination of the cell-type origin of primary cancers. Most breast cancers originate from the luminal epithelium and express luminal cell-specific cytokeratins. It is estimated, however, that 3–15 % of all breast cancers seem to originate from basal-like epithelium and express basal-specific cytokeratins as discussed by Schneider et al. [287]. Perou and colleagues, using complementary DNA microarrays, first proposed a molecular classification of breast cancers based on variations in global gene expression patterns [95, 97, 100].

Following these original reports, several other groups have confirmed that individual cancers could be categorized, based on their gene signature, to at least five distinct subtypes: luminal A, luminal B, normal-like, HER2-like, and basal-like. Normal-like tumors resemble normal breast tissue; HER2-like are characterized by HER2 overexpression; luminal A and B are estrogen receptor-positive; and basal-like are triple negative (estrogen receptor-negative, progesterone receptor-negative, and HER2-negative). BRCA1-associated breast cancers cluster within the basal-like subtype [288], which suggests that host germ line genetics may determine the tumor subtype. These subtypes have been shown to correlate with clinical outcomes [97, 103, 171]. The luminal A and B subtypes have the best prognosis, whereas HER2-like and basal-like have the worst prognosis, although the inclusion of trastuzumab in primary therapy for HER2-positive breast cancer has led to vast improvements in outcomes for patients with HER2-positive breast tumors. Immunohistochemical markers have been used to define these subtypes with similar prognostic value [289, 290], which allows for breast cancer subtype assignment in epidemiologic studies and clinical practice.

The distribution of breast cancer subtypes has been reported to vary across populations [291–294]. Estrogen receptor-positive, luminal A breast cancers were predominant in Asian, white, and postmenopausal African American populations, about 40 % in premenopausal African Americans, and only 27 % in indigenous Africans. In contrast, the proportion of the estrogen receptor-negative, basal-like subtype was 27 % in indigenous Africans and premenopausal African Americans, about 15 % in postmenopausal African Americans and premenopausal European Americans, and only about 10 % in other populations. There is also a clear gradient in the proportion of estrogen receptor-negative unclassified breast tumors across populations, with Africans having the highest proportion. Interestingly, the proportion of HER2-positive tumors (luminal B and HER2-positive/estrogen receptor-negative subtypes combined) was about 15 % in all populations except the Japanese. As discussed by Garcia-Closas and Chanock [13], genomic risk factors for these subtypes are also likely to vary across populations, which suggest that an integrative epidemiology approach [295] must be applied to drug development at both the population and individual levels. Breast cancer clinical trials must be conducted in populations in which the drugs are to be used, as one can no longer assume that drugs that are developed in predominantly European populations will produce similar outcomes in populations of Asian or African ancestry.

Genomic Therapeutic Targets and Biomarkers

Given the heterogeneity of expression and genomic composition within the distinct subclasses, it might also be expected that there will be distinct clinical responses to chemotherapeutic and targeted agents within the different subclasses. If agents can be identified that show enhanced efficacy against specific subclasses, this might represent the first step toward more personalized medicine for the treatment of breast cancer. Such class distinctions have already been identified, in particular for HER2-positive tumors. Targeted therapeutic agents, such as the monoclonal antibody trastuzumab or the small-molecule tyrosine kinase inhibitor lapatinib, have both been approved for use in treating patients with HER2-positive breast cancer, leading to improved survival in these patients [154, 296, 297]. Given that HER2-directed therapies were developed before the identification of breast cancer subclasses, it is now of interest to see if additional subclass-specific agents can be identified.

Recent studies have demonstrated that inhibitors of poly (ADP-ribose) polymerase (PARP), an important transducer of BRCA-mediated DNA damage response, selectively target BRCA1 and BRCA2 mutant breast cancer [298]. Numerous recurrently aberrant genes associated with reduced survival duration and other aspects of breast cancer pathophysiology have been suggested as therapeutic targets in breast cancer. Amplified genes implicated as therapeutic targets include ERBB2 [299], TOP2A [300], CCND1 and EMS1 [301], MYC [302], ZNF217 [303], RAB25 [304], MDM2 [305], TBX2 [306] RPS6KB1, and the microRNA mir-21 [307].

More recently, correlative analyses of gene expression and high-level amplification have identified 66 genes in regions of amplification that are associated with reduced survival duration that are candidate therapeutic targets, nine of which (FGFR1, IKBKB, ERBB2, PROCC, ADAM9, FNTA, ACACA, PNMT, and NR1D1) were predicted to be drug able [109]. Recurrent mutations or deletions in breast cancers include TP53 [308], PIK3CA [309], PTEN [310], BRCA1 [311], and BRCA2 [312]. Recurrent genomic aberrations in breast cancer are attractive targets because they are events for which strong evidence indicates positive selection, so the tumors may be addicted to the aberration. In addition, aberrations are not present in normal tissues so that therapies against them are likely to be relatively nontoxic. ERBB2 is the prototypic genome-based therapeutic target [313, 314]. This receptor tyrosine kinase is highly amplified in about 30 % of human breast cancers, and the antibody, trastuzumab [315], and the small-molecule inhibitor, lapatinib [316], have proved to be clinically effective against tumors in which ERBB2 is amplified.

One of the advantages of aberration-targeted therapies is that markers can be readily developed to identify tumors carrying the aberration that are most likely to respond to therapy. In the case of ERBB2-targeted therapies, FISH with probes to ERBB2 readily identify the tumor to be treated [317]. Following this lead, therapies directed against tumors with aberrations involving TP53 [318], MDM2 [319], TOP2A [320], PI3-kinase mutations (PTEN and PIK3CA) [321], and BRCA1 [322] are now being developed or tested. Clearly, the development of aberration-targeted therapy is only beginning.

Biomarker discovery for breast cancer is still very much in its early phase. Multiple approaches have been developed that hold promise for the identification of serum biomarkers [323]. A biomarker can be a substance that is introduced into an organism as a means to examine organ function or other aspects of health. Dowsett and Dunbier discuss the important role of biomarkers in the optimal selection of treatment for breast cancer [324]. For example, two multigene expression profiles have shown the ability to outdo the traditional prognostic and predictive factors. Onco*type* Dx, a 21-gene reverse transcription PCR-based assay [165], has already proved successful in identifying subsets of node-negative, estrogen receptor-positive patients who will benefit from the addition of chemotherapy to adjuvant antiestrogen therapy and those who will not. MammaPrint, a 70-gene signature, was developed from studying tumors of women with node-negative disease, unselected for estrogen receptor status [325, 326]. Tumors from patients who remained disease-free for 10 years were found to

have distinct profiles compared with patients who experience early relapse. Both Onco*type* Dx and MammaPrint have the potential to identify patients with node-negative tumors who do not require additional therapy, thus sparing these women from unnecessary and potentially toxic therapy. Both of these assays are Food and Drug Administration-approved and are currently undergoing prospective validation to more clearly define their roles in the optimization of therapy for node-negative breast cancer patients.

The next decade will surely witness the further explosion of predictive and prognostic genomic markers, and older biomarkers such as Ki67 will assume more prominence. Expanding on the success of Onco*type* Dx and MammaPrint, investigators are trying to identify signatures that predict response to specific therapies. Hess and colleagues have used transcriptional profiling to identify genomic signatures, which predict for response to the T-FAC adjuvant chemotherapy regimen [327].

An important advance in the last decade is the incorporation of trastuzumab into adjuvant therapy for HER2-positive breast cancer. Trastuzumab, a monoclonal antibody to HER2, in combination with standard adjuvant chemotherapy, has decreased the risk of recurrence by more than 50 % [328]. Targeting HER2 is a true success story and shows how identifying and inhibiting a target can transform a very aggressive phenotype into one with a favorable outcome. Prior to the use of targeted therapy for HER2-positive breast cancer, this form of the disease was associated with a high risk of relapse and a uniformly poor prognosis. Understanding the biology of HER2-positive breast cancer and the development of a tailored therapeutic approach has led to a cure for many women with this form of the disease.

Drug Development Through a Genomic Approach

Pharmacogenomics is the science that investigates how individuals react to medication. Although the recent sequencing revealed the 99.9 % semblance in human DNA makeup, the 0.1 % single-nucleotide polymorphism is about 1.4 million cells [329]. Pharmacogenomics is particularly attractive in oncology as most chemotherapy agents have a narrow therapeutic window, with severe drug toxicities that can be potentially life-threatening. Although advances in adjuvant chemotherapy for breast cancer have led to marked reductions in recurrence and mortality, women are still concerned about the short- and long-term toxicities associated with treatment. Moreover, improvements in chemotherapy have effects that are much more striking in women with estrogen receptor-negative tumors than in women with estrogen receptor-positive tumors, some of whom can now be spared chemotherapy. For example, the risk of death for dose-dense doxorubicin/cyclophosphamide followed by dose-dense paclitaxel (as in INT C9741) when compared with low-dose cyclophosphamide/doxorubicin/5-fluorouracil was reduced by 55 % (38–69 %) in women with estrogen receptor-negative tumors versus only 23 % (−17 to 49 %) in estrogen receptor-positive tumors [330]. Thus, it is conceivable that given the right combination of highly effective and less toxic chemotherapy, women with hormone receptor-negative breast cancer might be expected to have a more favorable outcome. In addition, hormonal therapies might be more effective if they are given in appropriate doses, taking into account the genetic variants that affect metabolizing enzymes as discussed in the review by Tan et al. [187]. Dr. Elias Zerhouni, the former director of the National Institutes of Health, stated in his "new strategic vision for medicine that the shift from a late curative paradigm to an early preemptive one is becoming increasingly possible" [331]. This is one mechanism of containing the escalating cost of cancer treatment nationwide. Electronic health records are another technology that can help to identify early onset and thus preempt the trajectory of cancer pathology and the ominous consequences of metastasis.

Genomic Epidemiology

Genomic epidemiology is defined as the investigation of the actions of genetic factors in determining health and disease onset in families and

populations and the interplay of genetics and environmental characteristics of people. Genomic epidemiology is the link uniting the intersection between genetic and molecular epidemiology. It focuses on the determinants and distribution of diseases and injuries in human populations [332, 333]. There are interindividual and interethnic variabilities of drug pharmacokinetics and pharmacodynamics, which may be caused by commonly occurring genetic polymorphisms of drug-metabolizing enzymes and transporters [334]. Spitz et al. recently proposed a unifying premise of integrative epidemiology and suggested that the same genes that are implicated in cancer risk may also be involved in a person's propensity to carcinogenic exposure and/or to modulation of therapeutic outcome [295]. Examples include glutathione-related transporter genes and the cytochrome P450 enzymes, such as CYP3A4 and CYP191A variants. Variants in these genes have been implicated in cancer risk and are important pathways for metabolizing antineoplastic drugs.

Tamoxifen, a selective estrogen receptor modulator, is commonly used for both the chemoprevention of breast cancer and the treatment of early- and advanced-stage estrogen receptor-positive breast cancer. Failure of tamoxifen therapy has long been attributed to intrinsic or acquired resistance of the tumor to the effects of estrogen receptor blockade. Tan and colleagues show that interindividual genetic variability plays a critical role not only in determining toxicity from therapy but also in determining benefit, in some cases [187]. Tamoxifen undergoes extensive metabolism via the CYP pathway to several primary and secondary metabolites, some of which exhibit more potent antiestrogenic effects than tamoxifen itself on breast cancer cells. CYP2D6 is one of the key enzymes in this pathway that metabolizes tamoxifen to a more active metabolite, endoxifen. There are several variants in the CYP2D6 gene that result in the poor metabolizer phenotype which, in turn, has been shown to correlate with worse outcomes. The majority of variations in drug-metabolizing enzymes identified to date have been through a "candidate gene" approach. It is unlikely, however, that drug efficacy and toxicity is a polygenic trait not attributable to a single gene.

Genome-wide association studies are making the study of multiple genes and SNPs involved in drug toxicity and efficacy possible. Several groups have developed genome-wide approaches to identify germ line polymorphisms that correlate with cytotoxicity. Huang et al. have developed a preclinical model that uses the International HapMap Project lymphoblastoid cell lines [334]. The HapMap Project contains lymphoblastoid cell lines from four distinct ethnic populations: Caucasians (Utah, United States), Africans (Yorubas from Nigeria), Japanese (Tokyo), and Chinese (Han from Beijing). These cell lines are an invaluable resource because they have been extensively genotyped. When lymphoblastoid cell lines are treated in vitro with a chemotherapeutic agent, individual cytotoxicity phenotypes are identified, which can then be combined with cell line-specific genotype data to do genetic association studies. This information can then be correlated to gene expression data, the so-called "triangular" approach, to allow the identification of SNPs that may explain variation in drug sensitivity. Importantly, the approach allows for simultaneous discovery of multiple SNPs involved in susceptibility, without bias for any particular gene.

Disparities in Clinical Outcomes Through a Genomic Approach

Racial disparities have been demonstrated in breast cancer mortality and are associated with differences in access to care and biological characteristics [335–337]. Poor access to care can lead to diagnosis at a more advanced stage, delays in starting treatment, failure to complete treatment, and receipt of inferior chemotherapy regimens [338–342]. Differences in tumor biology can result in more aggressive cancers, such as those that are negative for estrogen and progesterone receptors, as well as human epidermal growth factor receptor 2 (HER2)/*neu*, so-called triple-negative cancers. Black patients are more likely to be diagnosed with breast cancer that is

hormone receptor-negative and at a more advanced stage than white patients, and they are more likely to receive inferior care [336, 338, 343]. Hispanics fare better than black patients, but their rate of overall survival seems to be lower than that of non-Hispanic white patients [344].

For more than a decade, a prominent question in health services research has been how to best understand the relative contribution of these different causes of disparities in cancer survival rates, including breast cancer. Differences in treatment patterns have been a major focus of these efforts. Studies have shown that black patients are more likely than white patients to receive treatment that leads to poorer clinical outcomes, including nonstandard adjuvant chemotherapy regimens, first-cycle dose reductions (regardless of body mass index) [345], delays in radiation therapy [50, 342], and delays in and early termination of chemotherapy [341].

More recently, the list of factors known to worsen outcomes for minorities has expanded to include lower levels of English proficiency in some immigrant groups, lower education levels, and poor health literacy. One manifestation is that patients with low levels of English proficiency may be less able to participate in healthcare decisions, which may affect the treatment that they receive [346]. Population differences in the distribution of variants in drug-metabolizing enzymes and transporters might be relevant in addressing differences in outcomes in diverse populations and specifically in addressing health disparities. For example, women of African ancestry have a lower overall incidence of breast cancer, but a higher overall mortality compared with white women [347, 348]. The disparity in outcomes may be partly related to lower tolerance for side effects of treatment. Recent studies focus on disparities in treatment outcomes because differences in socioeconomic factors and tumor biology do not entirely account for the disparity in clinical outcomes. Several studies have documented differences in the receipt of cancer treatment by race [345, 349–351], and large clinical trials have established that dose delays, reductions, or early termination of chemotherapy greatly reduce treatment benefit [352–354].

Despite the data, undertreatment is common, especially among African American women. In a retrospective study of more than 20,000 women treated in community practices, Lyman and colleagues found that 36.5 % of patients received <85 % of their planned chemotherapy [355]. In a study of 472 women with early-stage breast cancer, Hershman et al. found that a substantial fraction of women terminated their chemotherapy prematurely and that early termination was significantly associated with both poorer survival and black race [341]. Suboptimal delivery of chemotherapy dose and intensity has been associated with decreased efficacy and poorer survival [356–358]. For these reasons, future drug development in breast cancer should incorporate genomic markers to identify interindividual and interethnic variability of drug pharmacokinetics and pharmacodynamics. With the development of more efficacious and less toxic drug regimens, one can expect to see a reduction in health disparities for the most vulnerable patients, especially women of African ancestry.

Breast Cancer Epigenome

DNA methylation is one of the three known layers of epigenetic control of germ line and tissue-specific gene expression. Hypermethylation plays an integral role in genomic imprinting, wherein one of the two parental alleles of a gene is silenced in order to establish monoallelic expression; X-chromosome inactivation in females occurs through a similar imprinting mechanism [359, 360]. Stated simply, DNA methylation is a heritable, epigenetic change that alters gene expression and is confined to the addition of a methyl group to the 5-carbon position of cytosine in a CpG dinucleotide. In the vertebrate genome, CpG dinucleotide sequences have been severely depleted to approximately 20 % of the predicted frequency during evolution, and among the remaining CpG dinucleotides, over 70 % are methylated [361]. A study of the human genome revealed that the distribution of CpG dinucleotides is not random and some of them cluster together to form CpG-rich DNA regions called

CpG islands. CpG islands are mostly located in the upstream promoter and exon 1 region of over half of human genes [362]. In normal cells, CpG islands in actively expressed genes are unmethylated. However, during neoplastic transformation, DNA methylation in cancer cells exhibits inverse profiles compared with normal cells; focal hypermethylation of CpG islands of 5′-end regions of many genes is observed [286, 363–365]. Thus, a change in DNA methylation profile is a hallmark of almost all human cancers, including breast cancer.

Much of the focus in breast cancer research over the past few decades has been on breast cancer genetics: identifying mutations and characterizing their roles in carcinogenesis. In the last several years, studies have shown that epigenetics plays a key role in tumor progression. Epigenetic changes are defined as heritable and reversible changes in gene expression that are not accompanied by changes in DNA sequence. In cancer, the main epigenetic mechanisms underlying abnormal gene expression include aberrant CpG island promoter methylation of specific tumor suppressor genes, global changes in genomic DNA methylation, and alterations in histone modification (deacetylation and methylation; Fig. 4.1). These abnormalities can be reversed by inhibitors of both DNA methyltransferases and histone deacetylases [366]. It has been postulated that promoter methylation may serve as the second "hit" in the Knudson two-hit model in sporadic cancer through inactivation of the normal allele of a tumor suppressor gene, implicated in hereditary cancer syndrome such as BRCA1 [367]. Hypermethylation of BRCA1 at promoter CpG islands occurs in 15–31 % of sporadic breast tumors and may play a critical role in sporadic breast cancer by inactivating one BRCA1 allele followed by loss of the wild-type BRCA1 [368]. BRCA1 methylation in sporadic breast cancer seems to result in a similar tumor phenotype to that seen in BRCA1 mutation carriers [367–370]. These findings are consistent with our study of C-MYC amplification in BRCA1-deficient breast cancers (BRCA1-mutated hereditary and BRCA1-methylated sporadic), in which we showed that the pattern of CMYC amplification in BRCA1-methylated cases resembles that of BRCA1-mutated cases rather than that of sporadic cancers [371].

We and others have offered a model of breast carcinogenesis in which BRCA1 promoter methylation serves as a "first hit," much like an inherited germ line mutation, and the "second hit" results in reduced BRCA1 copy number and/or chromosome 17 aneusomy. In this model, BRCA1 promoter hypermethylation occurs early and, when complete, causes defects in chromosome structure, cell division, and viability [371]. A BRCA1-deficient cell must acquire additional alterations, such as TP53 mutations or MYC amplification, that overcome these problems and force tumor progression down the same limited set of molecular pathways, similar to the progression of hereditary BRCA1 mutated tumors. Because the majority of BRCA1-mutated breast cancers are basal-like, Foulkes has hypothesized that the key function of BRCA1 is to be a stem cell regulator and promote the differentiation of glandular epithelium [372]. For this reason, in a BRCA1-deficient cell, this transition can fail or abort, and the basal cell phenotype gene expression pattern would be retained. Interestingly, Liu et al. have recently confirmed that inactivation of BRCA1 in breast epithelial stem cells restricts subsequent progenitor cells to a basal-like cell subtype [373]. Collectively, these observations suggest that inactivation of BRCA1 by mutation or methylation promotes breast cancer with basal tumor phenotype.

In contrast, loss of BRCA2 expression via aberrant promoter methylation does not seem to occur in sporadic cancers. Functional equivalency between the effect and significance of the epigenetic silencing of BRCA1 in sporadic breast cancer and genetic suppression of the gene in BRCA1 mutation carriers has implications for clinical practice. Lafarge and colleagues showed that decreased expression of BRCA1 in the HBL100 breast cancer cell line led to increased sensitivity to the DNA-damaging agent cisplatin [374]. As previously discussed, a number of studies have also correlated loss of BRCA1 with defects in DNA repair and cell cycle checkpoints. Tumors lacking functional BRCA1 protein show

a high frequency of chromosomal aneuploidy, characteristic of a defective G2-M checkpoint. Sudo and colleagues have shown that paclitaxel sensitivity is dependent on an intact checkpoint function [375], thus implying that any interference with the spindle assembly checkpoint would generate paclitaxel resistance. BRCA1-deficient cells secondary to mutation are relatively resistant to paclitaxel [374, 376–378]. Together, these data suggest that loss of normal BRCA1 expression by mutation or epigenetic regulation may confer a unique chemosensitivity profile. Thus, BRCA1 deficiency secondary to promoter methylation may represent a novel therapeutic target for the management of a subset of basal-like or triple-negative breast cancers.

Both inherited and sporadic breast cancers can also exhibit variable estrogen receptor-a expression. Interestingly, estrogen receptor-a coding region mutations seem to be quite rare [379, 380], although there is convincing evidence that estrogen receptor-a is an epigenetically regulated gene that can undergo promoter methylation in a significant proportion of breast cancers [381] and can strongly associate with BRCA1 promoter methylation [382]. An alternative epigenetic mechanism underlying the loss of estrogen receptor-a expression has been suggested by the results of cell-based assays analyzing histone function as a determinant of gene expression [383]. Restoration of estrogen receptor-a expression by histone deacetylase inhibitors suggests that reorganizing the heterochromatin-associated proteins, without demethylation per se, can restore functional estrogen receptor-a expression [383]. This possibility is being explored clinically in an ongoing phase II trial of a new-generation histone deacetylases inhibitor, vorinostat. The investigators of this trial will determine whether or not, following vorinostat treatment, a tumor becomes sensitive to hormone therapy and/or exhibits increased expression of estrogen receptor-a.

In addition, there have recently been a number of phase I/II trials initiated to investigate combining different classes of histone deacetylases inhibitors with traditional therapies for the treatment of breast cancer [384]. DNA methylation of certain genes (e.g., RASSF1A, CYP26A1, KCNAB1, SNCA, HIN-1, TWIST, and Cyclin D2) occurs in both premalignant lesions, such as atypical hyperplasia, and carcinoma of the breast [385, 386]. These findings suggest that epigenetic changes occur early in breast tumorigenesis and may serve as potential markers for early detection or risk assessment. Moreover, specific epigenetic changes may have prognostic and/or predictive value [387]. These observations are being translated into clinical care. For example, the National Cancer Institute is sponsoring a study of women at high risk of developing breast cancer who, following surgical resection for ductal carcinoma in situ or stage I, II, or III invasive breast cancer, are treated with simvastatin. Simvastatin belongs to the statin family of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, drugs that lower cholesterol in patients with cardiovascular disease; these agents have a theoretical role in chemoprevention through downregulating Ras, upregulating p27, and altering estrogen receptor levels. The change in methylation status across a panel of genes (estrogen receptor-a and estrogen receptor-b, cyclin D2, RAR-b, Twist, RASSF1A, and HIN-1) that are known to be frequently and specifically hypermethylated in breast cancer will be evaluated and correlated with changes in hsCRP, lipid profile, contralateral breast density, and estrogen concentration.

Conclusion and Future Perspective

The command of contemporary genome analysis tools, especially massively similar sequencing, is increasing at a remarkable rate. It seems clear that the “thousand-dollar genome” is nearly at hand. Modern genome-wide, high-resolution breast cancer analyses so far have focused mostly on evaluation of aberrations in invasive breast tumors. This provides a functioning aberration pieces list and recognizes aberrations that may be useful as prognostic markers or as therapeutic targets. They have not, though, provided considerable information about how these aberrations occur and evolve throughout development. This

information will come from longitudinal incorporated "omic" analyses of genome aberration appearance during cancer development. Studies in mouse models can provide some information, but studies of changes during the evolution of individual human cancers will be invaluable. This will entail a long-term, continued endeavor from the breast tumor research community to gather the obligatory samples and sustain refinement of large-scale omic investigation machineries so they are capable of investigating the petite quantities of neoplastic tissue that may be obtainable at premature stages of evolution.

Fewer than 30 % of women with metastatic breast tumor will live 5 years. This is in comparison to the cure of premature disease, where results have been enhanced greatly over the last decade. Dozens of subsequent generation therapies are being designed to target these aberrations. The best developed of these for breast tumors are trastuzumab and lapatinib, which target ERBB2-positive tumors. The responses to these agents, even when combined with predictable chemotherapeutic agents, are not, though, durable in patients with metastatic sickness, and long-term survival is uncommon.

It looks likely that recent cure strategies fail because these strategies do not take into account the genomic and epigenomic aberrations that contribute to resistance in metastatic breast tumors; resistance-related homeostatic, or feedback loops induced by pathway-targeted therapies; and factors unique to the metastatic microenvironments in the bone marrow, brain, liver, and lung that contribute to therapeutic resistance. Improvement of a meticulous "omic" considerate of drug-resistant, metastatic tumors will greatly facilitate cure of this important aspect of the disease. This will necessitate development of clinical trials in which samples of metastatic breast tissue suitable for large-scale omic investigation are acquired before and after development of medicine resistance, as well as the development of experimental model systems that mirror the aberrations that contribute to resistance.

It looks reasonable to anticipate that comprehensive cancer scans of genomic and epigenomic aberrations will become an important and routine part of cancer management. The challenges will be to manage the information so that it is generally accessible to the scientific and medical communities and to interpret it in ways that lead to improved cancer management. This will require a substantial investment in functional studies, since the roles of most genomic and epigenomic aberrations in cancer pathophysiology are not understood. It will also require full development and deployment of large-scale information management and interpretation systems. Success in these areas will allow the potential of cancer genome and epigenome in treatment personalization to be fully realized.

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5 Epigenomics of Breast Cancer

Kursat Oguz Yaykasli, Ertugrul Kaya, and Emine Yaykasli

Abstract

Breast cancer is the second most common malignant cancer and accounts for 1.38 million of the total new cancer cases and 458,400 of the total cancer deaths reported in 2008. Breast cancer with several subtypes is an extremely heterogeneous disease caused by interaction of both genetic and environmental risk factors. In order to understand the etiology of this heterogeneity, new perspectives like epigenetics are needed.

The term *epigenetics* was coined by Conrad Hal Waddington in the early 1940s. It refers to the study of gene function and regulation alterations without changes in the DNA sequence of the genome. The main epigenetic modifications are DNA methylation, histone modifications, and small noncoding RNAs (miRNAs). DNA methylation is the first to be associated with cancer and the most widely studied among epigenetic modifications. It regulates the gene expression by modifying the accessibility of DNA to the transcriptional machinery.

The importance of histone modification has been realized during the last 10 years, after identification of the coexistence of histone modifications. From the dynamically changing pattern of histone modification has emerged a new concept termed "histone cross talk." The epigenetic modifications are faster and reversible than mutation and easily affected by

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aging, environmental stimuli, and food in heritable manner. These characteristics provide a vital position in the etiology of diseases. After several investigations, it is well understood that the epigenetic modifications are involved in not only many biological processes such as X-chromosome inactivation, genomic imprinting, RNA interference, and programming of the genome but also several disease like breast cancer. Today we realize that the accumulation of epigenetic modifications occurs in the development of breast cancer. In addition, the epigenetic modifications improve our knowledge about the biology and heterogeneity of breast cancer by large-scale methods. Therefore, the researchers focused on epigenetic alterations-based breast cancer therapy, and it is speculated that epigenetic modifications may be markers for breast cancer. It is likely that epigenetics-based therapy will become a reality in the near future.

Keywords

Epigenetics • Breast cancer • DNA methylation • Histone modification • miRNA

Introduction

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. It is estimated that about 12.7 million cancer cases and 7.6 million cancer deaths occurred in 2008. Among them, 56 % of the cases and 64 % of the deaths occurred in economically developing countries [1]. In order to effectively combat it, we must understand the basic principles and processes of cancer. At the cellular level, we must understand the complex circuitries that dictate the cell-division cycle, survival, migration, and invasion. At the tissue level, the susceptible target cell population and the interactions between cancer cells and the microenvironment must be understood. Finally, the complex features that establish cancer "organ" at primary and distant sites, including metabolic and physiological effects and the establishment of a blood nutrient supply (angiogenesis), must also be clarified [2]. It had traditionally been considered that the underlying foundation of the mechanism of cancer development is the accumulation of genetic mutations. However, this paradigm has now been expanded to incorporate the distribution of epigenetic regulatory mechanisms that are prevalent in cancer [3–7].

Breast cancer is the second most common malignant cancer and the leading cause of cancer death in women, with increasing age bringing a sharp rise in incidence [1, 8]. Breast cancer constituted 23 % (1.38 million) of the total new cancer cases and 14 % (458,400) of the total cancer deaths reported in 2008. About half the breast cancer cases and 60 % of the deaths are estimated to occur in economically developing countries. In general, incidence rates are high in Western and Northern Europe, Australia/New Zealand, and North America; intermediate in South America, the Caribbean, and Northern Africa; and low in sub-Saharan Africa and Asia [9]. The American Cancer Society estimates that approximately 230,480 new cases of invasive breast cancer and 39,520 breast cancer deaths occurred among US women in 2011 [10].

Breast cancer is an extremely heterogeneous disease with molecular, histological, and phenotypic diversity caused by interaction of both inherited and environmental risk factors (age, obesity, alcohol intake, lifetime estrogen exposure, and mammographic density). Breast cancer can be classified into five major subtypes that differ significantly with regard to both molecular and clinical features. These subtypes are luminal A, luminal B, triple negative/basal like, HER2

enriched, and normal like [11, 12]. However, the main classification of breast cancer is based on the presence or absence of the estrogen receptor (ER), and investigations have been done regarding these subtypes [13]. Estrogens, sex steroid hormones, are responsible for the development of sex characteristics like breasts. Estrogens have also been recognized as the major factor in the development of breast cancers. The activity of estrogens is mediated by the two main isoforms of intracellular estrogen receptors (ERs): ERα and ERβ. The major breast cancer subtypes are ER-positive and ER-negative tumors [14, 15]. Our knowledge about breast cancer subtypes has increased since developing new high-throughput molecular techniques, such as microarray, next-generation sequencing, etc. [16, 17], and new perspectives like epigenetics [18–20].

Applications of Epigenomics of Breast Cancer

In the early 1940s, Conrad Hal Waddington coined the term epigenetics as "the causal interactions between genes and their products, which bring the phenotype into being." Nowadays, epigenetics refers to the study of gene function and regulation alterations in heritable manner. Unlike the genotoxic mechanism involving changes in genomic DNA sequences leading to mutations, the epigenetic modifications modulate the gene expression directly without changes in the DNA sequence of the genome. Epigenetic mechanisms coordinate biological processes such as X-chromosome inactivation, genomic imprinting, RNA interference, and programming of the genome during differentiation and development leading to gene silencing. Both the genetic and epigenetic events change the function and regulation of the gene products or lead to gain/loss of function of genes.

It is now acknowledged that genetic alterations are not the only path to gene disruption; reversible epigenetic modifications are increasingly being considered in cancer [18, 21–23]. In cancer cells, oncogenes are activated by mutations or overexpression, whereas tumor-suppressor genes become silenced. Accumulation of epigenetic modifications is also associated with oncogenesis. The epigenetic modifications occur early during carcinogenesis as potentially initiating events for cancer development, they have been identified as promising new targets for cancer prevention strategies [24]. Nowadays, the epigenetic mechanisms are known to be involved in several cancer types and diseases [25–34]. The epigenetic mechanisms also explain how two identical genotypes can give rise to different phenotypes in response to the same environmental stimulus [35].

Epigenomic Markers for Breast Cancer Diagnosis

The geographical variation of the incidence of breast cancer ratio indicates a significant role of factors affecting the epigenetic mechanism for the breast cancer risk [36]. Because epigenetic modifications are significant factors in the development of breast cancers, the assessment of the breast cancer in terms of the epigenetics could strongly improve our understanding of the biology and heterogeneity of breast cancer [37, 38]. The best-known epigenetic markers are DNA methylation, histone modifications and chromatin remodeling, and miRNAs.

DNA Methylation and Breast Cancer

The first and most widely studied epigenetic modification in mammals is DNA (cytosine) methylation [39, 40]. DNA methylation plays a crucial role in modulating the expression of the genetic information by modifying the accessibility of DNA to the transcriptional machinery, generally resulting in transcriptional gene silencing (activation of the gene is also possible in some cases). It involves several mechanisms like imprinting, X-chromosome inactivation, and inhibition of repeat elements and transposons transcription [41, 42]. DNA methyltransferases (DNMTs) catalyze the addition of the methyl group (-CH_3) using S-adenosylmethionine (SAM) as a methyl donor to dC residues in DNA.

Although most cytosine methylation occurs in the sequence CpG dinucleotides, the cytosine nucleotide of the CpA and CpT dinucleotides may also be methylated in some cases. CpG dinucleotides are found throughout the genome, but largely concentrated in small regions termed "CpG islands" [43, 44]. CpG islands are short sequences (length of 0.5 kilobases to several kilobases) of genomic DNA with a G+C content of at least 50 % and a ratio of observed to statistically expected CpG frequencies of at least 0.6. It is found in approximately 60–70 % of gene promoters commonly 5′-regulatory (promoter) regions of many "housekeeping" genes (which are essential for general cell function) and some tissue-specific genes [45–47]. CpG islands also can be found in the 3′-region of the gene and within the body of the genes (referring to exonic CpG island) [48]. Recent studies showed that not only methylation of CpGs in promoter but also methylation of CpGs within the gene bodies associate with transcriptional activation [49]. In contrast to expectation (the methylation level negatively correlates with the gene expression level), there is a positive correlation between gene-body methylation and gene activity in humans. It is proposed that the gene-body methylation may repress transcriptional noise, inhibit antisense transcription, and relate to replication timing [21, 50]. Intragenic methylation is also found at repetitive sequences in human DNA [51]. To date, several DNMTs (DNMT1p, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMTB3a, DNMT3b, and DNMT3L) have been identified. Among them only DNMT1, DNMTB3a, and DNMT3b have catalytic methyltransferase activity [52]. DNMT1 recognizes established hemimethylated DNA (the one strand of the CpG dinucleotides methylated, the other one not) and then methylates newly synthesized CpG dinucleotide whose partners on the parental strand are already methylated [53, 54]. Besides the capability of methylating hemimethylated DNA, the primary function of DNMT3a and DNMT3b is capable of de novo methylation patterns (both strands of the CpG dinucleotides are not methylated) during embryogenesis [55, 56]. Several methyl-binding proteins such as MBD1, MBD2, MBD3, and MeCP2 provide a platform for the DNA methylation [57], and it has already been determined that the mutations in DNMTs and MBDs contribute to diseases like acute myeloid leukemia (AML) [58, 59].

There are three types of DNA methylation: hypermethylation, hypomethylation, and loss of imprint. The CpG islands in the promoter region are commonly unmethylated (genes active) in normal tissues. In hypermethylation the CpG islands in the promoter region are aberrantly methylated, leading to gene silencing through the inhibition of transcription via recruitment of chromatin remodeling corepressor complexes. The loss of DNA methylation occurs in many gene-poor genomic areas including repetitive elements, retrotransposons, and introns at hypomethylation. It causes genomic instability and leads to reactivation of the genes. Loss of imprinting could be explained as the loss of specific monoallelic expression of genes in a parent-origin-specific manner [12, 35, 37].

DNA methylation is the first epigenetic mechanism to be associated with cancer after demonstration of global DNA hypomethylation and CpG-island hypermethylation in cancer tissues compared to normal tissues [60]. Global and gene-specific DNA hypomethylation and site-specific hypermethylation are common features in tumorigenesis [61]. The most extensively studied epigenetic alteration in cancer is DNA methylation of CpG islands. When the CpG islands of important genes like tumor-suppressor genes are hypermethylated, the tumor-suppressor genes become inactive and cancer emerges [62–64]. Nowadays, the next-generation sequencing (NGS) platform gives us enormous data relating genome-wide maps of CpG methylation. It is demonstrated that 5–10 % of normally unmethylated CpG promoter islands become abnormally methylated in various cancer genomes, and hypermethylation of promoter region also affects expression of various noncoding RNAs, some of which have a role in malignant transformation [5, 64]. DNA hypomethylation is observed in several tumor types, such as colorectal and gastric cancers, melanomas, etc. [65]. Decreased DNA methylation is thought to promote chromosomal instability, eventually leading

to carcinogenesis. Genome-wide DNA hypomethylation also affects transcription through loss of imprinting and upregulation of silent genes, all of which might induce tumor development [66]. During tumor progression, the degree of hypomethylation of genomic DNA increases as the lesion derives from a benign proliferation of cells to an invasive cancer [67].

Because of the high histological and molecular heterogeneity, the assessment of breast cancer in terms of the epigenetic aspects, especially DNA methylation, helps us to clarify the breast cancer mechanism. It is speculated that changed DNA methylation pattern of global or specific genes, such as RASSF1A, GHSR, etc., may be markers for breast cancer, after their appearance in several studies [68–71]. To find a reliable biomarker, several changed methylation pattern genes in breast cancer have been reported during the last decade, based on the tumor's clinicopathological characteristics, such as hormonal receptor status [72–76]. The methylated RASSF1A, CCND2, GSTP1, and TWIST genes for ER-positive breast cancers and PGR, TFF1, and CDH13 genes, predominantly for ER-negative breast cancers, have been linked [12, 37].

The involvement ERα in breast cancer is already known, and ERα is expressed approximately in 65–75 % of diagnosed breast tumors. ERα is encoded by the estrogen receptor 1 (ESR1) gene. The promoter region and first exon of the ESR1 gene contain five CpG islands [77, 78]. Several mechanisms relating the lack of ERα expression in ER-negative breast cancer have been proposed to date. Among them, the suppression of the ESR1 gene by hypermethylation of CpG islands has been investigated [79]. DNMTs are responsible for this methylation, and it has been demonstrated that the re-expression of the ER gene is possible by a DNMT1 inhibitor (5-aza-2′-deoxycytidine) or antisense oligonucleotide for inhibiting DNMT1 specifically [80, 81]. A recent study also showed that ER promotes genomic methylation through upregulation of DNMT1 in ER-positive breast cancer cells [82]. Another important molecule in breast cancer, E-cadherin, is responsible for maintaining the normal differentiated state of the mammary gland epithelium. Similarly, the loss of E-cadherin expression in all tumor stages of breast cancer has been observed due to hypermethylation of CpG islands. Therefore, epigenetic suppression of ERα and E-cadherin may occur prior to invasion and then increases as cells acquire invasiveness and metastatic potential [18, 78].

Cancer is a disease characterized by uncontrolled cell division due to checkpoints damaged by several factors such as chemical, UV, etc. [83, 84]. Despite the fact that the exact role of the BRCA1 protein is not clarified in detail, BRCA1 protein is known to be a tumor-suppressor gene. It involves several important biological processes, such as DNA repair damage, induction of apoptosis, etc. [85–87]. The mutations on BRCA1 and BRCA2 genes increase the development of familial breast cancers [88, 89]. The other mechanism of suppression of BRCA1 expression is hypermethylation of promoter region of genes. Recent studies have shown that suppression of BRCA1 expression by hypermethylation is involved not only in breast and ovarian cancer but also lung and oral cancers [90, 91].

Hypermethylation of CpG islands resulting from overactivity of DNMTs occurs in many cancers. Several studies reported that DNMTs are also overexpressed in breast cancer [92, 93]. A recent study in Tunisian breast cancer showed overexpression of three hypermethylating enzymes (DNMT1, DNMT3a, and DNMT3b) by immunohistochemistry. They found that overexpression of various DNA methyltransferases might be involved in epigenetic inactivation of multiple tumor-suppressor genes, leading to the development of aggressive forms of sporadic breast cancer [94]. However, re-expression of promoter-methylated genes can be achieved after DNMT inhibitor treatment, such as 5-aza-2′-deoxycytidine treatment [95, 96].

The other epigenetic mechanism, hypomethylation, also is involved in activating genes in breast cancer. The promoter region of the MDR1 gene is always highly methylated in normal conditions, while its hypomethylation occurs during tumorigenesis, and it might be a putative implication in biological aggressiveness of tumors [97]. Several hypermethylated and hypomethylated

genes are involved in biological functions linked to breast cancer. The demonstrated genes are in listed Table 5.1.

Moreover, global hypomethylation can be seen in breast cancer. It is widely assumed that global hypomethylation activates the gene expression. However, it might decrease the gene expression when accompanied by a gain of repressive chromatin. Taken together, it has been found that the global hypomethylation silences tumor-suppressor genes via repressive chromatin domains in breast cancer [98].

Male breast cancers often differ from female breast cancers in several respects. Kornegoor et al. studied the comparison of male and female breast cancers in terms of the DNA methylation patterns. The methylation patterns of the most frequently methylated genes (MSH6, WT1, PAX5, CDH13, GATA5, and PAX6) were found to be similar in male and female breast cancer. On the other hand, methylation occurred less often in male breast cancer when compared to female breast cancer [99].

Histone Modifications in Breast Cancer

The chromatin is a highly organized structure of DNA and protein. The organization of DNA in chromatin (euchromatin, active; heterochromatin, inactive) has many functions, such as packaging DNA into smaller volume, preventing DNA damage, and controlling DNA replication, transcription, and repair [100]. The fundamental unit of chromatin is the nucleosome, an octomeric structure containing two copies each of histones (H3, H4, H2A, and H2B) around which 147 base pairs of DNA are wrapped [101]. The states of chromatin are controlled by chemical modification of histone tail (N-terminus) via posttranscriptional including acetylation, methylation, phosphorylation, sumoylation, poly(ADP)-ribosylation, and ubiquitination and histone composition in conjunction with other nonhistone proteins [102, 103].

It was first proposed in 1964 that histone modifications may affect the regulation of gene expression, after demonstrating acetylation of the ε-amino group of lysine residues on histones

Table 5.1 Hypermethylated and hypomethylated genes in human breast cancer

Gene (description)	Function	Sample obtained	Case #	Methy. status	Marker	Reference
14-3-3-σ/stratifin (SFN)	Cell cycle regulation	Cell lines, tissue	20	Hyper	Therapeutic	Ferguson et al. [202]
14-3-3-σ/stratifin (SFN)å	Cell cycle regulation	Serum	100	Hyper	Diagnostic, prognostic	Mirza et al. [203]
ESR1 (estrogen receptor 1) or 14-3-3-σ/stratifin (SFN)	Cell cycle regulation	Serum	106	Hyper	Diagnostic	Martínez-Galán et al. [204]
RASSF1A (ras association domain family protein1)	Cell cycle regulation	Cell lines, tissue	45	Hyper	Therapeutic	Dammann et al. [205]
APC (adenomatous polyposis of the colon)	Inhibitor of β-catenin	Tissue	50	Hyper	Therapeutic	Jin et al. [206]
RASSF1, APC, DAPK1		Serum	34	Hyper	Diagnostic	Dulaimi et al. [207]
RARβ (retinoic acid receptor β)	Cell cycle regulation	Cell lines, tissue	24	Hyper	Therapeutic	Sirchia et al. [208]
RASSF1A and RARβ	Cell cycle regulation	Serum	20	Hyper	Diagnostic, prognostic	Shukla et al. [209]
RASSF1A or ATM	Cell cycle regulation	Plasma	50	Hyper	Diagnostic	Papadopoulou et al. [210]
RASSF1, RARB, MGMT, APC		Serum, tissue	33	Hyper	Prognostic	Taback et al. [211]
TMS1 (target of methylation-induced silencing-1)	Involved in apoptosis	Cell lines, tissue	27		Therapeutic	Conway et al. [212]

Table 5.1 (continued)

Gene (description)	Function	Sample obtained	Case #	Methy. status	Marker	Reference
TMS1, BRCA1, ERα, and PRB		Serum	50	Hyper	Diagnostic	Mirza et al. [173]
CCND2 (cyclin D2)	Cell cycle regulation	Tissue	106	Hyper	Diagnostic, prognostic	Evron et al. [213]
CCND2, CDKN2A, and SLIT2		Serum, tissue	36	Hyper	Diagnostic, prognostic	Sharma et al. [214]
CDH1 (E-Kadherin)	Cell adhesion and invasion	Tissue	151	Hyper	Prognostic	Shinozaki et al. [215]
CDH1 (E-Kadherin)	Cell adhesion and invasion	Tissue	79	Hyper	Prognostic	Caldeira et al. [216]
CDKN2A (cyclin-dependent kinase inhibitors)	Cell cycle regulation	Plasma	35	Hyper	Diagnostic	Silva et al. [217]
CDKN2A or CDH1		Serum	36	Hyper	Diagnostic, prognostic	Hu et al. [218]
CDH 13 (H-Kadherin)	Cell adhesion and invasion	Cell lines, tissue	55	Hyper	Therapeutic	Toyooka et al. [219]
BRCA1 (breast cancer 1)	DNA repair and recombination	Tissue	143	Hyper	Diagnostic	Birgisdottir et al. [220]
BRCA1, CDKN2A, or 14-3-3σ		Serum	38	Hyper	Diagnostic	Jing et al. [221]
APC, RASSF1, or ESR1		Serum	79	Hyper	Prognostic	Van der Auwera et al. [222]
GSTP1 (glutathione-S-transferase P1)	Carcinogen detoxification	Tissue	77	Hyper	Prognostic	Esteller et al. [223]
GSTP1, RARB, RASSF1, or APC		Plasma	47	Hyper	Diagnostic	Hoque et al. [224]
TWIST (TWIST homology of drosophila)	Involved in cell death	Mammary ducts' fluid	72	Hyper	Therapeutic	Vesuna et al. [225]
CCND2, RARB, TWIST1, or SCGB3A1		Plasma	34	Hyper	Diagnostic	Bae et al. [226]
RUNX3 (run-related transcription factor 3)	Transcriptional regulation	Cell lines, tissue	44	Hyper	Diagnostic	Lau et al. [227]
RUNX3, CDKN2A, RASSF1, or CDH1		Serum	19	Hyper	Diagnostic, prognostic	Tan et al. [228]
MDR1 (multidrug resistance 1)	Transmembrane efflux pump	Serum, tissue	100	Hypo	Prognostic	Sharma et al. [97]
CAV1 (Caveolin 1)	Cell invasion, metastasis	Cell line	30	Hypo	Prognostic	Rao et al. [229]
NAT1 (N-acetyltransferase type 1)	Cell invasion, metastasis	Tissue	103	Hypo	Prognostic	Kim et al. [230]
UPA (Urokinase)	Cell invasion, metastasis	Cell line	1	Hypo	Therapeutic	Pakneshan et al. [231]

[104]. After nearly half a century, it is has been elucidated that the posttranscriptional modifications of histone tails determine not only transcriptional activity but also all DNA-templated processes. The identification of the coexistence of histone modifications associated with activation or repression led to the proposal that the modification constitutes a code that could be recognized by transcription factors to determine the transcriptional state of a gene 10 years before [105]. However, these patterns appear to be not static, and a dynamically changing and

complex landscape via the chromatin signaling pathway led to the new concept termed "histone cross talk." This term represents the influence one or more coexisting histone modifications have on the deposition, interpretation, or erasure of other histone modifications [5, 106]. The recent investigations showed that histone cross talk mechanisms commonly seen and have a great importance for biological processes in organism [107, 108].

Histone modifications affect the chromosome function via several mechanisms. Generally it is believed that histone modifications cause structural changes in histone. This structural change may act as specific binding sites for protein domains (e.g., bromodomains, chromodomains, tudor domains) [109, 110]. Among the epigenetic mechanisms, histone modifications have further grown over the last decade with the discovery and characterization of a large number of histone-modifying molecules and protein complexes. The deregulation of these molecules or complexes may lead to deregulation of the control of chromatin-based processes by changing histone modifications and may have been associated with a large number of human malignancies. Genome-wide studies revealed that the histone modifications of malignant cells patterns disrupted when compared to healthy cells [111]. The posttranslational modification at amino acid tail of histone protein may result in changed transcription of important genes such as tumor suppressors. Changed patterns of histone modifications are a hallmark of cancer, and great amount of histone modifications have been linked to several cancer types to date [112]. The most well-known histone modifications types are acetylation/deacetylation and methylation/demethylation [113].

Histone Acetylation/Deacetylation in Breast Cancer

Histone acetylation/deacetylation status regulates several important regulatory proteins and transcription factors and is controlled by the interplay of histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. HATs transfer acetyl groups from acetyl-CoA to the amino group of lysine residues in histone tail. It removes the positive charges, thereby reducing the affinity between histones and DNA. This makes RNA polymerase and transcription factors easier to access the promoter region. So histone acetylation facilitates gene expression by allowing transcription factors to access the DNA. In contrast, the HDACs remove the acetyl group from histones to coenzyme A (CoA), resulting in coiling of chromatin, which inhibits transcription [22, 103].

At least 25 HATs and 18 HDACs have been identified in humans [114]. HATs were the first enzymes shown to modify histones [115]. There are two major classes of HATs: type A and type B. The type A HATs are nuclear proteins and can be grouped into at least three families—Gcn5/PCAF, MYST, and p300/CBP—depending on amino acid sequence homology [116]. In contrast to type A HAT, the type B HATs are predominantly cytoplasmic and show similar highly conserved primary structures, with acetylate-free histones but not those already deposited into chromatin, and newly synthesized histones H4 at K5 and K12. This pattern of acetylation is important for deposition of the histones [117]. The HDACs also have critical importance in the regulation of expression of genes involving cell survival, proliferation, differentiation, and apoptosis and can be divided into four major groups depending on sequence homology and target both histone and nonhistone proteins. Class I includes HDACs 1, 2, 3, and 8; class II includes HDACs 4, 5, 6, 7, 9, and 10; and class IV includes HDAC 11. In contrast to other HDACs, class III HDACs consist of NAD^+-dependent sirtuin family 1–7 [5]. HDACs also regulate the expression of tumor-suppressor and specific cell cycle regulatory genes. It has been observed that high HDAC expression level and hypoacetylation can be seen in several cancers. So HDAC inhibitors have been targeted for cancer therapy [118, 119]. The mechanism of the antiproliferative effects of HDAC inhibitors is complex. The target of HDAC inhibitors is the zinc cofactor at the active site of the HDACs to change chromatin structure and cause re-expression of aberrantly silenced genes [120].

Histone Methylation/Demethylation in Breast Cancer

Besides the gene promoter regions, the methylation/demethylation can occur on histone protein residues. DNA methylation at CpG islands of

promoter regions generates long-term gene silencing and makes the majority chromatin inaccessible for transcription, but histone methylation results in short-term inhibition of gene expression. Methylation, unlike acetylation and phosphorylation, does not alter the overall charge of the molecule [5, 18]. Histone methylation takes place at lysine and arginine residues by histone methyltransferases (HMTs). HMTs transfer a methyl group from the cofactor S-adenosyl methionine to lysine or arginine residues on histone tails, which play important roles in chromatin remodeling and transcriptional activity. The methylation at arginine residue of histone tails usually activates the gene transcription, although it may be involved in transcriptional repression in some cases. The methylation at lysine residue of histone tails can contribute to either activation or repression of transcription, depending on the position of methylation, and adjacent modifications [121, 122]. Some lysine methylases (like H3K4, H3K36, H3K79) often activate genes in euchromatin, while others (like H3K9, H3K27, and H3K20) are associated with heterochromatin regions of the genome. The methylation status (mono-, di-, or trimethylation) also alters gene expression. For example, the monomethylations of H3K27, H3K9, H4K20, H3K79, and H2BK5 are all linked to gene activation, whereas trimethylations of H3K27, H3K9, and H3K79 are linked to repression [123]. Histone demethylases (HDMs), discovered nearly 7 years ago, have been classified into two groups depending on their mechanism of action [124].

Several HMTs and HDTs relevant to cancer development have been identified to date [125]. The EZH2 one of the HMTs acts mainly as a gene silencer; it is the major enzyme that methylates lysine-27 of histone H3 (H3K27). EZH2 can add up three methyl groups to the ε-amino group of the lysine side chain, leading to chromatin condensation [126, 127, 128]. The overexpression of EZH2 is seen in many cancer types, including prostate and melanoma [129, 130]. The elevated EZH2 levels are associated with breast cancer [131]. It also correlates with tumor aggressiveness and poor prognosis, which suggests that EZH2 was an oncogene [132, 133]. However, loss-of-function mutations in EZH2 gene have described several malignancies, suggesting EZH2 was tumor-suppressor gene [134, 135]. In addition, some chemicals like diethylstilbestrol (DES) or bisphenol A (BPA) contribute to the formation of breast cancer by increasing EZH2 expression [136].

Another study relating EZH2 to breast cancer concluded that the overexpression of EZH2 regulates BRACA1 gene expression and genomic instability mediated by PI3K/Akt-1 pathway [137]. These investigations suggest that EZH2 histone methyltransferase is involved in breast cancer etiology.

The HMT G9a methylates at the ε-amino group of lysine 9 residues of histone 3. It has also been proven that G9a is involved in Snail-mediated E-cadherin repression by interacting with Snail in human breast cancer [138]. Another study proposed that G9a contributes to the estradiol (E2)-dependent induction of some endogenous target genes of estrogen receptor (ER)α in MCF-7 breast cancer cells [139]. Other lysine HMTs (NSD1, NSD3L, and SMYD3) are overexpressed in several cancers [125, 140]. Unlike lysine HMT, arginine HMTs have not been as well characterized. Arginine HMTs catalyze methylation of nitrogen of arginine residues, called protein arginine methyltransferases (PRMT). The 10 PRMTs are nearly identified and categorized into two groups based on the type's methylarginine products they produced [141]. Among PRMTs, the altered PRMT1 gene expression has been investigated in breast cancer [142].

Several types of histone lysine demethylases (HDMs) have been identified, but the pathological roles of their dysfunction in human disease have not been clarified. Among them, lysine-specific demethylase (LSD1) is the first identified histone lysine demethylase. LSD1 specifically demethylates histone H3 lysine 4, which is linked to active transcription [143]. After discovery of LSD1, the concept of histone methylation changed, and it is understood that histone methylation is a dynamically regulated process under enzymatic control rather than chromatin marks that could only be changed by histone replacement [19]. It has been reported that the expression level of LSD1 is elevated in human bladder [144],

small cell lung, colorectal, and neuroblastoma cancers, and the mutation of LSD1 gene causes prostate cancer [145].

In breast cancer, LSD1 expression has been found to be strongly upregulated in ER-negative breast cancer; it makes LSD1 a putative biomarker for aggressive tumor biology and a novel attractive therapeutic target for treatment of ER-negative breast cancer [146]. It is also demonstrated that LSD1 inhibits the invasion of breast cancer cells in vitro and suppresses breast cancer metastatic potential in vivo [147]. Other histone demethylase genes GASC1, PLU-1, and JMJD2B are involved in human breast cancers. The GASC1 gene may be linked to the stem cell phenotypes and show oncogene properties in human breast cancer [148]. PLU-1 is an H3K4 demethylase and plays an important role in the proliferative capacity of breast cancer cells through repression of tumor-suppressor genes, including BRCA1 [149]. The methylation status of histone H3 lysine 4 (H3K4) and of H3K9 is mutually exclusive, and H3K9 trimethyl demethylase JMJD2B is an integral component of the H3K4-specific methyltransferase MLL2. It has been demonstrated that the JMJD2B/MLL2 complex interacts to define the methylation status of H3K4 and H3K9 in ERα-activated transcription, and JMJD2B itself is transcriptionally targeted by ERα and may thus form a feed-forward regulatory loop in promoting hormonally responsive breast carcinogenesis [150]. JMJD2B also functions as coregulator of ERα signaling in breast cancer growth and mammary gland development [151]. And the histone protein LSD1 is able to demethylate nonhistone proteins, such as p53 and DNMT1 [152, 153].

miRNA in Breast Cancer

Scientists have long been aware of the existence of noncoding RNAs (ncRNAs). In spite of the great amount of knowledge about the function and types of ncRNAs, we are still far from fully knowing the role of large fractions of the transcriptome that do not encode for proteins [154]. Among ncRNAs, microRNAs are 18–25 nucleotides-long RNA molecules encoded in the genome that are transcribed by RNA polymerase II and important regulators of protein of gene expression that control both physiological and pathological processes, such as DNA methylation, development, differentiation, apoptosis, and proliferation [155, 156]. miRNAs are synthesized and processed in the nucleus, exported to the cytoplasm, and then bind to the target mRNA. The regulation of RNA transformation by miRNA is accomplished through RNA-induced silencing complex (RISC). miRNAs can inhibit mRNA translation or degrade mRNA [157, 158]. Major mechanisms of miRNA deregulation include genetic and epigenetic alterations as well as defects in the miRNA processing machinery. Each miRNA regulates multiple mRNAs and, conversely, each mRNA may be targeted by multiple RNAs (several hundreds). They can act as oncogenes or tumor suppressors and have been implicated in cancer initiation and progression, and the profiles of miRNA expression differ between normal and tumor tissues and between tumor types [159–161]. To date, several investigations relating to miRNA profiling has led to the identification of miRNAs' changed expression level in human breast cancer [162, 163]. The expression level of these miRNAs was correlated with specific breast cancer biopathological features, such as estrogen and progesterone receptor expression, tumor stage, vascular invasion, or proliferation [164]. miRNAs act as tumor suppressors and are oncogenic in breast cancer like other cancer types. So, tumor formation may arise from the overexpression (or amplification) of oncogenic miRNA and/or reduction (or deletion) of a tumor-suppressor miRNA [165].

miRNA-21 is overexpressed in breast cancer like in other cancer types [164, 166]. p53 and programmed cell death 4 (PDCD4) are tumor-suppressor proteins, and the deregulation of them may lead to cancer development. miRNA have been linked to breast cancer by targeting these proteins in breast cancer cells [167].

Epigenomic Markers for Breast Cancer Prognosis

Despite the extreme heterogeneity of breast cancer, global breast cancer survival rates have increased during the past decades due to advances

in the central role of genetic alterations in the diagnosis, treatment, prevention of breast cancer, and prognosis [2, 168]. Survival rates should be further improved by finding epigenetic molecular markers associated with risk assessment and/or prognosis of breast cancer. The knowledge about epigenetic alterations profiles in detail might prove vital in many respects. First, it might help us to estimate breast cancer risk and take precautions before breast cancer develops. In addition, there are several subtypes of breast cancer and corresponding therapies currently used. Each subtype, even each individual, has unique molecular epigenetic characteristics. The elucidating of epigenetic characteristic might contribute to a better estimation of breast cancer prognosis and lead to the choice of the most useful therapy [169]. In this way, patients will not be exposed to ineffective toxins associated with expensive therapy. Several reports have proposed that hypermethylation or hypomethylation of specific genes and global methylation status might be useful epigenetic markers for breast cancer prognosis. The recent studies also included miRNAs' expression profiles into putative epigenetic markers of breast cancer.

The major breast cancer subtype is ER-positive, and it has generally had a more favorable prognosis than ER-negative tumors. It is well established that ERα and E-cadherin are frequently involved in pathogenesis of breast cancer. The aberrant methylation of these genes is associated with malignant progression in human breast cancer [170]. ERα expression level is also regulated by miRNAs in the context of breast cancer. miRNA-206 [171] and miRNA-221/222 [77] target and regulate human ERα. miRNA-206 was upregulated in ERα-negative breast cancer. Another study found that miRNA-206 inhibits the expression of ESR1 mRNA through two binding sites in the ESR1 3′-untranslated region (3′-UTR). The researchers also found other miRNAs (miRNA-18a, miRNA-18b, miRNA-193b, and miRNA-302c) targeting to ESR1 mRNA in breast cancer cells [172]. Therefore, the aberrant methylation of the ESR1 gene and certain miRNAs altering the ESR1 gene expression might be putative epigenetic markers for human breast cancer prognosis.

BRCA1-associated breast cancer, hereditary or nonhereditary, occurs at early age due to involvement of the cellular DNA repair machinery. The inactivation of the BRCA1 by hypermethylation has been suggested to be the putative prognostic marker in breast cancer [173]. Besides the methylation, BRCA1 expression level could be regulated by miRNA-335. Overexpression of miR-335 resulted in an upregulation of BRCA1 mRNA expression, suggesting a functional dominance of ID4 signaling [174].

RASSF1A (Ras association domain family 1 isoform A) is a recently discovered tumor-suppressor gene. The protein encoded by RASSF1A interact is involved in the regulation of the cell cycle, apoptosis, and genetic instability. Thus, loss or altered expression level of the RASSF1A gene has been associated with several cancers. After illustrating the association between inactivation of the RASSF1A gene and the hypermethylation of its CpG-island promoter region, the RASSF1A gene has become the attractive biomarker for early cancer detection, diagnosis, and prognosis in many cancer types [175, 176]. The increased methylation level of the RASSF1A gene was observed in tumor size and lymph node status in breast cancer [177]. Similar results have been obtained by a meta-analysis of published data conducted with 1795 breast cancer patients. They concluded that RASSF1A promoter hypermethylation associates with worse survival in breast cancer patients [178]. These findings have indicated the great potential for the methylation of the RASSF1A gene in terms of the prognostic value of the breast cancer.

EZH2, histone-lysine N-methyltransferase acts as gene silencer by methylation and is related to several cancers. The overexpression of EZH2 is associated with aggressive breast cancer because of the enhanced cancer cell proliferation and a marker of poor prognosis in many solid tumor carcinomas including breast [179–181].

It has been investigated that several miRNAs are involved in breast cancer pathogenesis like cell regulation, and it has been proposed to be a prognostic factor for breast cancer. The miRNA-17-5p and miRNA-17/20 have been reported to be involved in breast cancer cell proliferation [182, 183]. miRNA-21 also could be a molecular

prognostic marker for breast cancer and disease progression because of its association with advanced clinical stage, lymph node metastasis, and patient poor prognosis [184].

Another strategy to clarify the role of miRNA in breast cancer is the analysis of DNA methylation and expression miRNAs in combination. Alteration of methylation in the promoters of miRNAs has also been linked to transcriptional changes in cancers. Morita et al. found that DNA methylation in the proximal promoter of miRNAs is tightly linked to transcriptional silencing [185].

Applications of Epigenomics in Breast Cancer Therapy

Cancer emerges not only because of the accumulation of genetic mutations, but also because of the reversible epigenetic changes. The dynamic alterations of the epigenetic mechanisms offer us a new field for developing novel cancer drugs that can react to epigenetically silenced tumor-suppressor genes [186]. So histone deacetylases and DNA methyltransferases have become the main targets for cancer therapy. In breast cancer, epigenetic silencing of tumor-suppressor genes due to alteration in both HATs and HDACs (histone modification) in combination with DNA hypermethylation is commonly observed [187]. The clarification of the epigenetic dysregulation mechanism in breast tumorigenesis has great importance in terms of the development of new therapies for breast cancer patients.

Aberrant HDAC activity has been investigated in several cancer types, especially in breast cancer. HDAC-1 expression and HDAC-3 protein expressions were analyzed immunohistochemically on a tissue microarray containing 600 core biopsies from 200 patients by Krusche et al. They found that moderate or strong nuclear immunoreactivity for HDAC-1 was observed in 39.8 % and for HDAC-3 in 43.9 % of breast carcinomas. HDAC-1 and HDAC-3 expressions correlated significantly with estrogen and progesterone receptor expression [188]. Another study concentrated on HDAC-6 expression levels in breast cancer has been done by Zhang et al. They also found that HDAC-6 mRNA expression is at significantly high levels in breast cancer patients with small tumors measuring less than 2 cm, with low histological grade, and in estrogen receptor α- and progesterone receptor-positive tumors. However, multivariate analysis concluded that the mRNA and protein of HDAC-6 were not independent prognostic factors for both overall survival and disease-free survival [189]. These studies led to the development of new therapies for breast cancer by finding suitable HDAC inhibitors. To date, a number of HDAC inhibitors have been designed and synthesized based on their chemical structure and are generally divided into four groups including hydroxamic acids, benzamides, cyclic peptide, and aliphatic acids (small chain fatty acids). The potential use of these inhibitors for breast cancer therapy has been investigated, as shown in Table 5.2.

Among them, some HDAC inhibitors like vorinostat (SAHA) and romidepsin (FK-228) have already been approved by the US Food and Drug Administration for clinical treatment of cutaneous T-cell lymphoma. Vorinostat is the first HDAC inhibitor and currently under evaluation in several phase II trials in breast cancer. It is already shown that vorinostat has profoundly antiproliferative activity and inhibits proliferation of both ER-positive and ER-negative breast cancer cell lines [190]. Entinostat (MS-275) and panobinostat (LBH-589) HDAC inhibitors are in phase I and II studies in combination with endocrine therapies, chemotherapeutic agents, or novel targeted therapy in women with breast cancer [12, 120]. A recent phase II study relating to the HDAC inhibitor vorinostat combined with tamoxifen for the treatment of patients with ER-positive metastatic breast cancer using 43 patients has been done. Even though the number of patients was small, they concluded that the combination of vorinostat and tamoxifen is well tolerated and exhibits encouraging activity in reversing hormone resistance. HDAC inhibitor with tamoxifen may restore hormone sensitivity by causing re-expression of a silenced ER gene [191].

In addition to phase trials, preclinical investigations have been widely done. The other idea for treatment of ER-negative breast cancer cells is using the synergistic effects of a combination

Table 5.2 The investigations of HDAC inhibitors in breast cancer

Agent(s)	Alternative name	Class	Study design	Samples	Case #	Reference
Vorinostat	SAHA, suberoylanilide hydroxamic acid	Hydroxamic acid	Preclinic	Human breast cancer cells		Munster et al. [190]
Vorinostat			Phase II	Metastatic breast cancer	14	Luu et al. [232]
Vorinostat + tamoxifen			Phase II	ER-positive metastatic breast cancer	43	Munster et al. [191]
Vorinostat + paclitaxel + bevacizumab			Phase I–II	Metastatic breast cancer	54	Ramaswamy et al. [233]
Panobinostat	LBH-589	Hydroxamic acid	Preclinic	Human breast cancer cells		Chen et al. [234]
Panobinostat			Preclinic	ER-negative human breast cancer cells		Zhou et al. [194]
Panobinostat			Preclinic	Human breast cancer cells		Rao et al. [235]
Panobinostat			Preclinic	Triple-negative breast cancer cells		Tate et al. [236]
Entinostat	MS-275, SNDX-275	Benzamide	Preclinic	Human breast cancer cells		Lee et al. [237]
Entinostat			Preclinic	Human breast cancer cells		Huang et al. [238]
Entinostat			Preclinic	ERα-negative human breast cancer cells		Sabnis et al. [239]
Entinostat + trastuzumab			Preclinic	Human breast cancer cells		Huang et al. [120]
Romidepsin	Depsipeptide (FK-228), FR901228	Cyclic peptide	Preclinic	Human breast cancer cells		Hirokawa et al. [240]
Valproic acid	–	Aliphatic acids	Preclinic	Human breast cancer cells		Jawed et al. [241]
Valproic acid + tamoxifen			Preclinic	Human breast cancer cells		Hodges-Gallagher et al. [242]
Valproic acid + trichostatin A			Preclinic	Human breast cancer cells		Reid et al. [243]
Valproic acid + retinoic acid + 5-aza-2′-deoxycytidine			Preclinic	Human breast cancer cells		Mongan et al. [244]
Phenylbutyrate	–	Aliphatic acids	Preclinic	Human breast cancer cells		Dyer et al. [245]

treatment of HDAC inhibitors and DNMT inhibitors (demethylating agents). Fan et al. and Sharma et al. used 5-aza-2′-deoxycytidine (AZA) as a DNMT1 inhibitor and trichostatin A (TSA) as a HDAC inhibitor to investigate this synergistic effect. Both studies have shown the reactivate ERα and PR gene expression in ER-negative breast cancer cell lines, which are known to be aberrantly silenced in breast cancer [192, 193]. Other studies have shown that the HDAC

inhibitors lead to reactive of ERα and PR expression by inhibition of the HDAC activity in breast cancer cells [194–196].

The other enzyme families to target for cancer therapy are HMTs and HDMs, previously implicated in cancer, inflammation, and diabetes [197]. The gene expressions level of the histone-modifying enzymes (HDMs and HTMs) are specific to cell types and highly correlated with target gene expression [198]. A recent study examined the expression profiles of 16 different histone-modifier genes including HATs, HDACs, and HDMs in breast cancer. They found that significantly different expression levels of histone-modifier genes exist between breast tumors and normal tissue, and their findings were significantly associated with conventional pathological parameters and clinical outcomes. So, it appears that histone-modifier enzymes offer utility as biomarkers and potential for targeted therapeutic strategies [199].

After these recent findings, miRNAs also have become the target for developing therapies for breast cancer. The miRNA-based treatments, in combination with traditional chemotherapy, may be a new strategy for the clinical management of drug-resistant breast cancers in the near future [200]. One of the initial studies has concluded that miRNA-221/222 confers breast cancer fulvestrant resistance by regulating multiple signaling pathways [201].

Conclusion and Future Perspective

A new field has been opened to developing effective clinical therapies now that we understand the importance of epigenetic alterations. In contrast to genetic code, the epigenetic codes may be easily affected by aging, environmental stimuli, and food in heritable manner. Breast cancer is a multifactorial disease with molecular, histological, and phenotypic diversity caused by the interaction of both inherited and environmental risk factors. The importance of epigenomics for breast cancer development has been realized after gaining of great amount of knowledge by large-scale methods. Epigenetics-based therapy for breast cancer will most likely become a reality in the near future.

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Nutrigenomics in Breast Cancer

6

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Abstract

Environmental factors and genetic makeup play a central role in phenotypic appearance of a trait via central dogma of biology. Epigenetic, transcriptomics, and proteomics are the key players of the expression biology which make the difference in structure and function. Environmental factors include the various exposure factors by oral intake, air, and via the skin commonly. Several bioactive food components, including both essential and nonessential nutrients, can regulate gene expression patterns. Thus, nutrigenomics is providing the effects of ingested nutrients and other food components on gene expression and gene regulation, i.e., diet–gene interaction in order to spot the dietetic components having beneficial or detrimental health effects. Nutritional genomics (nutrigenomics), the junction between health, diet, and genomics, is influenced via epigenetic, transcriptomics, and proteomics processes of biology. Thus, it will help in determining the individual nutritional requirements based on the genetic makeup of the person (personalized diet) as well as the association between diet and chronic diseases like cancer, opening new vistas to understanding the complexity of breast cancer and leading to its better management.

Keywords

Nutrigenomics • Breast cancer • Carcinogenesis • Isoflavones • Vitamins • Lycopene

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Introduction

Worldwide, breast cancer accounts for 22.9 % of all cancers (excluding nonmelanoma skin cancers) in women. In 2008, breast cancer caused 458,503 deaths worldwide (13.7 % of cancer deaths in women) [1]. Among all breast cancer cases, only 5–10 % are believed to be due to inherited susceptibility, and thus environmental

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factors including one's eating habits are likely major contributors to risk.

Current cancer models include those that are inherited through the germ line and represent only ~5 % of total cases of human cancers. These tumors initiate because of mutational events. The remaining ~95 % originate as sporadic events and grow as a result of exposure to the environment, which includes exposure to both environmental contaminants and dietary agents. The etiology of cancers, and particularly breast cancer, is still not very clear. Several researchers have provided various mechanisms of carcinogenesis, but since it is very complex and interwoven with multiple factors, so the current knowledge is still in infancy [2]. It is estimated that a third of all cancer deaths can be attributed to dietary factors. This is due to both a lack of intake of protective natural components in an individual's diet, such as polyphenols, sterols, flavonoids, and carotenoids, as well as exposure to natural carcinogens in the diet, such as aflatoxin, fumonisin, and heavy metals. Bioactive food components can influence a number of physiological processes: apoptosis, metabolism, cell differentiation and growth, DNA repair, hormone regulation, inflammation, etc.

Nutrigenomics includes the determination of individual nutritional requirements based on the genetic makeup of the person, as well as the association between diet and chronic disease. Nutrigenomics is part of a broader movement toward personalized medicine, focusing on a personalized diet. Nutrigenomics is linked to nutrigenetics, which studies the genetic basis of the different individual response to the same nutritional stimulus. This phenomenon arises from gene polymorphism. As a consequence, genes are important in determining a function, but nutrition is able to modify the degree of gene expression [3]. The risk of certain cancers such as breast cancer increases in association with Western diets as compared to the Mediterranean or native Mexican diets. Therefore, nutrition strategies need to be developed to prevent the effects of carcinogenic agents, target and eliminate premalignant lesions at the early stages, and antagonize (i.e., induce apoptosis) the proliferation of clonal neoplastic populations [4].

The ultimate goal of nutrigenomics is that of developing genomics-based biomarkers that help in the early detection and prevention of diet-related diseases, including cancer. To reach this goal, it is essential to develop tissue-specific dietary responses that can be used as signatures or fingerprints to estimate risk [5]. The availability of nutritional biomarkers at early stages (e.g., initiation) may be used as prognostic tools. A complicating factor is that the diet contains a large number of compounds and that each nutrient has different gene targets and affinities. An example is the cross talk of estrogens and isoflavones with estrogen receptors and how this interaction may affect the development and prevention of breast cancer [6].

Nutritional genomics (nutrigenomics), the junction between health, diet, and genomics, can be seen as the combination of molecular nutrition and genomics. The diverse tissue- and organ-specific effects of bioactive dietary components include gene expression patterns (transcriptome); organization of the chromatin (epigenome); protein-expression patterns, including post-translational modifications (proteome); as well as metabolite profiles (metabolome) as represented in Fig. 6.1, which shows bioactive food components and their integration with various molecular streams in phenotype development and progression. Table 6.1 represents the factors affecting nutrigenomics and phenotype. Nutrigenomics will promote an increased understanding of how nutrition influences metabolic pathways and homeostatic control and how this regulation is concerned in the early phases of diet-related disease and the extent to which individual sensitizing genotypes contribute to such diseases. Nutrigenomics will also identify the genes involved in physiological responses to diet and the genes in which small changes, called polymorphisms, may have significant nutritional consequences and the influence of environmental factors on gene expression [7].

Exploitation of this genomic information, along with high-throughput "omic" technologies, allow the acquisition of new knowledge aimed at obtaining a better understanding of nutrient–gene interactions depending on the genotype, with the ultimate goal of developing personalized nutrition strategies for optimal health

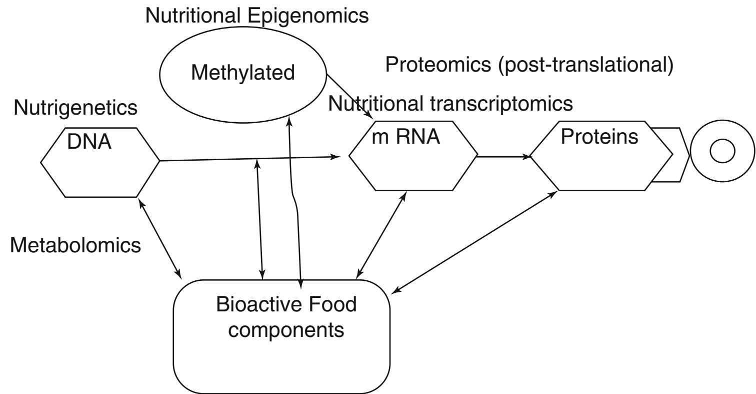

Fig. 6.1 Bioactive food components and its integration with various molecular streams in phenotype progression and development

Table 6.1 Factors affecting nutrigenomic research and phenotype

Approach	Definition and factors
Transcriptomics	The branch of molecular biology that deals with the study of messenger RNA molecules produced in an individual or population of a particular cell type
	Identification of transcription factors that respond to nutrients and gene targets
	RNA amplification and procedure (quantity, quality, replicates, real-time PCR, high-density analysis)
	Quantity of starting tissue/cell material
	Fold change in expression
	Intraindividual and interindividual variations in healthy and diseased subjects and Identification of single genes or group of genes that are regulated (up or down) in a particular disease or nutritional condition
	Heterogeneity of cell populations and single cell gene expression profiling
	Combination of gene variants (SNPs)
	Data processing and interpretation
Epigenetics	It is defined as heritable changes in gene expression, which are not due to any alteration in the DNA sequence
	Characterization of chromatin modifications that influence gene expression and impact of nutrients
	Histone modifications
	DNA methylation
	Nucleosome organization
	Order, interdependence and intradependence, and reversibility of histone modifications
	Cross talk and mutual dependency between histone modifications, DNA methylation, and methyl-binding proteins
	Help in exploring the evolutionary origin of cell differentiation and change in cancer cells
Proteomics	It is the large-scale study of proteins, particularly their structures and functions
	Linking gene expression studies with protein functions
	Tissue and cellular localization
	Plasma levels
	Expression levels
	Posttranslational modifications
	Protein–protein interactions
	Cellular function
	Bioinformatics and data interpretations
Metabolomics	It is the scientific study of chemical processes involving metabolites
	Linking exposure to biological effects induced by metabolites
	Interindividual differences in metabolisms and disposition
	Measurements of metabolite in specimens
	Recovery methods from tissue/plasma

Adapted from Milner et al. [2]

and disease prevention [8]. There are three central factors that underpin nutrigenetics and nutrigenomics as a central science. First there is great diversity in the inherited genome between ethnic groups and individuals which affects nutrient bioavailability and metabolism. Second, people differ greatly in their food/nutrient availability and choices depending on cultural, economic, geographical, and taste perception differences. Third, malnutrition (deficiency or excess) itself can influence gene expression and genome stability; the latter leading to mutations at the gene sequence or chromosomal level, which may cause abnormal gene dosage and gene expression leading to adverse phenotypes during the various life stages [9].

The decisive goal is to (1) match the nutriome (i.e., nutrient intake combination) with the current genome status (i.e., inherited and acquired genome) so that genome maintenance, gene expression, metabolism, and cell function can occur normally and in a homeostatically sustainable manner [8] and (2) provide better interpretation of data from epidemiological and clinical intervention studies regarding health impacts of dietary factors that may help to revise recommendations for personalized nutrition [10].

The fundamental hypotheses reinforcing the science of nutrigenetics and nutrigenomics are the following [9]:

- Nutrition may apply its impact on health outcomes by directly affecting expression of genes in critical metabolic pathways and/or indirectly by affecting the incidence of genetic mutation at the base sequence or chromosomal level which in turn causes alterations in gene dosage and gene expression.
- The health effects of nutrients and nutriomes (nutrient combinations) depend on inherited genetic variants that alter the uptake and metabolism of nutrients and/or the molecular interaction of enzymes with their nutrient cofactor and hence the activity of biochemical reactions.
- Better health outcomes can be achieved if nutritional requirements are customized for each individual, taking into consideration both his/her inherited and acquired genetic characteristics depending on life stage, dietary preferences, and health status.

Genomic and epigenomic processes likely do not utterly account for the ability of dietary factors to influence phenotypic changes, since changes in the rate of transcription of genes (transcriptomics) can also be fundamental to cellular processes [11]. Multiple pathways appear to overlap as a cause of multiple diseases [12]. Thus, the examination of these pathways via transcriptomic profiles may simultaneously provide important hints about multiple disease risks. Noteworthy, several bioactive food components, including both essential and nonessential nutrients, can control gene expression patterns. Their influence on gene transcription and translation is not only concentration dependent but also time dependent [13]. Yet these changes may provide significant insights about the specificity of individual food components to influence one or more biological processes, including those involved in the risk of cancer development and/or tumor behavior.

This chapter will try to clarify the current research updates on interaction of diet with genetic backgrounds and how diet is involved in the development of one of the most common cancers afflicting females today globally. Simultaneously we will pinpoint the various diets that can be used in curing or decreasing the risks, although the nature of these interactions is indeed very complex.

Nutrigenomic Diseases

Diseases that are known to be associated with the interactions between multiple genetic and environmental factors such as diet include many cancers, diabetes, heart disease, obesity, and some psychiatric disorders. Therefore, both disciplines aim to unravel diet–genome interactions; however, their approaches and immediate objectives are distinct. Nutrigenomics will unravel the optimal diet from within a series of nutritional alternatives, whereas nutrigenetics will yield critically important information that will assist clinicians in identifying the optimal diet for a

given individual, i.e., personalized nutrition [10]. The following five tenets of nutritional genomics serve as a conceptual basis for understanding the focus and promise of this budding field: [7]

1. Under certain conditions and in some individuals, diet can be a serious risk factor for a number of diseases.
2. Universal dietary chemicals can act on the human genome, either directly or indirectly, to alter gene expression or structure.
3. The degree to which diet influences the balance between healthy and disease states may depend on a person's genetic makeup.
4. Some diet-modulated genes (and their normal, common variants) probably play a role in the onset, incidence, progression, and/or severity of chronic diseases.
5. Dietary intervention based on the knowledge of nutritional requirements, nutritional status, and genotype (i.e., personalized nutrition) can be employed to prevent, mitigate, or cure chronic disease.

Nutrigenomics and Carcinogenesis

Cancer is a process composed of multiple stages in which gene expression, and protein and metabolite function, begins to operate aberrantly [14]. In the post-genomic era, the cellular events mediating the onset of carcinogenesis, in addition to their modulation by dietary factors, have yielded significant information in understanding of this disease [15]. Inherited mutations in genes can increase one's susceptibility for cancer. Evidences of genome and epigenome damage biomarkers, in the absence of overt exposure of genotoxins, are themselves sensitive indicators of deficiency in micronutrients required as cofactors or as components of DNA repair enzymes, for maintenance methylation of CpG sequences and prevention of DNA oxidation and/or uracil incorporation into DNA [16]. Diet is considered as a source of either carcinogens (intrinsic or cooking generated) present in certain foods or constituents acting in a protective manner (vitamins, antioxidants, detoxifying enzyme-activating substances, etc.) [17]. It is clear that carcinogen metabolism-affecting polymorphisms may modify the chance of contact between carcinogens and target cells, thus acting at the stage of cancer initiation. Influences of polymorphisms of gene encoding factors involved in hormonal regulation are most strongly manifested in hormone-dependent tumors such as breast, prostate, ovarian, and endometrial cancers. Polymorphisms in sex hormone receptor genes comprising those encoding estrogen receptor, progesterone receptor, and androgen receptor have been shown to be associated with cancer risk modulation [18]. Dietary factors can undoubtedly interact with hormonal regulation. Obesity strongly affects hormonal status. At the same time, some food components, such as phytoestrogens, are known to be processed by the same metabolic pathways as sex hormones [19]; thus their cancer-preventive effect can be modulated by the polymorphisms.

Epigenetic Link with Nutrigenomics

An important emerging part of nutrient–gene interaction studies with the potential for both intra- and transgenerational effects is epigenetics [20]. Epigenetics refers to the processes that regulate how and when certain genes are turned on and off, while epigenomics pertains to analysis of epigenetic changes in a cell or the entire organism. Epigenetic processes have a sturdy influence on normal growth and development, and this process is deregulated in diseases such as cancer. Diet on its own or by interaction with other environmental factors can cause epigenetic changes that may turn certain genes on or off. Table 6.2 provides a brief description of the nutrients and chemicals involved in DNA methylation. Epigenetic silencing of genes that would usually protect against a disease, as a result, could make people more susceptible to developing that disease later in life. The epigenome, which is heritable and modifiable by diet, is the global epigenetic pattern determined by global and gene-specific DNA methylation, histone modifications, and chromatin-associated proteins that control expression of housekeeping genes and restrains the expression of parasitic DNA such as

transposons. Table 6.3 represents the dietary chemicals, DNA methylation, and its mechanism of action.

One study has demonstrated that sulforaphane, butyrate, and allyl sulfur are effective inhibitors of histone deacetylase (HDAC). HDAC inhibition was associated with global increases in histone acetylation, enhanced interactions of acetylated histones with the promoter regions of the P21 and BAX genes, and higher expression of p21Cip1/Waf1 and BAX proteins [33]. Importantly, sulforaphane has been reported to reduce HDAC activity in humans [33]. Future research likely needs to relate HDAC changes in humans to a change in a cancer-related process. Furthermore, since acetylation is only one method to regulate histone homeostasis [34], greater concentration needs to be given to how nutrition might influence the other types of histone modifications.

Table 6.2 Nutrients and chemicals involved in DNA (hyper-/hypo-) methylation

Nutrient	Chemicals
Alcohol	Genistein
Arsenic	Methionine
Betaine	Nickel
Cadmium	Polyphenol
Choline	Selenium
Coumestrol	Vitamin A
Equol	Vitamin B6
Fiber	Vitamin B12
Folate	Zinc

Adapted from Trujillo et al. [21], Davis and Uthus [22]

The field of nutrigenomics harnesses multiple disciplines and includes dietary effects on genome stability (DNA damage at the molecular and chromosome level), epigenome alterations (DNA methylation), RNA and micro-RNA expression (transcriptomics), protein expression (proteomics), and metabolite changes (metabolomics), all of which can be studied independently or in an integrated manner to diagnose health status and/or disease trajectory. However, of these biomarkers, only DNA damage is a clear biomarker of fundamental pathology that may

Table 6.3 Dietary chemicals, DNA methylation, and its mechanism of action

Dietary chemicals	Mechanism of action	Phenotype/outcome	Reference
Alcohol	Affects folate metabolism, altering DNA methylation	Cancer susceptibility	[23]
Arsenic	Compete with cytosine, DNA methyl transferase and selenium for methyl donation from S-adenosil-1-methionine	Global hypomethylation in the liver, cancer susceptibility	[24]
Choline	Deficiency in diets has been associated with decreased tissue S-adenosil-1-methionine	Hepatic tumorigenesis, cancer susceptibility	[25]
Folate	Its deficiency has complex effect on DNA methylation depending on cell type, organ, and development stage	Cancer susceptibility	[26]
Genistein	Dietary genistein can migrate tumorigenic process via promoter modulation of gene expression	Mitigates tumorigenesis	[27]
Lycopene	It has direct DNA demethylating activity. It migrates tumorigenic processes via promoter methylation modulation of gene expression	Mitigates tumorigenesis	[28]
Methionine	Its deficiency decreases tissue SAM resulting in global DNA hypomethylation and HCC in rodents	HCC	[29]
Nickel	Environmental carcinogen, induce de novo methylation of tumor-suppressor genes Suppressive effect on histone H4 acetylation in mammalian cells	Cancer susceptibility	[30]
Selenium	Its deficiency decreases DNA methylation. Low intake influences the activity of selenoproteins, causing changes in mRNA levels for the encoding genes	Cancer susceptibility	[31]
Vitamins	Vitamins (B2, B6, and B12) are necessary cofactors in one carbon (methyl metabolism)	Affect several metabolic pathways, cancer susceptibility	[32]

be mitigated by promotion of apoptosis of genetically aberrant cells or by reducing the rate of DNA damage accumulation. Changes at the epigenome, transcriptome, proteome, and metabolome levels may simply reflect modifiable homeostatic responses to altered nutritional exposure and on their own may not be sufficient to indicate definite irreversible pathology at the genome level.

DNA damage can be diagnosed in a number of complementary ways as follows: (1) damage to single bases (e.g., DNA adducts such as the addition of a hydroxyl radical to guanine caused by oxidative stress), (2) basic sites in the DNA sequence (measurable by use of the aldehyde-reactive probe), (3) DNA strand breaks (commonly measured using the Comet assay), (4) telomere shortening (measured by terminal restriction fragment length analysis, quantitative PCR, or flow cytometry), (5) chromosome breakage or loss (usually measured using micronucleus cytome assays or metaphase chromosome analysis), and (6) mitochondrial DNA damage (usually measured as deletions or base damage in the circular mitochondrial DNA sequence). These DNA damage biomarkers are presently at different levels of validation based on evidence relating to the association with nutrition (cross-sectional epidemiological and intervention studies) and disease (cross-sectional epidemiology and prospective cohort studies) [35]. The micronucleus assay in cytokinesis-blocked lymphocytes is currently the best validated biomarker for nutritional genomic studies of DNA damage.

Given the advances in diagnostic technologies assessing DNA damage, it has now become feasible to determine dietary reference values for DNA damage prevention and to start translating into practice the Genome Health Clinic concept of DNA damage prevention [35]. The latter is based on the recognition that damage to the genome is the most elementary cause of developmental and degenerative diseases, which can be accurately diagnosed and prevented by appropriate diet and lifestyle intervention at a genetic subgroup and personalized level. The ability of diet to affect the flow of genetic information can occur at multiple sites of regulation [10]. Advances in genomics, transcriptomics, proteomics, and metabolomics have enabled a more rapid and comprehensive understanding of how bioactive compounds affect human health. Dietary bioactive compounds can be tested for their potential health-promoting properties by applying these different technologies to cell culture, and animal or human studies. Each experimental approach offers unique strengths and has certain limitations.

Current Updates of Nutrigenomic Studies in Breast Cancer

Several studies of sporadic breast cancers have shown that fruits and vegetables [36], fish, monounsaturated and polyunsaturated fatty acids [37], vitamin D, calcium, and phytoestrogens may reduce the risk of breast cancer, although there are inconsistencies in the literature. High intake of meat, poultry, total energy, and total fat and saturated fatty acids has been reported to be associated with increased risk for breast cancer [38]. Malmö Diet and Cancer cohort examined the association between dietary folate equivalents (DFE) and breast cancer among carriers of two genetic polymorphisms for MTHFR gene (MTHFR 677C/T and 1298A/C). A positive association between DFE and breast cancer among women carriers of MTHFR 677CT/TT-1298AA occurred while an inverse association was observed in 677CT-1298 AC women [39]. In a nested case-control study, Maruti et al. reported that postmenopausal women with two copies of variant T alleles (TT genotype) had increased risk of breast cancer. In addition, the intake of other B vitamins may influence the relationship between the MTHFR genetic variants and breast cancer risk. It has been found that the most pronounced MTHFR-breast cancer risk was observed among women with the lowest intakes of dietary folate and vitamin B6 [40].

In a nested case-control study within the Singapore Chinese Health Study, it has been observed that there is an inverse relationship between breast cancer risk and low folate intake and weekly/daily green tea intake compared with

reduced green tea intake. Also, women carrying the high-activity MTHFR/TYMS genotypes with 0–1 variant allele and weekly/daily green tea intake had a lower breast cancer risk, especially women who also had low folate intake. No association was observed among women carriers of two variant alleles. These findings suggest one of the mechanisms through which green tea can provide protection against breast cancer is through folate regulation [41]. The anticancer effect of the EGCG (tea polyphenol (−)-epigallocatechin-3-gallate) may be mediated by the regulation of epigenetic processes. It was found that EGCG can lead to ERα reactivation in the ERα-negative breast cancer cell lines by remodeling the chromatin structure of the ERα promoter by the alteration of the histone acetylation and methylation status [42]. Further, it has been reported that EGCG inhibits the telomerase by decreasing the hTERT promoter methylation and ablating the histone H3 Lys9 acetylation in the MCF-7 cell lines [43].

Another study reported the inverse relationship between green tea intake and breast cancer risk among Asian-Americans [44] as a function of the catechol-*O*-methyltransferase (COMT) genotype. This enzyme is recognized to be involved in the metabolism of the tea polyphenols. More specifically, a reduced risk of breast cancer was observed only among tea (green and black) drinker carriers of at least one low-activity COMT allele. These findings of reduced breast cancer risk with tea catechins, especially in women who had the low-activity COMT alleles, suggest that these women were less efficient in eliminating tea catechins, therefore optimizing the benefits from the tea and its associated bioactive constituent [45].

The Shanghai Breast Cancer Study examined the relationship between breast cancer risk, GSTP1 genetic variants, and other diet components, the cruciferous vegetables. The GSTP1 Val/Val genotype was associated with increased breast cancer risk, especially in premenopausal women with low intake of cruciferous vegetables. Thus, cruciferous vegetable intake with high isothiocyanates may reduce breast cancer risk and modify the effect of the GSTP1 genotype [45], and not necessarily be beneficial to all women.

Finally, the response to marine *n*-3 fatty acids with breast cancer risk may depend on genetics. Women with genetic variants that encode lower or no enzymatic activity of GSTT1 have a 30 % lower breast cancer risk from the marine *n*-3 fatty acids, when compared with women with high-activity genotypes. These data suggest that the peroxidation products of *n*-3 fatty acids may be involved in the protection against breast cancer [46]. This is not that unusual since other food components have been reported to inhibit tumors by generating free radicals [47]. Watercress, a rich source of phenethyl isothiocyanates (PEITC), has been proposed to have anticancer activity. A crude watercress extract was reported to inhibit cancer cell growth and hypoxia-inducible factor (HIF) activity and reduced angiogenesis by decreasing the phosphorylation of the translation regulator 4E binding protein 1 (4E-BP1) [48].

In a breast case-control study that examined the association of the 5-lipoxygenase gene (ALOX) and 5-lipoxygenase-activating protein gene (ALOX5AP) polymorphisms and dietary linoleic acid intake with breast cancer risk, it was found that women carriers of two variant alleles for the ALOX5AP 4900 A/G who had a diet rich in linoleic acid had a greater breast cancer risk compared with women carrying AG or GG genotypes. These results propose that genetic predisposition related to *n*-6 polyunsaturated fatty acid metabolism should be taken into account when the relation between dietary fat and breast cancer risk is examined [49]. Furthermore, dietary omega-3 polyunsaturated fatty acids downregulate the expression of the polycomb group (PcG) protein, enhancer of zeste homologue 2 (EZH2) in breast cancer cells. This study reported a decrease in histone 3 lysine 27 trimethylation (H3K27me3) activity of EZH2 and upregulation of E-cadherin and insulin-like growth factor-binding protein 3. The treatment with omega-3 PUFAs led to decrease in the invasion capacity of the breast cancer cells [50].

Additionally, a nested case-control study of postmenopausal women examined the interaction between oxidative stress-related genes

including catalase (CAT) C262T, myeloperoxidase (MPO) G463A, endothelial nitric oxide synthase (NOS3) G894T, and heme oxygenase-1 (HO-1) GT (*n*) dinucleotide length polymorphism and level of vegetable and fruit intake on breast cancer risk. The study found that women with low intake of vegetables and fruits and the low-risk CAT CC genotypes appeared to be associated with increased breast cancer risk, especially those women with four or more low-risk alleles, suggestive of the role of endogenous and exogenous antioxidants in breast carcinogenesis [51].

Iwasaki et al. examined the effect of four SNPs in cytochrome P450c17alpha (CYP17), aromatase (CYP19), 17beta-hydroxysteroid dehydrogenase type I (17beta-HSD1), and sex hormone-binding globulin (SHBG) genes on the association between isoflavone intake and breast cancer risk. The study identified an inverse association between isoflavone intake and breast cancer risk among women with at least one variant allele for the 17beta-HSD1 polymorphism and among postmenopausal Japanese women with GG genotype for the SHBG gene, indicating that genetic variants of the 17beta-HSD1 and SHBG genes may modify the relationship between isoflavone intake and breast cancer risk [52]. The association between isoflavones and breast cancer risk may be explained by the antiestrogenic effect of the isoflavones and the effect on the DNA methylation. It has been reported that the daily administration of isoflavones to healthy premenopausal women led to dose-specific changes in RARbeta2 and CCND2 gene promoter methylation, changes that correlated with genistein levels. Additionally, an inverse correlation between estrogenic marker complement C3 and genistein was observed, suggesting an antiestrogenic effect [53]. Furthermore, genistein has been associated with specific epigenetic changes. For example, the long-term exposure to genistein of the MCF-7 breast cancer cell lines has been found to lead to reduced expression of the acetylated histone 3 (H3). Additionally, this exposure was associated with alteration in growth responses to mitogenic factors and histone deacetylase inhibitors [54].

Dietary berries have been suggested to influence breast cancer risk (AICR Report), although considerable variability in response is observed. Preclinical studies demonstrate that tumor formation is suppressed as a result of the levels of E(2)-metabolizing enzymes during the early phase of E(2) carcinogenesis [55].

A nested case-control study within the Singapore Chinese Health Study Cohort showed a significant interaction between the level of green tea drinking and the activity of the angiotensin-converting enzyme (ACE) with respect to breast cancer risk depending on ACE gene polymorphism [56]. Even the BRCA breast cancer-associated gene is affected by diet. A diet rich in fruits and vegetables protects a woman from the BRCA gene becoming activated [57]. Olive oil is an integral ingredient of the "Mediterranean diet" and accumulating evidence suggests that it may have a potential role in lowering the risk of several types of cancers. A number of epidemiological studies have linked consumption of olive oil with a reduced risk of cancer, and researchers are increasingly investigating this association further in laboratory studies. The mechanisms by which the cancer-preventing effects of olive oil as having novel anticancer actions may relate to the ability of its monounsaturated fatty acid (MUFA) oleic acid (OA; 18:1n-9) to specifically regulate cancer-related oncogenes. Exogenous supplementation of cultured breast cancer cells with physiological concentrations of OA was found to suppress the overexpression of HER2 (Her-2/neu, erbB-2), a well-characterized oncogene playing a key role in the etiology, progression, and response to chemotherapy and endocrine therapy in approximately 20 % of breast carcinomas.

OA treatment was also found to synergistically enhance the efficacy of trastuzumab (Herceptin) a humanized monoclonal antibody binding with high affinity to the ectodomain (ECD) of the Her2-coded p185(HER2) oncoprotein. Moreover, OA exposure significantly diminished the proteolytic cleavage of the ECD of HER2 and, consequently, its activation status, a crucial molecular event that determines both the aggressive behavior and the response to

trastuzumab of Her2-overexpressing breast carcinomas. Recent findings further reveal that OA exposure may suppresses HER2 at the transcriptional level by upregulating the expression of the Ets protein PEA3 -a DNA-binding protein that specifically blocks HER2 promoter activity in breast, ovarian, and stomach cancer cell lines. This anti-HER2 property of OA offers a previously unrecognized molecular mechanism by which olive oil may regulate the malignant behavior of cancer cells. From a clinical perspective, it could provide an effective means of influencing the outcome of Her-2/neu-overexpressing human carcinomas with poor prognosis. Indeed, OA-induced transcriptional repression of HER2 oncogene may represent a novel genomic explanation linking olive oil and cancer, as it seems to equally operate in various types of Her-2/neu-related carcinomas [58].

In another study, OA treatment in Her-2/neu-overexpressing cancer cells was found to induce upregulation of the Ets protein polyomavirus enhancer activator 3 (PEA3), a transcriptional repressor of Her-2/neu promoter. Also, an intact PEA3 DNA-binding site at endogenous Her-2/neu gene promoter was essential for OA-induced repression of this gene. Moreover, OA treatment failed to decrease Her-2/neu protein levels in MCF-7/Her2-18 transfectants, which stably express full-length human Her-2/neu cDNA controlled by a SV40 viral promoter. OA-induced transcriptional repression of Her-2/neu occurs through the action of PEA3 protein at the promoter level [59].

Various Diet Components and Their Cellular/Molecular Effects on Breast Cancer

All main signaling pathways are deregulated in cancer, including cell proliferation, apoptosis, DNA repair, carcinogen metabolism, inflammation, immunity, differentiation, and angiogenesis. Increasingly, evidence points to each of these as molecular targets for cancer prevention. Since several of these sites appear to be tailored by multiple dietary components, it becomes challenging to tease apart nutrient–nutrient interactions and thus what constitutes an ideal diet for health promotion [2]. For example, the apoptosis or programmed cell death that is essential in the fight against cancer through two pathways—either the intrinsic, mitochondrial-mediated pathway, or the extrinsic, death receptor-mediated pathway—could be a target for dietary bioactive agents including genistein, curcumin, resveratrol, luteolin, lupeol, indole 3-carbinol, etc. [4]. Dietary components can modulate apoptosis through effects at different levels, all of which culminate in changes in gene expression. Table 6.4 provides details on transcription factor pathways mediating nutrient–gene interactions.

Table 6.4 Transcription factor pathways mediating nutrient–gene interactions [60]

Macronutrients	Compound	Transcription factor
Fats	Fatty acids Cholesterol	PPARs, SREBPs, LXR, HNF4, ChREBPs, LRs, FXR
Carbohydrates	Glucose	USFs, SREBPs, ChREBP
Proteins	Amino acids	C/EBPs
Micronutrients		
Vitamins	Vitamin A Vitamin D Vitamin E	RAR, RXR, VDR, PXR
Minerals	Calcium	Calcineurin/NF-ATs
	Iron	IRO1, IRP2
	Zinc	MTF-1
Other food components		
Soy	Flavonoids	ER, NF-Kb, AP1
	Xenobiotics	CAR, PXR

Compounds such as Japanese knotweed (*Polygonum c.*), 20 % resveratrol, ginger, (*Zingiber off.*) 5 % gingerols, Rosemary (*Rosemarinus off.*), 6 % carnosic acid, 1 % rosemarinic acid, 1.5 % ursolic aicd, etc have demonstrated broad-spectrum, multi-targeting, anticancer effects, as well as disease-preventive and health-promoting benefits. The other important aspect of these compound-rich foods, spices, and herbs is that have been regularly used by many cultures throughout the world. Cancer prevention studies have exposed that all of the major signaling pathways deregulated in different types of cancer are affected by nutrients. Pathways studied

include carcinogen metabolism, DNA repair, cell proliferation/apoptosis, differentiation, inflammation, oxidant/antioxidant balance, and angiogenesis [61].

So far, more than 1,000 different phytochemicals have been identified with cancer-preventive activities [62]. Dietary fibers have a protective effect against bowel cancer. Long-chain polyunsaturated fatty acids (LC-PUFA) beneficially affect physiological processes, including growth; neurological development; lean and fat mass accretion; reproduction; innate and acquired immunity; infectious pathologies of viruses, bacteria, and parasites; and the incidence and severity of virtually all chronic and degenerative diseases including cancer, atherosclerosis, stroke, arthritis, diabetes, osteoporosis, neurodegenerative, inflammatory, and skin diseases [63]. Fish oil, rich in omega-3 fatty acids, inhibits the growth of colonic tumors in both in vitro and in vivo systems [64].

Bioactive components present in fruits and vegetables can prevent carcinogenesis by several mechanisms, such as blocking metabolic activation through increasing detoxification. Plant foods can modulate detoxification enzymes as flavonoids, phenols, isothiocyanates, allyl sulfur compounds, indoles, and selenium [65]. As a result of carcinogen activation, covalent adducts with the individual nucleic acids of DNA or RNA are formed. It has also been found that reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals attack DNA bases, resulting in potential mistranscription of DNA sequence [66]. Such disruptions can interfere with DNA replication and thus produce mutations in oncogenes and tumor-suppressor genes. ROS can also result in breakage of DNA strand, resulting in mutations or deletions of genetic material [67].

Vitamin D

Few findings demonstrate that primary circulating form of vitamin D $1,25(OH)_2D$ acts in a cell type and tissue-specific manner. For example, $1,25(OH)_2D$ inhibits cell growth of both normal and tumor cells by inhibiting the transition for the G_1 to the S phase of the cell cycle [68]. This effect was mediated by increased expression of cyclin A1 in ovarian cancer cells [69], whereas breast cancer cells had increased expression of cyclin D2 [70]. It has also been suggested that the enzyme responsible for the degradation of vitamin D metabolites, CYP24, can also be influenced by cancer. The CYP24 gene was amplified in breast tumors [71].

The majorities of established breast cancer cell lines express transcriptionally active VDR and undergo growth inhibition in response to 1,25D [72]. In general, VDR expression and sensitivity to 1,25D-mediated growth arrest is higher in the less aggressive, estrogen receptor (ER)-positive breast cancer cell lines such as MCF-7 than in ER-negative cell lines. Tumor cells derived from VDR null mice were used to conclusively demonstrate that 1,25D mediates effects in breast cancer cells via the nuclear VDR [73]. Screening for molecular changes induced by 1,25D or vitamin D analogs in various breast cancer cells has identified scores of VDR regulated genes and proteins in diverse pathways, indicating a broad range of downstream [74] involved in cell cycle (cyclins, cyclin-dependent kinases and their inhibitors), apoptosis/autophagy (bcl-2 family, caspases, cathepsins), and inflammation (NFkB, prostaglandins, cox-2). The net effect of these changes is to block mitogenic signaling, including that of estrogen, EGF, IGF-1, and KGF, and to enhance the effects of negative growth factors such as TGFb. In many breast cancer cell lines, 1,25D-mediated growth arrest is associated with the induction of differentiation markers such as casein, lipid droplets, and adhesion proteins [75]. Notably, 1,25D exerts additive or synergistic effects in combination with other triggers of apoptosis, such as ionizing radiation and chemotherapeutic agents [76].

Collectively, these studies indicate that a wide variety of signaling pathways, cell cycle and apoptotic regulatory proteins, and proteases contribute to the antiproliferative, pro-differentiating, and apoptotic effects of 1,25D depending on the specific breast cancer cell line and/or context. In primary cultures of normal human mammary epithelial (HME) cells, vitamin D signaling also mediates growth arrest and induction of

differentiation markers such as E-cadherin, but apoptosis has not been observed [77]. In contrast to breast cancer cells, non-transformed mammary cells retain expression of CYP27B1 and generate 1,25D when incubated with physiological concentrations of 25D. Many breast cells also express the megalin–cubilin complex, which mediates internalization of 25D bound to the vitamin D binding protein [78]. Autocrine metabolism of 25D triggers chemopreventive effects in breast epithelial cells including growth inhibition, differentiation, and protection from various cellular stresses [77].

In the intact mammary gland, the epithelium is surrounded by stromal fibroblasts and adipocytes, which provide critical growth factor signals for development and also impact on carcinogenesis. Recent evidence suggests that breast adipocytes express CYP27B1 and generate 25D, which signals via adipocyte VDR to release inhibitory factors that regulate mammary epithelial cell growth [79]. Since vitamin D metabolites are stored in fat tissue, the contribution of adipocyte signaling to the tumor-suppressive actions of vitamin D in mammary gland are likely of physiological importance and require further study. As in colon cancer, acquisition of the transformed phenotype in breast cells is associated with deregulation of the vitamin D pathway [80]. In HME cells, introduction of SV40 large T antigen and/or oncogenic ras induces transformation and reduces responsiveness to 25D in association with downregulation of VDR and CYP27B1 [81]. Oncogenes and tumor-suppressor genes that impact on VDR expression in breast cells include ras, p53, and slug, which act via diverse mechanisms including transcriptional regulation and mRNA instability [82]. The mechanism by which transformation abrogates CYP27B1 expression in breast cells is not yet known.

Green Tea

From a nutrigenomic perspective, the use of green tea as a nutraceutical or functional food has shown anticancer potential, in particular for breast cancer, although more studies are needed. High levels of angiotensin II have been associated with an increased risk of breast cancer development in humans [56]. The angiotensin I-converting enzyme (ACE) gene encodes or activates the enzyme that converts angiotensin I to the active angiotensin II. A low conversion rate is linked with lower rates of breast cancer in women than in those who have a high conversion rate. In those with the high-activity genotype, a high consumption of green tea has resulted in a dramatic drop of one-third in the risk of developing breast cancer. The authors concluded that the antioxidant properties (particularly of the EGCG) are protective against the reactive oxygen species (or free radicals) generated by the high levels of angiotensin II. No such association was made in women with the low levels of angiotensin. Epigallocatechin gallate (ECGC) and other green and black tea polyphenols inhibit cancer cell survival. EGCG suppressed androgen receptor expression and signaling via several growth factor receptors. Cell cycle arrest or apoptosis involved caspase activation and altered Bcl-2 family member expression. EGCG inhibited telomerase activity and led to telomere fragmentation. While at high concentrations polyphenols had pro-oxidative activities, at much lower levels, antioxidative effects occurred [83].

Another study conducted at the University of Southern California looked at green tea ingestion and activity of the catechol-O methyl transferase (COMT) gene [45]. Dr Wu's group also found an association between green tea intake and a cancer-protective effect in those individuals with at least one copy of the low conversion COMT gene. This means that the beneficial catechins remained in circulation for a longer time period and reduced the risk of breast cancer. It is important to note that the study was only conducted in Asian-Americans and needs to be reproduced in a wider population group.

Soy and Isoflavones

The interest on isoflavones in breast cancer prevention derives from the fact that breast

Table 6.5 Various studies on isoflavones and nutrigenomic approaches in breast cancers

Experimental model	Dietary bioactive compounds	Reference
Human MCF-7 breast cancer cells	Natural estrogens (1,7 beta estradiol, estriol, estrone, genistein)	[85]
Human MCF-7 breast cancer cells	Isoflavones (genistein, daidzein, glycitein, biochanin A, and ipriflavone), flavones (chrysin, luteolin, and apigenin), flavonols (kaempferol and quercetin), and coumestan, flavanone and chalcone (coumestrol, naringenin, and phloretin, respectively)	[86]
Human MCF-7 breast cancer cells	Genistein	[87]
Human MCF-7, T47D breast cancer cells	Genistein	[88]
FVB female mice	Isoflavones, of which 66.5 % was genistein, 32.3 % daidzein, and 1.2 % glycitien	[89]

cancer risk for women residing in geographical areas of high consumption of soy products during puberty is lower compared to that of women living in Western countries, and Asian women who had a low soy intake [84]. Table 6.5 gives details on various studies on isoflavones and nutrigenomic approaches in breast cancers. However, clinical trials reported small [90] or no effect of supplementation with isoflavones on breast cancer risk [91], and administration of isoflavones elicited in some cases an estrogen-like effect. Other studies indicated that the reduction in breast cancer risk due to soy intake was limited to Asian populations [92]. A case-control study conducted in Southeast China in 2004–2005 reported that premenopausal and postmenopausal women in the highest quartile of total isoflavone intake had a reduced risk for all receptor (ER/PR) status of breast cancer with a dose–response relationship. The protective effect was more pronounced for women with ER+/PR+and ER–/PR– breast tumors [93].

Several factors may be accountable for the inconsistent effects of soy-related diets on cancer outcome. These include age, reproductive history, genetic background, dose and timing of exposure, and dietary patterns. For example, because of their binding affinity for the ER, isoflavones may function as agonists or antagonists depending on the concentration. The differential binding of isoflavones to the ER may interfere with or activate the genomic actions of the ER. Moreover, the agonist/competing effects of isoflavones for the ER may be modified by interactions with polymorphisms for the ER [94]. For example, polymorphisms in the ERβ have been shown to modify the association between isoflavone intake and breast cancer risk [95]. Given the role of cross talk between ER and isoflavones in breast cancer risk, genome-wide studies are required to examine the effects of isoflavones and exposure levels on promoter sequences that are targeted by the ER. DNA microarray technologies have been used to monitor genome-wide effects by isoflavones.

Soy and its processed products (tofu, tempeh, miso, natto, soy milk, and soy-based yogurts and desserts) are the only sources providing high quantities of isoflavones in the human diet. Soy intake has been reported in some cases to be linked to a reduction in breast cancer risk. Recently, Satih et al. identified 278 and 334 differentially expressed genes after treatment with two soy constituents, genistein and daidzein, respectively, in estrogen-positive (MCF-7) and estrogen-negative (MDA-MB-231, MCF-10a) cells [96]. Isoflavone intake has been estimated to be 25–50 mg/day in Asian countries, and there is a lower risk of breast cancer in areas of high soy and isoflavone intake, especially in Asia [97]. A smaller risk reduction for breast cancer (odds ratio 0.86, 95 % CI 0.75–0.99), stronger for premenopausal women, was found in a meta-analysis compiling 6 cohort and 12 case-control studies [90]. Asian women whose soy intake was high during puberty experienced lower risk for

breast cancer than women who did not consume soy products or did so only as adults [98].

Messina et al. recently discussed the published and ongoing clinical breast cancer studies [91]. Three double-blind randomized controlled trials reported no effect of a 1–2-year isoflavone supplementation on mammographic density used as a marker of breast cancer risk [99]. A 2-week administration of a soy supplement (45 mg/day isoflavones) increased epithelial cell proliferation and progesterone receptor (PR) expression in normal breast tissue, suggesting an estrogen agonist effect [100]. Mechanism of isoflavones as anticancerous have been reported to modulate steroid biosynthesis, transport and metabolism, as well as carcinogen activation and detoxification, to inhibit cell proliferation induced by growth factors, to induce cell cycle arrest or apoptosis, to favor cell differentiation, to reduce oxidative stress, or to inhibit angiogenesis, cell invasiveness, and metastasis [101]. They may act through modulation of cell signaling (direct binding to nuclear receptors, modification of the phosphorylation state of some signal transduction proteins), regulation of gene expression, and/or specific inhibition of some key enzyme activities. In addition to the inhibition of cell proliferation, isoflavones can induce apoptosis in the human breast and prostate cells at concentrations over 25 and 20 mM, respectively [102]. Catechins, for example, belong to the flavonoid family, which are polyphenolic compounds available in foods of plant origin, and there is much research into their beneficial effects as well as multi-mechanisms. Several epidemiological studies have reported that consumption of flavonoids, and especially catechins, might function as chemopreventive agents against cancer [103].

Gamma-Linolenic Acid (GLA)

GLA is an essential omega-6 fat that is found in evening primrose, black currant seed, borage oil, and pine seed oil and can inhibit the action of the cancer gene Her-2/neu. This gene is responsible for almost 30 % of all breast cancers. When cancer cells that overexpress the Her-2/neu gene are treated with GLA, it not only helps suppress the cancer-causing gene but also causes up to a 40-fold increase in response to the drug Herceptin (trastuzumab), which is used as part of breast cancer treatment. GLA also selectively affects cancer cells without damaging normal cells. This is especially good news because patients who possess the HER2/neu gene also typically have an aggressive form of the disease and a poor prognosis. GLA is one of two essential fatty acids, which are necessary for the normal functioning and growth of cells, nerves, muscles, and organs [104].

Data derived from epidemiological and experimental studies suggest that alpha-linolenic acid (ALA; 18:3n-3), the main omega-3 polyunsaturated fatty acid (PUFA) present in the Western diet, may have protective effects in breast cancer risk and metastatic progression. A recent pilot clinical trial assessing the effects of ALA-rich dietary flaxseed on tumor biological markers in postmenopausal patients with primary breast cancer demonstrated significant reductions in tumor growth and in HER2 (erbB-2) oncogene expression. The molecular mechanism by which ALA inhibits breast cancer cell growth and metastasis formation may involve a direct regulation of HER2, a well-characterized oncogene playing a key role in the etiology, progression, and response to some chemo- and endocrine therapies in approximately 20 % of breast carcinomas. In a recent study, ALA exposure was found to dramatically repress the activity of HER2/neu. Moreover, the nature of the cytotoxic interaction between ALA and trastuzumab revealed a significant synergism. Omega-3 fatty acids suppress overexpression of the HER2 oncogene at the transcriptional level, which, in turn, interacts synergistically with anti-HER2 trastuzumab-based immunotherapy [59].

Caffeine

Knowledge gained by incorporating genetic variation into a nutrition study not only provides a more rational basis for giving personalized dietary advice but will also improve the quality of

evidence used for making population-based dietary recommendations for the prevention of specific diseases. Caffeine is metabolized primarily by the cytochrome P450 1A2 (CYP1A2) enzyme, and a polymorphism in the *CYP1A2* gene determines whether individuals are "rapid" caffeine metabolizers (those who are homozygous for the −163 A allele) or "slow" caffeine metabolizers (carriers of the −163 C allele) [105].

A similar concept was utilized in an observational study of coffee and breast cancer. The study associated a lower risk of breast cancer among slow metabolizers [106]. No protective effect was observed among fast metabolizers, implicating caffeine as the protective component of coffee. This study also suggested that caffeine protects against breast cancer in women with a BRCA1 mutation and illustrated the importance of integrating individual genetic variability when assessing diet–disease associations. This is consistent with findings from animal studies showing that caffeine inhibits the development of mammary tumors [107].

Alcohol

Alcohol has long been considered a risk factor for breast cancer in women [108]. The International Agency for Research on Cancer has declared that there is enough scientific evidence to classify alcoholic beverages a group 1 carcinogen that causes breast cancer in women [109]. Group 1 carcinogens are the substances with the clearest scientific evidence that they cause cancer, such as smoking tobacco. A study of more than 1,280,000 middle-aged British women concluded that for every additional drink regularly consumed per day, the incidence of breast cancer increases by 1.1 % [110]. Approximately 6 % (between 3.2 and 8.8 %) of breast cancers reported in the UK each year could be prevented if drinking was reduced to a very low level (i.e., less than 1 unit/week). Among women, breast cancer comprises 60 % of alcohol-attributable cancers [111]. A study of 17,647 nurses found that high drinking levels more than doubled risk of breast cancer with 2 % increase risk for each additional drink per week consumed. Binge drinking of 4–5 drinks increases the risk by 55 % [112]. Moreover, alcohol consumption is related to promoter methylation of E-cadherin in breast cancer [113].

There is growing evidence to indicate that dietary fiber, in particular digestion-resistant starch, promotes bowel health, and one of the areas of focus for experimental research is its potential protection against the development of colorectal cancer [114]. Additional studies have shown that butyrate, one of the predominant short-chain fatty acids produced from the fermentation of resistant starch by the gut bacteria, may be responsible for its physiological effects [115]. While the cellular effects of butyrate are well documented, numerous studies have been conducted in order to explain the mechanisms by which butyrate may elicit its antitumorigenic effects.

There is substantial evidence that alcohol consumption increases breast cancer risk. In a pooled analysis of the six largest cohort studies with data on alcohol and dietary factors [116], the risk for breast cancer increased monotonically with increasing intake of alcohol. For a 10 g/day increase in alcohol, breast cancer risk increased by 9 % (95 % CI = 4–13 %). Adjustment for other breast cancer risk factors had little impact. Beer, wine, and liquor all contribute to the positive association, strongly suggesting that alcohol per se is responsible for the increased risk. In an intervention study, it is reported that consumption of approximately one to two alcoholic drinks per day increased estrogen levels in premenopausal and postmenopausal women [117], suggesting a mechanism by which alcohol may increase breast cancer risk.

In several large prospective studies, high intake of folic acid appeared to mitigate completely the excess risk for breast cancer due to alcohol [118, 119]. This relationship was recently confirmed using plasma folic acid levels [120]. The public health recommendations for alcohol are complicated because consumption of one to two alcoholic beverages per day probably protects against cardiovascular disease. Because cardiovascular disease is the leading cause of death among women, moderate drinking is associated

overall with a modest reduction in total mortality [121]. However, avoiding alcohol appears to be one of relatively few methods for reducing breast cancer risk, whereas many methods exist to reduce risk for cardiovascular disease. For women choosing to consume alcohol regularly, use of a multivitamin to ensure adequate folic acid intake may decrease breast cancer risk.

Lycopene

Using pangenomic array technology, it has been demonstrated that lycopene supplementation (10 μM–48 H) modulates many molecular pathways by affecting the expression of apoptosis and cell cycle-related genes [122], as well as xenobiotic metabolism, fatty acid biosynthesis, and gap junctional intercellular communication. Further, one other study emphasized that lycopene may be a significant dietary element involved in breast cancer prevention. This study observed upregulation of apoptosis-related genes such as PIK3C3 and Akt1. PIK3C3 belongs to the phosphoinositide (PI)3-kinase family involved in both receptor-mediated signal transduction and intracellular trafficking. PI3K proteins generate specific inositol lipids involved in the regulation of cell growth, proliferation, survival, differentiation, and cytoskeletal changes. This study also showed upregulation of MAPK-related genes such as heat shock protein *HSPA1B*, the fibroblast growth factor *FGF2*, and *FOS*, a major component of the activator protein-1 (AP-1) transcription factor complex, which includes members of the JUN family [123]. The AP-1 transcription factor is a regulator of processes essential for normal growth and development as well as carcinogenesis. One of the best-characterized targets of PI3K lipid products is the protein kinase Akt, or protein kinase B (PKB) [124]. Serine/threonine protein kinase Akt mediates signals from epidermal growth factor receptor to the apoptosis-related genes including BRCA1 through activated Ras and PI3K in the estrogen and other signaling pathways [125]. Moreover, Viglietto et al. demonstrated that Akt regulates cell proliferation in breast cancer cells by preventing $p27^{Kip1}$-mediated growth arrest [126].

Vitamin E

Vitamin E has inhibited mammary tumors in rodents in some experiments [127]. In1984, Wald et al. demonstrated a prospective study of 5,004 women. Thirty-nine of these women who developed breast cancer had lower levels of plasma E in the blood that had been collected between 1968 and 1975 than did 78 controls [128]. Researchers declared a clear association of the lower vitamin E levels with a higher risk of breast cancer. A similar prospective study by Willett et al. claimed no such association with cancer when the slightly lower plasma vitamin E levels, in those who later developed cancer, were adjusted for serum cholesterol levels [129]. A strong request to support studies to validate the hypothesis that vitamin E can reduce a woman's risk for development of breast cancer has been made by London et al. [130]. It has been suggested that γ-TmT, γ-tocopherol, and δ-tocopherol may be involved in inhibiting tumor formation. Probable mechanism of actions in inhibiting breast cancer could be inducing PPARγ expression and consequently reducing the expression of ERα; inducing Nrf2, which consequently reduces inflammation and oxidative stress; and inhibiting cell proliferation while inducing apoptosis [131].

Vitamin A

Vitamin A consists of preformed vitamin A from animal sources, and carotenoids found mostly in fruits and vegetables. Many carotenoids are potent antioxidants and may provide a defense against reactive oxygen species that damage DNA. Vitamin A also regulates cell differentiation and may thus prevent carcinogenesis. In a cohort of Canadian women (519 cases) [132], a marginally significant protective association between total vitamin A intake, preformed vitamin α- and β-carotene, and breast cancer was seen. With 14 years of follow-up in the Nurses'

Health Study (2,697 cases) [133], an inverse association with total vitamin A was seen only among premenopausal women. This inverse association was mainly accounted for by intakes of β-carotene and lutein/zeaxanthin and was strongest among women with a family history of breast cancer. However, in an extended follow-up of the Canadian cohort (1,452 cases) and in a Swedish cohort (1,271 cases), little overall association was seen between intake of carotenoids and breast cancer [134, 135].

An alternative to the dietary assessment of vitamin A intake is the measurement of vitamin A compounds in the blood. In the two largest studies based on blood samples collected before diagnosis [136, 137], low levels of β-carotene and other carotenoids were associated with an approximately twofold increase in risk for breast cancer. Thus, available data from observational studies suggest a possible protective effect of vitamin A intake, particularly carotenoids, on breast cancer risk, particularly in premenopausal women. Ideally, the effect of vitamin A supplements should be evaluated in randomized trials. However, the β-carotene arm of the Women's Health Study (a breast cancer-prevention trial conducted in 40,000 women) was terminated in 1996 after reports that β-carotene supplements appeared to increase the risk for lung cancer among smoking men. Thus, data from randomized trials on specific carotenoids and breast cancer risk may never be available.

Types of Fat

Specific types of fat could differentially influence the risk for breast cancer. In most animal studies, diets high in polyunsaturated fat, but typically at levels beyond human exposure, have evidently increased the occurrence of mammary tumors. A positive association has not been found in prospective epidemiologic studies [138]. In a pooled analysis of cohort studies [138], saturated fat (compared with carbohydrate) was weakly associated with higher risk for breast cancer (RR for 5 % of energy = 1.09; 95 % CI = 1.00–1.19). In a recent prospective study conducted in premenopausal women in the Nurses' Health Study II [139], intake of animal fat and high-fat dairy foods was associated with a 33–36 % increase in risk for breast cancer for the highest compared with the lowest quintile of intake. Total fat per se was not associated with breast cancer risk, suggesting that other constituents of dairy foods consumed early in adult life may increase breast cancer risk.

Total Fat

Preclinical and human ecological studies have suggested an association between increased dietary fat intake and breast cancer risk [140], while cohort studies revealed less consistent effects [141]. For instance, a case-control study (414 cases and 429 controls) established no association between breast cancer and dietary fat intake [142]. Similarly, observational studies on the influence of dietary fat on breast cancer recurrence have produced mixed results [143]. The variable associations may be due to differences of fat intake in the study population, difficulty in accurately measuring fat intake with diet-assessment methods, and high correlation between dietary fat and other diet and lifestyle variables [144]. Most investigators assume that any observed tumor-enhancing effect of dietary fat needs to be adjusted statistically for energy intake. However, this is not a clear-cut issue because a change in fat composition of the diet may cause alterations in energy intake. Thus, higher energy intake resulting from changes in fat intake may be considered as one of the mechanisms by which fat affects tumor development.

Dietary fat may also play a role in the development of breast cancer via hormone metabolism. This may be particularly relevant for ER-positive cancers, as an elevation of endogenous estrogen levels with increased fat intake is thought to be related to breast cancer [145]. Alternatively, any role for dietary fat in breast cancer may be less direct. For example, high-fat diets may lead to greater body mass or obesity, a probable risk factor for postmenopausal breast cancer. In postmenopausal women, high-fat intake may increase levels of bioavailable estrogens, thus elevating the risk of breast cancer.

Furthermore, higher-fat intake in childhood or adolescence may promote faster growth and earlier onset of menarche, both established risk factors for breast cancer [145].

Saturated Fat

Giving more weight to prospective studies, Wakai et al. reported no relationship between saturated fat intake and breast cancer risk in 26,291 subjects from the Japan Collaborative Cohort Study [146]. On the other hand, combined analysis of 12 case-control studies revealed an increased risk of postmenopausal breast cancer with higher saturated fat intake, giving an overall OR of 1.57 ($p < 0.0001$) for the uppermost quintile of intake; this estimate was adjusted for total fat intake, which was also associated with increased risk [147] and in view of the relationship between breast cancer and foods high in saturated fat, such as meat and dairy products [141, 147]. On the other hand, the observed associations for meat consumption may reflect a true effect of saturated fat [147].

Monounsaturated Fatty Acids (MUFAs)

Olive oil is a rich source of MUFAs, and in case-control studies, it has been revealed that the risk of breast cancer is decreased with the consumption of more than one teaspoon of olive oil per day (OR =0.75; 95 % CI: 0.57–0.98) [148]. Antioxidants present in olive oil, such as vitamin E, have been considered to be one of the protective constituents [149]. However, a meta-analysis of 17 case-control and eight cohort studies found no association between MUFAs and breast cancer risk [141]. The relationship between MUFAs intake and breast cancer risk appears to depend on the contributing foods.

Polyunsaturated Fatty Acids (PUFAs)

Nkondjock et al. conducted a case-control study of 414 cases and 429 population-based controls and observed no overall association between PUFAs and breast cancer risk [150]. Similarly, a combined analysis of 12 case-control studies indicated no statistically significant association between postmenopausal breast cancer risk and PUFA intake [147]. Furthermore, another cohort study noted a similar absence of association [139]. Diets high in PUFAs may not be associated with breast cancer risk independently of any contribution to total fat intake. Thus, contrary to data from animal experiments, human studies do not show an increase of breast cancer risk with PUFA intake. Estimating the risk associated with PUFA intakes remains difficult as food composition tables for these fatty acids are incomplete.

Zinc

Zinc (Zn) is an essential trace element required for maintaining both optimal human health and genomic stability. Zn plays a critical role in the regulation of DNA repair mechanisms, cell proliferation, differentiation, and apoptosis involving the action of various transcriptional factors and DNA or RNA polymerases. Zn is an essential component for more than 1,000 proteins including copper/Zn superoxide dismutase (SOD) as well as a number of other Zn finger proteins. Zn is an essential cofactor or structural component for important antioxidant defense proteins and DNA repair enzymes, such as Cu/Zn SOD, OGG1, APE, and PARP. Thus, it may play an important role in breast cancer prevention.

Recently, it was shown that the migration potential of MDA-MB-231 cells on fibronectin, demonstrated in the control, was not affected by a low level of zinc (2.5 μM), but was significantly inhibited by higher levels of zinc (5–50 μM). Zinc at 5–50 μM also reduced magnesium-dependent cell adhesion to fibronectin, likely through interfering with magnesium-dependent integrin activation, and induced cell rounding in the normally elongated, irregular-shaped MDA-MB-231 cell lines of human breast carcinoma [151].

Selenium

Studies conducted with methylseleninic acid (MSA), a synthetic mammary cancer chemopreventive agent, in the rat mammary tumor model point out that this form of selenium is able to block clonal expansion of premalignant lesions

and induce apoptosis [152]. Similar cellular responses are replicated with human premalignant breast cells grown in culture using MSA [153]. These investigators characterized the profile of gene expression changes after 4 weeks in the whole mammary tissue of rats treated with methylnitrosourea (MNU) and fed MSA [153].

In a follow-up study, Dong et al. further examined the cellular and molecular effects of MSA in premalignant human breast cells (MCF10AT1 and MCF10AT3B) [153]. MSA inhibited growth of both cell lines in a dose- and time-dependent manner, induced apoptosis, and blocked cell cycle progression at the G1 phase. These organoselenium compounds altered additional genes (BCL-2, BAD, CYCLIN D1, P27, APO1, P21, CASPASE-3, CMYC, PCNA) thereby leading to inhibition of cell proliferation and induction of apoptosis. As stated above, modulations of apoptosis and cell proliferation by selenium can account for chemoprevention during the post-initiation phase of mammary carcinogenesis. Clearly, using mammary adenocarcinomas, the results of this study showed that selenium has an impact on genes that are involved in the multistep carcinogenesis process [154].

Recently, human cellular glutathione peroxidase I was found not only to be a selenium-dependent enzyme that protects against oxidative damage and its peroxidase activity but also to be associated with cancer risk in the lung and breast [155].

Conclusion and Future Perspective

From the data discussed in this chapter, it is evident that elucidations of the mechanisms of action of bioactive compounds are complex and entail simultaneous examination of alternations in gene expression (transcriptomics), study of molecular relationships between nutrients and genes (nutrigenetics), influence changes in the profile of proteins (proteomics), study of multiple signaling and metabolic pathways (metabolomics), and associations of different nutritive compounds that exert synergistic, additive, or opposing effects. DNA methylation and histone modifications are epigenetic events that mediate heritable changes in gene expression and chromatin organization in the absence of changes in the DNA sequence. Examples of large-scale breast cancer studies based on modern highly sensitive and specific instruments like MS spectrometry microarray, real-time PCR on related genes like BRCA1 and other single nucleotide polymorphisms and apoptotic pathways, cell cycle, epigenetic modifications with nutritive compounds that may be negative, positive, or neutral response in their result of cancer treatment, risk prediction, prognosis, and diagnosis.

No single lab will be able to manage the concept of a personalized nutrition alone; rather, a collective effort by the scientific community to adhere to guidelines put forth regarding experimental designs, analysis, and data storage will generate a database that is readily available to researchers and clinicians alike. Thus, all records and databases of nutritional strategies targeted to the prevention of breast cancer may open new vistas in better management of breast cancer patients.

It is becoming increasingly evident that nutrigenomics is taking a central stage in the investigation of the effect of nutrition on health outcomes like breast cancer and that impacts of nutrients can be evaluated comprehensively by a multitude of modern “omic” technologies and biomarkers. In this view, the future lies not with the technologies, but with the storage, management, and interpretation of the immense quantity of metabolomic data. The realization that genetic background, gender, and life stage can have an impact on nutritional requirements is becoming increasingly evident; thus, we are progressing toward the personalize diet concept. Translation of this knowledge into recommendations based on genotype or at the individual level is only practical in those few cases (as galactosemia), when the effect of genotype clearly overpowers the impact of any other factor and is the ultimate determining factor of the nutritional and health status for an individual or genetic subgroup. Thus, nutrigenomic research is now providing a new ray of hope in better management of breast cancer.

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7 Long Noncoding RNAs in Breast Cancer: Implications for Pathogenesis, Diagnosis, and Therapy

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Abstract

Human genome mapping has revealed that protein-coding genes represent less than 2 % of the total genome sequence, and simultaneously more than 75 % of the genome is actively transcribed into RNA. Recent studies of the human transcriptome led to the discovery of new heterogeneous group of transcripts—noncoding RNAs. The major part of these ncRNAs consists of long noncoding RNAs (lncRNAs), which differ in size, location on genome, and biological functions. Generally, through distinct mechanisms, they affect a number of biological processes, such as modulation of protein activity, alternative splicing of mRNA, and epigenetic regulation or microRNA silencing, and play a key role in transcriptional and post-transcriptional gene expression regulation. Deregulated levels of lncRNAs were observed with a wide range of tumors, including breast cancer. Gene expression patterns of lncRNAs are able to distinguish normal and tumor tissue or even various breast cancer stages, which makes them a potential diagnostic and prognostic biomarkers or therapeutic targets.

Keywords

Breast cancer • Long noncoding RNA • lncRNA • lincRNA • Diagnosis • Prognosis • Therapeutic targets

Introduction

Breast cancer is the most frequently diagnosed cancer and also the leading cause of cancer death among females worldwide [1]. It is a heterogeneous disease, which can be divided by various approaches into many molecular subgroups. Despite the notable progress in diagnosis and therapy, a significant number of breast cancer patients with the same diagnostic profile indicate distinctly different clinical outcomes [2, 3].

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This diversity presents a challenge for better molecular classification and personalized therapy. Therefore, one of the current goals in breast cancer research is to find sensitive and specific noninvasive biomarkers, which can be used for early stage breast cancer detection, as well as for monitoring of the disease and response to therapy [4]. Such biomolecules include long noncoding RNAs, recently described in various types of cancer, including breast cancer.

Despite the central dogma of molecular biology, which understands RNAs as a tool for protein synthesis, there is a large number of RNAs which, instead of coding the protein, act as functional RNA. Moreover, with development of transcriptome analytical methods, it was found that these noncoding RNAs constitute the major part of the human genome [5]. Originally, these RNAs were considered as waste, but now it is clear that they significantly affect diverse cellular pathways. An important and biggest group of noncoding RNAs are the long noncoding RNAs, endogenous cellular molecules with a length of 200 nt to 100 kb [6]. lncRNAs are often caped, polyadenylated, and spliced, yet do not overlap other protein-coding genes [7]. Recent research has shown that lncRNAs plays a key role in transcriptional and posttranscriptional regulation of gene expression [8]. Unsurprisingly, as with other noncoding RNAs, different expressions of lncRNAs were observed in tumor and non-tumor tissue as well. This fact confirms involvement of long noncoding RNAs in tumorigenesis [9].

The regulatory function of RNAs was described in 1961 by François Jacob and Jacques Monod [10], whereas first individual lncRNAs H19 and XIST (X-inactive-specific transcript), which is critical to X chromosome inactivation, were identified a few decades later [11, 12]. However, these RNAs were only discovered to be non-protein coding; therefore, a true milestone was a study by Okazaki et al., who studied mouse genome using large-scale sequencing and defined lncRNAs as a separate class of transcripts [13]. It is estimated that the number of lncRNAs is 7,000–23,000; nevertheless, the list of functionally validated lncRNAs is much shorter (approximately 200lncRNAs) [14].

The aim of this chapter is to provide a short introduction to long noncoding RNAs classification and biology. We also describe basic methods for high-throughput and functional analysis of lncRNAs. The main part is focused on the roles of lncRNAs in pathogenesis and diagnosis of breast cancer and their potential usage in prediction of prognosis or targeted therapy.

Classification and Biology of Long Noncoding RNAs

A growing number of newly annotated long noncoding RNAs together with their varied length and biological functions explain the need for a clear categorization. Some classification approaches, like categorization by position relative to coding genes, splicing, and polyadenylation status or by molecular mechanism [15–17], are not accurate because individual lncRNAs may represent more subgroups. Although we currently lack satisfactory classifications for these transcripts, here we summarize recently discovered groups of lncRNAs as long intergenic noncoding RNAs, long intronic noncoding RNAs, long ncRNAs with dual functions, telomere-associated lncRNAs, pseudogene RNAs, and transcribed-ultraconserved regions [18] (See Table 7.1).

Long noncoding RNAs are a diverse group of transcripts, which differ in size, location in the genome, and other biological properties. Such diversity is also the reason why a wide range of functions was observed in lncRNAs. Long ncRNAs can affect gene expression via RNA polymerase II inhibition (B2 SINE) or chromatin modification (COLDAIR). They can also serve as precursors of siRNAs (H19) and other small ncRNAs (GAS5). By forming complexes with proteins, they may modulate its activity (SRA), influence structural and regulatory functions (XIST), change protein localization, or affect epigenetic processes (HOTAIR). They are also involved in alternative splicing of mRNA (MALAT-1) and are responsible for microRNA silencing (HULC) (See Fig. 7.1).

Table 7.1 Classification of lncRNAs: their characteristics and meaning in biological processes and diseases

Class	Symbol	Characteristic	Disease/biological function associations
Long intergenic noncoding RNAs	lincRNAs	Ranging from hundreds to tens of thousands nts; lie within the genomic intervals between two genes; transcriptional cis-regulation of neighboring genes	Involved in tumorigenesis and cancer metastasis/involved in diverse biological processes such as dosage compensation and/or imprinting
Long intronic noncoding RNAs		Lie within the introns; evolutionary conserved; tissue and subcellular expression specific	Aberrantly expressed in human cancers/possible link with posttranscriptional gene silencing
Telomere-associated lncRNAs	TERRAs	100 bp–9 kb; conserved among eukaryotes; synthesized from C-rich strand; polyadenylated; form inter-molecular G-quadruplex structure with single-stranded telomeric DNA	Possible impact on telomere-associated diseases including many cancers/negative regulation of telomere length and activity through inhibition of telomerase
Long noncoding RNAs with dual functions		Both protein-coding and functionally regulatory RNA capacity	Deregulation described in breast and ovarian tumors/modulate gene expression through diverse mechanisms
Pseudogene RNAs		Gene copies that have lost the ability to code for a protein; potential to regulate their homologous protein-coding genes; made through retrotransposition; tissue specific	Often deregulated during tumorigenesis and cancer progression/regulation of tumor suppressors and oncogenes by acting as microRNA decoys
Transcribed-ultraconserved regions	T-UCRs	Longer than 200 bp; absolutely conserved between orthologous regions of human, rat, and mouse; located in especially intra- and intergenic regions	Expression is often altered in some cancers; possible involvement in tumorigenesis/antisense inhibitors for protein-coding genes or other ncRNAs

Long Intergenic Noncoding RNAs

Long intergenic noncoding RNAs (also known as lincRNAs) were originally identified by Guttman et al., who used methods to reconstruct the transcriptome of a mammalian cell [19]. They described an evolutionary conserved group of noncoding RNAs with a range in length from a few hundred to tens of thousands of bases. Genes of lincRNAs are localized in regions of DNA between two protein-coding genes, but they lack any protein-coding capacity and open reading frames. To date, more than 8,000 lincRNAs have been identified, but most of them remain unannotated [8, 20], and therefore the functions of lincRNAs are largely unknown. However, the involvement of lincRNAs in biological processes has been found, including cell-cycle regulation, imprinting, embryonic stem cell pluripotency, and cell proliferation [21, 22].

The most unclear part of lincRNAs biology is the basis of their molecular mechanism of action. In this context, targeting of chromatin modification complexes (i.e., histone-modifying enzymes) is frequently mentioned, which directly leads to gene expression regulation [8]. Some lincRNAs interact with numerous effector proteins and thus control their levels (i.e., XIST). Others can affect alternative splicing (MALAT-1) by controlling levels of splicing factors [23]. Recently,

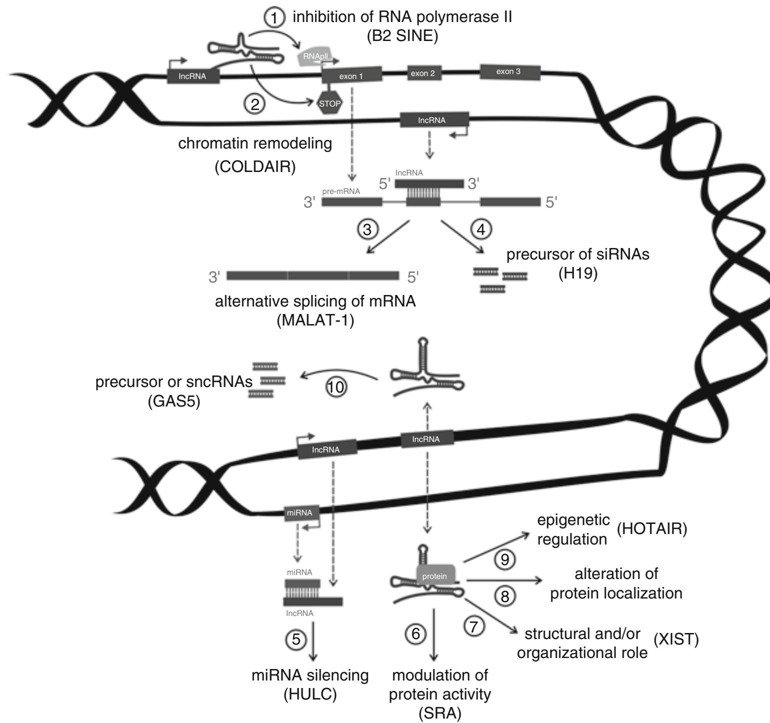

Fig. 7.1 Schematic illustration of lncRNAs functioning. lncRNA transcribed from an upstream noncoding promoter can negatively (*1*) or positively (*2*) affect expression of the downstream gene by inhibiting RNA polymerase II recruitment and/or inducing chromatin remodeling, respectively. lncRNA is able to hybridize to the pre-mRNA and block recognition of the splice sites by the spliceosome, thus resulting in an alternatively spliced transcript (*3*). Alternatively, hybridization of the sense and antisense transcripts can allow Dicer to generate endogenous siRNAs (*4*). The binding of lncRNA to the miRNA results in the miRNA function silencing (*5*). The complex of lncRNA and specific protein partners can modulate the activity of the protein (*6*), is involved in structural and organization roles of the cell (*7*), alters the protein localizes in the cell (*8*), and affects epigenetic processes (*9*). Finally, long ncRNAs can be processed to the small RNAs (*10*)

it has been demonstrated that lincRNAs can act as competitive inhibitors of microRNAs termed “microRNA sponges.” Such molecules contain binding sites for specific miRNAs and thus may regulate their level [24].

As mentioned, lincRNAs regulate significant number of genes, but their expression is under genetic control as well [25]. Individual lincRNAs are transcriptionally regulated by important transcription factors such as p53, NFκB, Sox2, Oct4, and Nanog [19]. Interestingly, Juan et al. suggest that some miRNAs may bind lincRNAs and cause their repression [26].

Relevance for translational medicine stems from the fact that long intergenic noncoding RNAs are remarkably tissue specific and deregulated within a large number of diseases, including cancer. Different levels of lincRNAs were observed at various stages of breast cancer, so based on these gene expression patterns they may serve as potential prognostic markers [9].

Long Intronic Noncoding RNAs

More than one-third of conserved noncoding regions in human genome consist of intronic regions. John Mattick first suggested that sequence of introns is not random and that introns may be involved in gene regulation [27]. According to this, it was found that about 81 % of human protein-coding genes have transcriptionally active introns [28]. Finally, discovery of numerous evolutionarily conserved regions

in introns that match the size of lncRNAs led to the identification of a new class of transcripts—long intronic noncoding RNAs. These RNAs are exclusively expressed in the nucleus, and it is expected that intronic ncRNA expression would be responsive to common physiological signals, e.g., hormones [29]. The biogenesis is poorly understood, but involvement of RNA polymerase II (RNAP II) is assumed. The presence of poly(A+) tail may serve as indirect evidence [18].

The main role of intronic noncoding RNAs is posttranscriptional regulation of gene expression. In his study, Louro et al. described some mechanisms by which RNAs can regulate gene expression [30]. Interestingly, it was found that intronic ncRNAs can serve as precursors of smaller noncoding RNAs. Another mechanism is a direct interaction with promoters, which decrease the expression of the protein-coding RNA. Intronic ncRNAs can also affect RNA alternative splicing by forming RNA–RNA duplexes. Finally, they are probably able to stabilize protein-coding RNA localized on the same locus [28].

Some oncogenes or tumor suppressor genes have noncoding RNAs transcribed from their introns. This might be one of reasons why altered expression of intronic ncRNAs with various malignancies was detected. Moreover, level of long intronic ncRNAs significantly correlates with different degrees in renal, prostate, and pancreatic carcinoma [31–33].

Long ncRNAs with Dual Functions

According to the central dogma of molecular biology, RNA was considered as an intermediated molecule required for the formation of protein. After the discovery of noncoding RNAs, which in many aspects resembled the mRNA, but lack protein-coding capacity, it was obvious that RNA can act either as a functional or protein-coding molecule. Hence, it was a big surprise when bifunctional RNAs were found. Such RNAs serve both as intermediate molecules translated into protein and as functional RNA [34].

Functions of RNAs are dependent on their secondary and tertiary structure, so the presence of isoforms can be important for bifunctional character [35]. Well described is the steroid receptor RNA activator (SRA), whose RNA is a noncoding RNA that coactivates several human hormone receptors like progesterone, estrogen, and androgen. Moreover, isoforms of SRA are also expressed to produce proteins. SRA transcripts have been identified in normal human tissues, and increasingly SRA RNA is expressed in breast and ovarian tumors. Interestingly, higher levels of noncoding isoforms of SRA were observed in tumor tissue [36, 37].

Telomere-Associated lncRNAs

Telomeres—heterochromatic complexes located on linear chromosome ends—are formed by tandem repeats of the TTAGGG sequence. With each run of cell division, one telomeric hexanucleotide is lost, which finally leads to chromosome destabilization. Therefore, telomeres protect chromosomes from degradation and repair activities [38, 39]. Until recently, it was assumed that telomeres are transcriptionally silent, but a recently discovered group of long noncoding RNAs confirmed that telomeres are transcribed into telomeric repeat-containing RNA (TERRA or Tel RNA). TERRA transcripts range between 100 bp and 9 kb and originate in the subtelomeres of telomeric C-rich strand [40, 41]. Based on RNA-FISH techniques, it was identified that TERRA associates with telomeric chromatin [42]. Other studies have suggested a likely role of TERRA in the enzyme telomerase regulation [43]. Finally, TERRA seems to be involved in negative regulation of telomere length [44].

Pseudogene RNAs

For a long time, pseudogenes were considered as failed copies of coding genes that had lost the capability to produce proteins. Nevertheless, recent research has revealed the ability of pseudogenes to regulate homologous protein-coding genes [45]. Pseudogenes may arise as a result of simple mutations or be generated by retrotransposition,

during which reverse-transcribed RNAs are integrated into the genomic sequence [46, 47]. Many pseudogenes are transcribed into RNA, which can be later processed into smaller RNAs. Thus, gene expression regulation is based on an RNA interference process. Interestingly, pseudogenes can affect gene expression regulation by acting as miRNA decoys [48]. Some studies provide evidence that pseudogenes (i.e., MYLKP1) are involved in cancerogenesis and suggest them as potential diagnostic and therapeutic targets in cancer [49].

Transcribed-Ultraconserved Regions

The last newly discovered class of ncRNAs is known as transcribed-ultraconserved regions (T-UCRs). Overall, 481 T-UCRs were annotated—all of them are genomic segments of more than 200 base pairs [50]. They are extremely evolutionarily conserved among mammals [51] and are localized especially in intra- and intergenic regions [52]. The degree of conservation may have a fundamental functional importance for ontogeny and phylogeny of mammals. Untranslated UCRs may serve as distal enhancers [53]; on the contrary transcripts they are involved in gene expression regulation as antisense inhibitors for protein-coding genes. Calin et al. found that the expression of many T-UCRs is altered in some types of cancer, especially in adult chronic lymphocytic leukemias, colorectal and hepatocellular carcinomas, and neuroblastomas [54]. Accordingly, it was found that T-UCRs are often located at fragile regions of chromosomes. Specific transcribed-ultraconserved regions are also associated with prognosis and response to therapy, which makes them promising targets in cancer research [53, 55].

Methods for High-Throughput Analysis of Long Noncoding RNAs in Cancer

The human genome mapping has revealed that more than 90 % of the genome is transcribed. Application of high-throughput techniques highlighted the complexity of mammalian transcriptome and led to the discovery of long noncoding RNAs, a class of regulatory noncoding RNAs [16, 56]. As a transcriptional class, lncRNAs were first described during the large-scale sequencing of full-length cDNA libraries in the mouse [13]. Such large-scale cDNA analysis and genome annotations can detect or predict thousands of lncRNAs, but their biological functions remain, in most cases, unknown [57]. Methods used in the study of lncRNAs can be thus divided by purpose into (1) high-throughput methods designed for lncRNAs identification (microarrays, RNA sequencing), (2) methods designed for verification of high-throughput data (qRT-PCR, northern blot, FISH, RNAi), and (3) methods designed for detection of RNA–protein interactions (RIP, RIP-CHIP); see Fig. 7.2 [58].

Microarrays

The structure and expression of long noncoding RNAs is very similar to mRNAs, even though lncRNAs lack open reading frames and other properties necessary for them to be translated into proteins [59]. One of the most common methods of identification is the microarray-based approach. Microarrays are based on nucleic acid hybridization between target molecules and probes and enable simultaneous monitoring of thousands of genes in a single experiment. However, this method shows only whether or not lncRNA is expressed and is therefore not suitable for the identification of novel transcripts [60]. On the other hand, microarrays allow us to detect differences in transcriptional profiles between different tissues and cell types or identify possible targets of lncRNAs [21, 58].

RNA Sequencing

The transcriptome includes all RNAs synthesized in an organism, including protein-coding, noncoding, alternatively spliced, alternatively polyadenylated, alternatively initiated, sense, antisense, and RNA-edited transcripts [13]. The most widely used method for qualitatively and quantitatively profiling the full set of transcripts is RNA sequencing

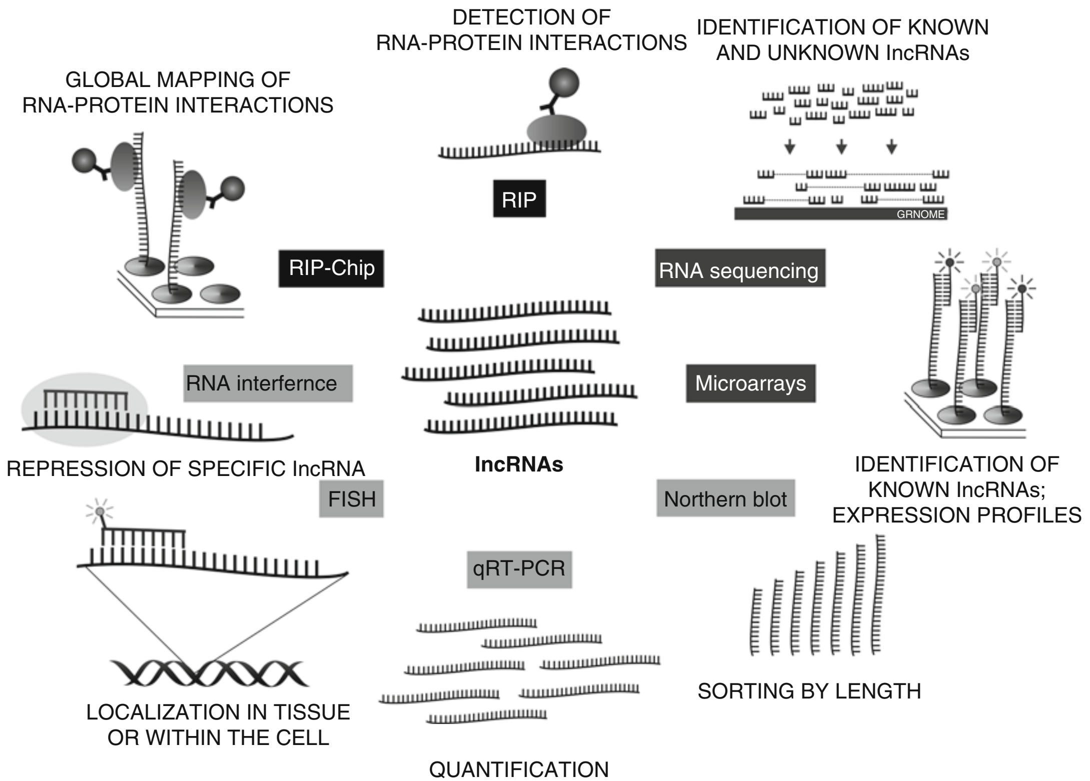

Fig. 7.2 Methods used in the study of lncRNAs can be divided into (1) high-throughput methods designed for lncRNAs identification (microarrays, RNA sequencing), (2) methods designed for verification of high-throughput data (qRT-PCR, northern blot, FISH, RNAi), (3) methods designed for detection of RNA–protein interactions (RIP, RIP-CHIP)

(RNA-seq), which is based on next-generation sequencing (NGS) [61]. RNA-seq works on a genome-wide scale at single nucleotide resolution and is not limited to detecting already known sequences. Thus, it can be used to discover previously unknown lncRNAs [62]. Considerable disadvantages of this approach are the time and cost related to the downstream analysis of the RNA-seq data [60]. After sequencing, the generated reads are used to assemble the transcriptome, and then novel lncRNAs can be identified and annotated via bioinformatic databases (i.e., FANTOM or ENCODE) [63]. After that, novel lncRNAs often undergo further scrutiny to verify that they are not transcriptional noise and that they indeed do not encode proteins. In the same way, candidate targets are required to be verified by other molecular biology methods as well.

qRT-PCR and Northern Blot

Quantitative real-time polymerase chain reaction (qRT-PCR) allows amplification and quantification of selected segments of the genome. This highly sensitive method is used for gene expression studies, but qPCR can be used even for analysis of lncRNAs. For this purpose, qPCR is combined with reverse transcription (RT), which ensures transcription of RNA into cDNA. For the verification of high-throughput data, qRT-PCR is frequently followed by northern blot analysis, which is the only direct method to prove the presence of RNA without the need of amplification. This combination was used to demonstrate the lengths of detected lncRNAs and their level of expression [64].

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) is a type of hybridization that uses a fluorescently labeled complementary DNA or RNA strand to localize a specific sequence on a chromosome, section of tissue (in situ) fixed on a slide or even cell [65]. In lncRNAs research, FISH was used to detect individual lncRNA or their localization within the cell [23, 66].

RNA Interference (RNAi)

The process of RNA interference involves the binding of short interfering RNA molecules to mRNAs, which leads to expression–repression of a gene of interest. The use of synthetic dsRNA allows also effective knockdown of specific lncRNAs and is very important for studying their functions [67].

RNA Immunoprecipitation and RIP-Chip

Long noncoding RNA may affect the regulation of gene expression also through the modification of chromatin via interactions with various proteins (i.e., transcription factors). Such RNA–protein interactions are easy to detect with RNA immunoprecipitation (RIP). It is an antibody-based technique in which the RNA-binding protein of interest immunoprecipitates together with its associated RNA and allows localize RNA-binding sites on the genome [68]. A number of lncRNAs, such as Xist and Tsix, were identified through this approach [69].

Major advances in the global analysis of subsets of mRNAs bound to RNA-binding proteins brought combinations of RIP and microarrays called RNA-binding protein immunoprecipitation-microarray (Chip) profiling or RIP-Chip [70]. Using this high-throughput method, it was revealed that a large number of lincRNAs associate with chromatin-modifying complexes to affect gene expression [8].

Long Noncoding RNAs in Pathogenesis of Breast Cancer

lncRNAs were found to be deregulated in several human cancers and show analogically to protein-coding genes tissue-specific expression. Functional studies elucidated a large range of molecular mechanisms used by lncRNAs in cancer cells. Till now, only a few lncRNAs were observed to have altered expression in breast cancer, including HOTAIR, MALAT-1, GAS5, ZFAS1, LSINCT5, SRA1, H19, XIST, and BC200, which are characterized in detail in Table 7.2. Here we discuss molecular functioning of these lncRNAs mainly in the context of typical hallmarks of cancer. Some important molecular mechanisms used by particular lncRNA are mentioned although they are observed in cancer types other than breast cancer.

HOTAIR

HOX transcript antisense intergenic RNA (HOTAIR) has very important role in cancer metastasis. It was discovered as a 2.2 kb-long ncRNA transcribed in antisense direction from the HOXC gene cluster [71]. HOTAIR functions in trans by interacting and recruiting the polycomb repressive complex 2 (PRC2) to the HOXD locus, which leads to transcriptional silencing across 40 kb. PRC2 complex consisted of H3K27 methylase EZH2, SUZ12, and EED (see Fig. 7.3) [72]. Polycomb group proteins are involved in repression of transcription of large groups of genes. This pathway influences differentiation, pluripotency, and cancer development [73]. Later it was found that HOTAIR interacts with a second histone modification complex, the LSD1/CoREST/REST complex, which coordinates targeting of PRC2 and LSD1 to chromatin for coupled histone H3K27 methylation and K4 demethylation [72]. Overexpression of HOTAIR in epithelial cancer cells alters H3K27 methylation via PRC2 and therefore alters target gene expression. This leads to increased cancer invasiveness and metastases. Therefore, HOTAIR depletion inhibits breast cancer invasiveness [73].

Table 7.2 Characteristics of lncRNAs deregulated in breast cancer

Gene	Locus	Length (kb)	Function in cancer	Association in hallmarks of cancer	Tumor types	References
HOTAIR	12q13	2.2	Oncogene	Activating invasion and metastasis	BC, CRC, PaC, HCC	[73]
MALAT-1	11q13	8.4	Oncogene	Activating invasion and metastasis; evading growth suppressors	BC, BlaC, HCC	[76, 107, 108]
GAS5	1q25	5.3	Tumor suppressor	Evading growth suppressors	BC, M, Ly, PC	[81, 83, 111]
ZFAS1	20q13	0.5	Tumor suppressor	Evading growth suppressors	BC	[84]
LSINCT5	5p15	2.6	Oncogene	Sustaining proliferative signaling	BC, OC	[85]
SRA1	5q31	0.87	Oncogene	Sustaining proliferative signaling	BC, OC, PC	[90, 92, 93]
XIST	Xq13	42	Oncogene	Activating invasion and metastasis	BC, Ly, OC, CRC	[96, 98]
H19	11p15	3.5	Oncogene, tumor suppressor	Sustaining proliferative signaling	BC, BlaC, CerC, CRC, HCC, LC, M	[100, 101]
BC200	2p21	0.26	Oncogene	Activating invasion and metastasis	BC	[105]

BC breast cancer, *CerC* cervical cancer, *CRC* colorectal cancer, *PC* pancreatic cancer, *HCC* hepatocellular cancer, *BlaC* bladder cancer, *M* melanoma, *Ly* lymphoma, *PC* prostate cancer, *OV* ovarian cancer

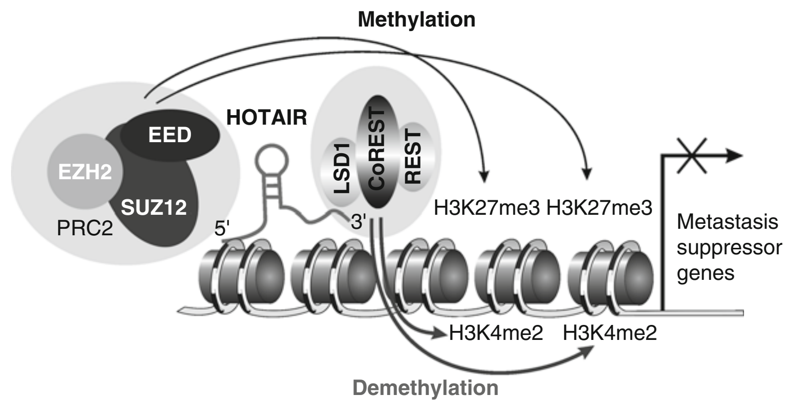

Fig. 7.3 Association of HOTAIR with the polycomb repressive complex 2 (*PRC2*) and LSD1/CoREST/REST complex

MALAT-1

Metastatic-associated lung adenoma transcript 1 (MALAT-1) is abundant in many human cell types. It is probably a very important transcript because its sequence is very conserved across many species. MALAT-1 is an 8,708 nt-long transcript occurring in the nucleus and frequently localized in nucleus speckles [74]. These structures play a role in pre-mRNA processing. Recently MALAT-1 has been shown to regulate alternative splicing of pre-mRNA by modulating the levels of splicing factors. These factors regulate tissue-specific alternative interactions with SR splicing factor, SRSF1, which affects the distribution of these and other splicing

factors in nuclear speckle domains. Depletion of MALAT-1 with antisense oligonucleotides or transient overexpression of SRSF1 changes the alternative splicing of the endogenous pre-mRNAs. Importantly, MALAT-1 controls cellular phosphorylation status of SR proteins, thereby regulating cellular ratio of phosphorylated versus dephosphorylated form of SR proteins [23], suggesting that MALAT-1 regulates pre-mRNA processing by modulating the levels of active SR proteins. Depletion of MALAT-1 alters the processing of a subset of pre-mRNAs, which play important roles in cancer biology [75].

Recent studies indicate additional functions for MALAT-1 in the nucleus. MALAT-1 was shown to interact with the unmethylated form of CBX4, which controls relocation of growth-control genes between polycomb bodies and interchromatin granules, places of silent or active gene expression, respectively. Altered expression levels of MALAT1 were detected in breast cancer tissue compared to normal breast tissue. Also MALAT-1 locus is frequently altered in breast cancer and other tumor types [76].

GAS5

Growth arrest-specific 5 (GAS5) is the host gene for many snoRNAs, which were found in GAS5 introns, dedicating GAS5 to be involved in the important cellular activities [77]. It was proved that GAS5 transcripts displayed many different patterns of alternate splicing, but there is no putative open reading frame [78]. GAS5 functions as "riborepressor" of the glucocorticoid receptor (GR), influencing cell survival and metabolic activities during starvation by modulating the transcriptional activity of the GR. Its transcript interacts with the DNA-binding domain of GRs and reduces the probability of steroids' interaction with their receptors [79]. In this way, GAS5 suppresses expression of several genes including cellular inhibitor of apoptosis 2 (cIAP2) and thus sensitizes cells to apoptosis. This induction of apoptosis is independent of other stimuli in several breast cancer cell lines [80]. It was also shown that silencing of endogenous GAS5 levels in breast cancer cells leads to resistance to apoptosis and various GAS5 transcripts stimulate apoptosis through different cellular signaling pathways [81]. Moreover, in leukemia cell models, GAS5 is required for normal functioning of mammalian target of rapamycin (mTOR) pathway that controls cell growth and proliferation also in breast cancer [82]. In addition, GAS5 locus was found to be frequently altered in many types of cancer (e.g., melanoma, lymphoma, prostate cancer) [83].

Zfas1/ZFAS1

Zinc finger antisense 1 (Zfas1) is a mouse antisense RNA to NFX-1 type containing zinc finger. Zfas1 is located close to a protein-coding gene and in its introns hosts three small nucleolar RNA (snoRNA) genes: Snord12, Snord12b, and Snord12c [84]. Knockdown of Zfas1 in a mammary epithelial cell line resulted in increased cellular proliferation and differentiation. But this knockdown did not substantially alter the levels of the SNORDs. Functional role for Zfas1 in the regulation of alveolar development and epithelial cell differentiation in the mammary gland, together with its dysregulation in human breast cancer, suggests ZFAS1 as a putative tumor suppressor gene. ZFAS1 is highly expressed in the mammary gland and is downregulated in breast tumors compared to normal tissue. While there is relatively low level of primary sequence conservation between Zfas1 and its human ortholog ZFAS1, secondary structures of Zfas1 and ZFAS1 transcripts share several similar features. It was proved that from ZFAS1 mRNA originates at least five different isoforms through alternative splicing.

LSINCT5

Long stress-induced noncoding transcript 5 (LSINCT5) is greatly overexpressed in many of the breast cancer cell lines [85]. LSINCT5 is polyadenylated RNA that is transcribed from the negative strand by RNA polymerase III with no open reading frame. When nuclear and cytoplasmic

LSINCT5 levels are compared, LSINCT5 indicates higher expression in the nuclear fraction. Instead of decrease in cellular proliferation, knockdown of LSINCT5 in cancer-derived cell lines causes expression deregulation of several genes including important kinase (PDPK1), nuclear assembly genes (NEAT1 and PSPC1), genes involved in membrane transport (HERC1), transcription factor (ANKF41), and genes associated with carcinogenesis (EPPK1), cellular stress (PRKAA1/AMPK), motility (ACTR2), and T-cell differentiation (CXCR4, MAPK9/JNK2) [86]. Moreover, LSINCT5 is overexpressed in breast and ovarian cancer tissue.

SRA1

Steroid receptor RNA activator (SRA) modulates activity of steroid receptors and other transcription factors both at the RNA (SRA) and the protein (SRAP) level [87]. SRA appears highly expressed in the liver, skeletal muscle, adrenal gland, and the pituitary gland, whereas intermediate expression levels are seen in the placenta, lung, kidney, and pancreas. Interestingly, brain and other typical steroid-responsive tissues such as the prostate, breast, uterus, and ovary contained low levels of SRA RNA [88]. SRA is a component of ribonucleoprotein complexes recruited to the promoter of regulated genes. These complexes may contain positive regulators, such as the steroid receptor coactivator 1 (SRC-1), the DExD/H box family of RNA-helicase members p68 and p72, or the pseudouridine synthases Pus1p and Pus3p. Negative regulators, such as SMRT/HDAC1-associated repressor protein (SHARP) and the recently identified SRA stem-loop interacting RNA-binding protein (SLIRP), can also interact with SRA to decrease its activity [89]. Elevated levels of SRA are found in breast tumors and the increased SRA levels might contribute to the altered ER/PR action that occurs during breast tumorigenesis. The SRA1 gene might not only act as an ncRNA but also codes a protein that acts as a coactivator or corepressor. The ratio between noncoding and coding transcripts of SRA1 characterizes specific tumor phenotypes but might also be involved in breast tumorigenesis and tumor progression by regulating the expression of specific sets of genes [90]. The sequence of the protein encoded by SRA, referred to as SRAP, is highly conserved in Chordata. The most conserved amino acids define two distinct domains (N- and C-terminal) that represent the typical signature of this new family of proteins, and which are likely both participating in SRAP function [91]. This protein is also ubiquitously found in human cancer cell lines derived from the breast [92], the prostate [93], and other tissues, even though levels of expression appear to vary from one cell type to another.

XIST

X-inactive specific transcript (XIST) is transcribed from the inactivated X chromosome, is involved in its inactivation, and exists in many types of isoforms [94]. On the active X allele, XIST is repressed by its antisense RNA, TSIX [95]. XIST contains a double-hairpin RNA motif in the RepA domain. It is located in the first exon, which is crucial for its ability to bind polycomb repressive complex 2 (PRC2) and propagate epigenetic silencing of the X chromosome [69]. Subsequently, the inactive X (Xi) acquires the typical features of heterochromatin: late replication, hypoacetylation of histones H3 and H4, methylation of histone H3 lysines 9 and 27, lack of methylation of H3 lysine 4, and methylation of DNA CpG islands. Initial studies suggested a role for XIST in hereditary BRCA1-deficient breast cancers [96], whereas data indicated that BRCA1 was not required for XIST to function in these cells [97]. Aberrant XIST regulation was also observed in other cancers, including lymphoma and male testicular germ-cell tumors [98].

H19

H19 is located in a cluster of imprinted genes on the human chromosome 11. The regulation of H19 is related to its closely linked and reciprocally imprinted neighbor IGF2. They are studied intensively both because of their role in human diseases

and as a model for understanding imprinting control mechanisms. Thereby H19 is transcribed only from the maternal allele, whereas IGF2 expression is exclusively paternal. H19 is considered a regulatory RNA [12]. It has been suggested that H19 functions in many different processes, ranging from transcriptional and posttranscriptional regulation of expression [99] to tumor suppression and oncogenesis, including breast cancer [100]. The expression of H19 is high during vertebrate embryo development, but is downregulated in most tissues shortly after birth with the exception of skeletal tissue and cartilage [101]. In breast cancer cell lines, c-Myc induces the expression of the H19 ncRNA and binds directly to DNA sequence elements called E-boxes close to the imprinting control region (ICR). Thus, c-Myc specifically binds and regulates the active maternal H19 allele and does not bind or affect the expression of the silenced paternal allele. In addition, c-Myc downregulates transcription of the reciprocally imprinted gene IGF2 [103]. H19 was also shown to be directly activated by the oncogenic transcription factor c-Myc in colon cancer, suggesting H19 may be an intermediate functionary between c-Myc and downstream gene expression [102]. The upregulation of H19 by c-Myc and correlation of c-Myc and H19 levels were observed in primary and established tumor cells derived from breast cancer patients [103]. The tumor suppressor gene p53 has been shown to decrease H19 levels. H19 transcripts also serve as a precursor for miR-675, the miRNA involved in the regulation of developmental genes. MiR-675 is processed from the first exon of H19 and leads to a decrease in the levels of tumor suppressor retinoblastoma gene 1 (RB1) [104].

BC200

BC200 RNA is a 200 nt-long RNA that is selectively expressed in the primate nervous system where it has been identified in somatodendritic domains of a subset of neurons [105]. BC200 RNA is not normally expressed in nonneuronal somatic cells [110]. It has been shown that it is expressed in germ cells and in cultured immortal cell lines of various nonneural cell types. In order to investigate whether the neuron-specific expression of BC200 RNA is also deregulated during tumorigenesis in nonneural human tissues, 80 different tumor specimens, representing 19 different tumor types, were screened for the presence of this RNA [106]. BC200 was detected in carcinomas of the breast, cervix, esophagus, lung, ovary, parotid, and tongue, but not in corresponding normal tissue. BC200 was not detectable in bladder, colon, kidney, or liver carcinoma tissues examined in this study. These results demonstrate that BC200 expression is deregulated under certain neoplastic conditions. The expression of BC200 RNA in nonneural tumors may indicate a functional interrelationship with induction and progression of these tumors [106].

Long Noncoding RNAs in Diagnosis of Breast Cancer

HOTAIR levels were up to a 2,000-fold increase in primary and metastatic breast cancer tissue compared with normal breast tissue [73]. In breast cancer tissue, moderate or high levels of MALAT-1 were also observed [76]. MALAT-1 levels were increased also in bladder cancer and hepatocellular carcinoma [107, 108]. LSINCT5 had increased expression in breast and ovarian cancer cell line and tumor tissue [85]. Elevated levels of SRA are found in breast tumors and the increased SRA levels might contribute to the altered ER/PR action that occurs during breast tumorigenesis. Relative expression of SRA varies between breast cancer cell lines with different phenotypes [91].

Another type of oncogenic lncRNA is XIST, which is typically expressed by all female somatic cells. But XIST expression has been found to be lost in female breast, ovarian, and cervical cancer cell lines [97]. There is a substantial body of evidence to suggest the occurrence of X chromosome inactivation alterations in breast cancer cells. Interestingly, in cell lines derived from the duct carcinoma of the kidney, XIST gene, along with several other chromosome X genes, was found being amplified [109]. BC200 was expressed in carcinomas of the breast, cervix,

esophagus, lung, ovary, parotid, and tongue but not in corresponding normal tissues [106]. In an independent study, it was shown that BC200 RNA is detectable at significant levels in a number of human tumors, including infiltrating ductal carcinoma of the breast, squamous cell carcinoma of the lung, and lung metastasis of melanoma. Corresponding normal tissue obtained from the same patient was found to be BC200 negative. BC200 RNA is expressed at high levels in high-grade ductal carcinoma in situ (HG DCIS) but not in non-high-grade ductal carcinoma in situ (NHG DCIS) [105]. High expression of BC200 RNA in carcinoma in situ is thus indicative of high grade.

H19 is upregulated by c-Myc and H19 levels were observed in primary and established tumor cells derived from breast cancer patients [103]. In comparison with normal breast epithelial tissue, reduced levels of GAS5 were detected in cancer tissue [80]. ZFAS1 is highly expressed in the mammary gland and is downregulated in breast tumors compared to normal tissue [84]. ZFAS1 expression is decreased in ductal carcinoma relative to normal epithelial cells.

lncRNAs with diagnostic potential in breast cancer are summarized in Table 7.2. The majority of lncRNAs that were identified as deregulated in breast cancer are oncogenes, and their levels in cancers are increased: HOTAIR, MALAT-1, LSINCT5, SRA1, XIST, BC200, and H19. Only two studied lncRNAs indicate properties of tumor suppressors: GAS5 and ZFAS1. At the moment, independent studies in large cohorts of breast cancer patients enabling detailed clinicopathologic correlations are needed to prove and define potential diagnostic usage of these promising lncRNAs.

Long Noncoding RNAs as Prognostic and Predictive Biomarkers in Breast Cancer

From the lncRNAs studied in breast cancer only five indicate the potential to be prognostic biomarkers. In human breast cancer, HOTAIR expression is increased in primary tumors and metastases, and its expression level in primary tumors positively correlates with the development of metastasis and poor outcomes [72, 73]. Also, MALAT-1 expression was remarkably increased in primary tumors that subsequently metastasized in contrast to primary tumors of patients with better outcomes [107].

Invasive breast cancer cell lines were shown to have higher levels of noncoding SRA than less invasive ones. This suggests that the expression of noncoding SRA in breast cells is probably associated with the ability for invasion [91]. Interestingly, the apparent overexpression of SRAP in some cases correlated with an overall survival in the breast cancer patients [93]. This also suggests that an increase in SRAP expression might characterize a less aggressive phenotype and it is possible that this protein contributes to the improved outcome after tamoxifen antiestrogen therapy [93].

Several studies noted that aggressive breast tumors do not show a detectable Barr body due the cytological examination of the Xi [111]. Decreased levels of XIST lead to reduced sensitivity to treatment with Taxol in ovarian cancer cell lines, suggesting that the expression of XIST may serve as a predictive biomarker of therapy response [112].

Only the level of GAS5 expression in the breast cancer cell lines showed a general inverse correlation with tumorigenic behavior [82]. Interestingly, in head and neck squamous cell carcinoma, a high level of GAS5 was associated with a good prognosis [113].

Recent evidence suggests that some lncRNAs deregulated in breast cancer tissue may serve as prognostic or predictive biomarkers in breast cancer patients, indicating their potential in translational oncology.

Long Noncoding RNAs as Potential Therapeutic Targets

Besides the imminent use of our knowledge of cancer-associated long ncRNAs for diagnosis, therapeutic applications may be possible in a more distant future. The use of lncRNAs

as therapeutic agents is only beginning to be explored. Although our understanding of the molecular mechanisms of lncRNA function is limited, some features of lncRNAs make them ideal candidates for therapeutic intervention. Many lncRNAs appear to have protein-binding or functional potential that is dependent on secondary structure; this may provide a means of intervention [114]. Preventing the interactions of HOTAIR with the PRC2 or LSD1 complexes, for example, may limit the metastatic potential of breast cancer cells [73]. The progress in the use of RNAi-mediated gene silencing for the treatment of different diseases is encouraging and could be applied to selectively silence oncogenic lncRNAs. Gene therapy could also be applied for the delivery to specific cells of tumor suppressor lncRNAs for the treatment of breast cancer. However, many technical challenges have to be overcome for a wider use of therapeutic RNAi and gene therapy [114]. The expression of the lncRNA H19 is increased in a wide range of human cancers, including breast cancer [100]. One promising therapeutic approach presents a plasmid vector carrying the gene for the diphtheria toxin, which is under the control of the H19 promoter. Intratumoral injections of this plasmid induced high expression levels of diphtheria toxin specifically in the tumor cells, resulting in a reduction of tumor size in human trials [115]. GAS5 expression induces growth arrest and apoptosis independently of other stimuli in some breast cancer cell lines [82]. Therefore, development of technology inducing GAS5 expression in tumors—or designing a vector that would induce the expression of GAS5 when delivered into the tumor cells—might provide an attractive therapeutic approach. Collectively, these advances indicate the significant potential in developing of lncRNAs-mediated therapies.

Conclusion and Future Perspective

There is much research still on the way toward a deeper understanding of regulation processes, in which lncRNA is one of the important players. Although long noncoding RNA expression profiles in tumor tissue highlighted the potential value of this class of noncoding RNAs as tumor biomarkers in diagnosis and prognosis of breast cancer patients, only studying the mechanisms of lncRNA involvement in oncogenic and tumor suppressive pathways could lead to the establishment of new diagnostic biomarkers and figure their potential usage as novel therapeutic targets. As the catalog of lncRNAs still grows, it will also become important to elucidate the genetic networks and signaling pathways regulated by the lncRNAs, which are abnormally expressed in breast cancer cells, to understand the role of these lncRNAs in the processes of malignant transformation.

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8 Breast Cancer MicroRNAs: Clinical Biomarkers for the Diagnosis and Treatment Strategies

Gowhar Shafi, Tarique N. Hasan, Naveed Ahmed Syed, Ananta Paine, Jesper Tegner, and Anjana Munshi

Abstract

Breast cancer is the second most common cancer in females that accounts for the highest cancer-specific deaths worldwide. Although recent advances in clinical management significantly reduced the mortality rate in breast cancer patients, the success rate of the effective therapy remains largely dependent on early detection. It has been demonstrated that gene expression profile may be a useful tool to define the signature of breast cancer as well as to predict the prognosis or response to treatment. The microRNA expression profile is gaining lots of attention to define various types of cancers since they play critical roles in many different cellular processes including metabolism, apoptosis, differentiation, and development. Several studies have shown that microRNA's signatures are associated with the staging, progression, and response to treatment in breast cancer. In addition to this microRNA has been shown to act as oncogenes and tumor suppressor genes.

Continued efforts to delineate the microRNA function in mammary physiological and pathological conditions will reveal novel insights into normal cells and breast cancer biology and ultimately provide a new molecular target for alternate therapy. The book chapter covers the role of microRNAs in the diagnosis, staging, progression, prognosis, and response to treatment of breast cancer.

Keywords

MicroRNA • Breast cancer • Diagnosis • Prognosis • Breast cancer staging • Treatment strategies

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Introduction

MicroRNAs (miRNAs) are small noncoding RNAs (ncRNAs), usually 20–25 nucleotides long and capable of regulating the gene expressions at the post-transcriptional level. miRNA regulates gene expression by translational repression, mRNA cleavage, and mRNA decay, which have been found to control cell division, differentiation, and death. Their regulatory activity brings about by their binding to the coding region as well as 3′ and 5′ untranslated regions (UTRs) of messenger RNAs (mRNAs). Such bindings result in either inhibition of translation or degradation of mRNAs [1–3]. As estimation, the human genome encodes about 1,500 miRNAs. It is believed that they regulate more than 30 % of protein-coding genes. Like one gene multiple polypeptide and multiple gene single polypeptide story, an individual miRNA can target multiple genes, and each protein-coding gene can be regulated by several miRNAs [4]. Their involvement has been reported in several biological processes, such as apoptosis, proliferation, differentiation, and metastasis [5, 6].

Breast cancer is the most common form of cancer in women and the second most common cause of cancer death for women worldwide [7]. The tools available for breast cancer diagnosis and prognosis are not yet satisfactory at the molecular level and require further improvements. The miRNA expression profiling of human breast cancer has led to the identification of signatures correlated with the diagnosis, staging, progression, prognosis, and response to treatment. MicroRNA fingerprinting can therefore be added to the diagnostic and prognostic tools in diseases including breast cancer used by medical experts.

MicroRNAs: The Discovery

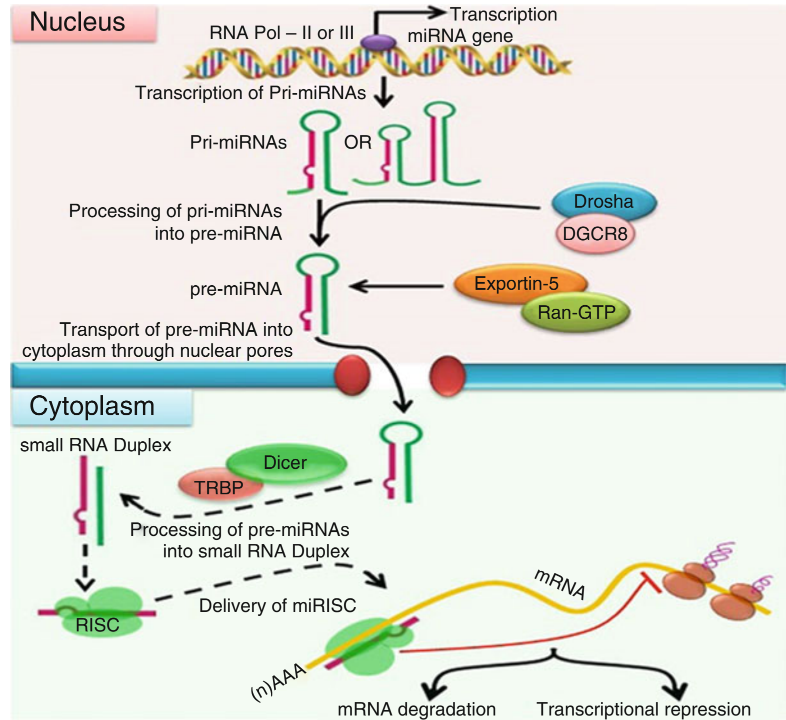
MicroRNAs were first discovered in 1993 by the joint efforts of Ambros and Ruvkun's laboratories [8]. They discovered that lin-4 in *C. elegans* does not code for a protein, but instead produced a pair of short RNA transcripts that each regulates the timing of larval development by translational repression of lin-14, which encodes for a nuclear protein. Hence, it was postulated that the regulation was due in part to sequence complementarity between lin-4 and unique repeats within the 3′ UTR of the lin-14 mRNA. The downregulation of lin-14 at the end of the first larval stage initiates the developmental progression into the second larval stage [9, 10]. It was only in 2000 when let-7 [11] was discovered to repress lin-41, lin-14, lin-28, lin-42, and daf-12 mRNA during transition in developmental stages in *C. elegans*. This function was phylogenetically conserved in species beyond nematodes, and it became apparent that the short noncoding RNA identified in 1993 was part of a wider phenomenon.

Since then, thousands of miRNAs have been identified in different organisms through random cloning and sequencing or computational prediction [12]. However, about 1,500 miRNAs are reported in the human genome. The identified miRNAs and their associated data are currently curated at the miRBase database. miRBase is hosted by the Sanger Institute as a publicly available repository (http://microrna.sanger.ac.uk/). Due to their abundant presence and far-reaching potential, miRNAs have all sorts of functions in physiology, from cell differentiation, proliferation, and apoptosis to the endocrine system, hematopoiesis, morphogenesis, etc. They display different expression profiles from tissue to tissue, reflecting the diversity in cellular phenotypes and as such suggest a role in tissue differentiation and maintenance. Figure 8.1 emphasizes the revolutionary studies that have significantly contributed to the history of miRNAs.

MicroRNAs: The Biogenesis

Various approaches have provided a basic understanding of the molecular details of miRNA biogenesis (Fig. 8.2) and it has long been viewed as linear and universal to all mammalian miRNAs. To understand miRNA biogenesis at a molecular level, we have classified miRNA biogenesis into the following three subheadings:

Fig. 8.1 Historical perspective of selected hallmarks on the evolution of microRNA history



Year	Hallmark
1969	Non-protein coding transcripts (activator RNAs) regulate gene activity (Britten and Davidson)
1993	Recognition of *lin-4* as non coding small RNA(Lee, Feinbaum, and Ambros)
2000	Discovery of *let-7* (Reinhart et al.) RNAi "unit": 21-23 nt (Zamore et al.)
2001	Large class of small RNA (miRNA) co-express and regulates the gene expression (Lau et al.; Lagos-Quintana et al.; Lee and Ambros)
2002	Discovery of miRNA and targets of miRNA in plants (Reinhart et al.; Rhoades et al.) Downregulation of miRNA (miR15 and miR16) in blood cancer cells (Calin et al.)
2004	Majority of miRNA genes are located in cancer-associated genomic regions (Calin et al.) miRNA as diagnostic/prognostic biomarker(Takamizawa et al.) Co-expression of miRNAs and their host genes (Rodriguez et al.)
2005	miRNA-target interaction relevant to cancer (Johnson et al.) Altered expression of miRNAs affects tumor formation/growth *in vivo* (He et al.) Association between miRNAs and the MYC oncogene (O'Donnell et al.) Inhibition of miRNA by antagomirs in mammals (Krützfeldt et al.)
2006	Epigenetic regulation of miRNAs (Saito et al.) molecule of the year -hsa-mir-155 and hsa-let-7a-2 (Yanaihara et al.)
2007	5'-UTR also may the target for miRNA (Lytle et al.) miRNAs are deregulated in cancer metastasis (Ma et al.) miRNAs can up-regulate mRNA expression and translation of proteins (Vasudevan et al.) miRNAs can reactivate of silenced tumor suppressor genes by affecting epigenetics (Fabbri et al.) miRNAs can regulate ncRNAs from the category of long ultraconserved genes (UCGs) (Calin et al.) miRNAs carrying hexanucleotide terminal motifs are enriched in the nucleus (Hwang et al.)
2008	miRNAs can transcriptionally silence gene expression (Kim et al.) Functional single nucleotide polymorphism (SNP) in the miRNA seed region (Shen et al.) miRNA binding sites located within mRNA-coding sequence (Tay et al.) Expression of miRNA in serum/plasma (Chim et al.)
2009	Proof of concept of miRNA delivery as cancer therapy (Kota et al.)
2010	miRNA as molecular decoys (Eiring et al.) miRNAs predominantly cause mRNA destabilization (Guo et al.) Overexpression of a single miRNA is sufficient to cause cancer (Medina et al.)
2011	Competing endogenous RNA (ceRNA) communicate with and regulate other RNA transcripts by competing for shared miRNAs(Salmena et al.)

Fig. 8.2 MicroRNA processing and activity. Depicts the formation of long primary microRNA (pri-miRNA) in the nucleus which is processed by the microprocessor complex (Drosha, an RNAse III enzyme, and Pasha, a double-stranded RNA-binding protein) into precursor microRNA (pre-miRNA) (70 nt stem-loop structure) and transported to the cytoplasm by Exportin-5-mediated export, where Dicer, an RNase II enzyme, cleaves it to 20–25 nt mature miRNA that integrates it into the miRNA-inducing silencing complex (miRISC), a complex of proteins that is responsible for regulation of gene expression either by translational inhibition or by target mRNA degradation

Nuclear Processing by Drosha

This canonical maturation includes the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 (Pasha) in the nucleus [13]. A primary transcript RNA (pri-miRNA) transcribed from a miRNA gene by RNA polymerase II or III is first processed into a stem-loop structure of about 70–80 nucleotides known as precursor miRNA (pre-miRNA) by a microprocessor enzyme comprising of a double-strand (ds)-RNA-specific ribonuclease, Drosha, with the help of its binding partner DGCR8.

Nuclear Export of Pre-miRNAs

The resulting precursor hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5–Ran-GTP [13]. Exportin-5 recognizes the pre-miRNA independently of its sequence or the loop structure. A defined length of the double-stranded stem and the 3′ overhangs are important for the successful binding to Exportin-5, ensuring the export of only correctly processed pre-miRNAs [13].

Cytoplasmic Processing by Dicer

In the cytoplasm, the RNase Dicer in complex with the double-stranded RNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RNA-induced silencing complex (RISC), where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression, or deadenylation, whereas the passenger strand is degraded [13].

MicroRNA Genes and Their Transcription

MicroRNA genes reside in regions of the genome as distinct transcriptional units as well as in clusters of polycistronic units—carrying the information of several microRNAs [10, 14–16]. Studies suggest that approximately half of known microRNA reside in non-protein-coding RNAs (intron) or within the intron of protein-coding genes [17].

The understanding of microRNA transcription is very important for determining their regulators as well as the specific role they may play in signaling cascades. The understanding of microRNA transcriptional regulation has great public health significance. The ability to understand how these post-transcriptional gene regulators function in cellular networks may provide new molecular targets for cures or therapies to a variety of human diseases.

Transcription of miRNA Genes

Little is known about the transcriptional regulation of these intergenic miRNAs, although RNA polymerase II appears to be involved in the process [18]. This suggests that miRNAs may have active promoter regions that contain cis-regulator elements similar to coding genes. miRNA genes are currently believed to be transcribed by RNA polymerase II (Pol II), [18] although a few may be transcribed by RNA polymerase III [19]. RNA polymerase II transcribes miRNA genes, generating long primary transcripts (pri-miRNAs) [20]. Subsequently, the process to yield mature miRNAs involves two steps involving RNase III enzymes and companion double-stranded RNA-binding domain (dsRBD) proteins. There are two different classes of miRNAs with respect to transcription mechanism—those found within annotated genes (intronic miRNAs) and those found in intergenic regions of the genome (intergenic miRNAs). It is presently believed that all intronic miRNAs are co-transcribed along with their host gene; this has been shown in both expression correlation studies [21] as well as PCR-based biochemical verification [17]. Intergenic miRNAs have been postulated to come from transcripts of up to 50 kb in length, allowing for the co-transcription of neighboring miRNAs (polycistronic miRNA clusters) [21]. Identification of the method by which miRNA genes are transcribed can lead to

the identification of the factors that are responsible for their regulation.

MicroRNAs in Breast Cancer

MicroRNAs have been concerned with an increasing number of neoplasia and biological process. The latest studies have shown a contribution for these regulatory molecules in breast cancer. For instance, microRNA profiling studies have identified microRNAs that are deregulated in breast cancer. Moreover, functional studies have revealed their roles in breast cancer as both oncogenes (e.g., hsa-*miR-21*) and cancer suppressor genes (e.g., hsa-*miR-335*). MicroRNAs deregulated in breast cancer control the translational regulation of entrenched regulative molecules, such as estrogen receptor-α, which are regulated by novel cancer-related molecules and *miR-206* whose functions are not yet completely understood.

MicroRNA in Cancer in General

miRNAs have been associated with the regulation of differentiation, proliferation, apoptosis, and even exocytosis [22]. Volinia et al. has demonstrated that the predicted targets for the differentially expressed miRNAs are significantly enriched for protein-coding tumor suppressors and oncogenes [22]. There is also confirmation to suggest that these miRNAs function in concert with classical tumor suppressors and oncoproteins to regulate key pathways involved in cellular growth control [23, 24]. miRNA profiling has the potential not only to classify tumors but also to augur patient outcome with high accuracy, but this approach needs more validation and detailed studies by using clinical samples [25]. Jian et al. showed that profile analysis with a probe set of 201 miRNAs achieved the similar discriminative potential as traditional gene array with 8,000 mRNA probes [26]. Consequently, this would mean that classification of tumors can be achieved with a more manageable amount of data and could potentially diminish the disparity that is often seen with mRNA-based classifier systems.

MicroRNAs Study in Breast Cells and Tissues

Since the study of mRNA, various technologies exist that allow the investigation of the expression of either profiling of a large number of miRNAs or individual miRNAs simultaneously. In general, these observational approaches have implicated individual or groups of miRNAs in pathological or physiological processes, as a result of the detection of changes in their expression, while additional functional experiments are required to gain further knowledge of their current roles. A few miRNA profiles have been developed using a large number of single miRNA detection experiments, such as Northern blotting [14], and these technologies remain the standard against which newer profiling methods are primarily compared. Nevertheless, oligonucleotide microarray-based detection platforms, with their associated ease of use and high-throughput nature, have largely supplanted this technique [27]. Microarrays have been used for miRNAs profiling from a wide range of breast tissue types and cell lines, including formalin-fixed paraffin-embedded (FFPE) clinical samples. It is essential to note that, due to the small size of miRNAs, they are comparatively insensitive to the damage that typifies mRNAs within FFPE. Accordingly, miRNAs present an invaluable new target for studies using archival clinical samples, which can often be linked to extensive clinical background and, more importantly, follow-up data or meta-analysis [28]. Multiplex real-time RT-PCR and liquid bead-based technologies are current alternative strategies for miRNA profiling, and it is claimed that they may have a higher sensitivity and specificity [29, 30]. Methodologies based on deep sequencing of small RNA libraries obtained from tissues may also allow miRNA profiling, with the supplementary advantage that these techniques are unbiased with respect to target sequences and may permit detection of novel miRNAs [31].

Profiling Data of miRNA in Breast Cancer

The expression of miRNAs has been investigated in an extensive range of breast cancer cell lines, tissues, and clinical normal. These metadata hint toward functional roles of various miRNAs by association with cellular behavior or particular molecular markers. To gain the best insight into breast cancer, it is also essential to understand miRNA function in normal mammary gland development, and work is underway to address this question in detail [32]. A potentially powerful empirical approach is to compare miRNA expression in normal breast versus breast cancer and thereby to differentiate those miRNAs expressed at different levels. Table 8.1 lists miRNAs identified as playing a role in breast cancer and their potential targets. Iorio et al. reported for 76 breast samples diagnosed for the expression of 246 miRNAs, out of which 29 miRNAs expression levels were found to be significantly different (i.e., p <0.05) in cancer versus normal clinical tissue. The majority consistently downregulated were has-*miR-10b*, has-*miR-125b*, and has-miR-145, while has-*miR-21* and has-*miR-155* were upregulated, suggesting that these may act as oncogenes or tumor suppressor genes, respectively [32]. They went on to examine whether the expression profile varied according to conventional clinical aspects: ER+ /ER−, PR+ /PR−, HER2+ /HER2−, positive and negative lymph node status, presence and absence of vascular invasion, high and the low proliferation index, and ductal/lobular histopathological subtype. The majority of comparisons discovered a small number of differentially expressed miRNAs, indicating that miRNAs may have roles in defining the differences between these pathological and molecular profiles. Yet, comparison between ductal/lobular carcinomas and HER2+ and HER2− tumors did not reveal differentially expressed miRNAs.

Similarly, specific miRNA profiles have been associated with breast cancer subgroups of distinct patterns of molecular marker expression. Profiling of 204 miRNAs has been shown sufficient to allow unsupervised clustering to be used to distinguish HER2+ /ER−, HER2+ /ER−, and HER2−/ER+ breast cancers within a cohort of 20 tumors. While this in itself is not an advance, since these cancers are routinely defined using immunohistochemistry, further supervised analysis of the profiles allowed distinct miRNA subsets to be identified that distinguished HER2+ from HER2− and ER+ from ER− breast cancers, independent of other clinically important parameters. Restricted subsets of miRNAs specific to HER2 status (*let-7f*, *let-7g*, *miR-107*, *miR-10b*, *miR-126*, *miR-154*, and *miR-195*) and specific to

Table 8.1 miRNAs whose expression is deregulated in breast cancer and their potential targets

MicroRNA	Expression pattern	Target
hsa-let-7 family	Down	IL-6, ESR1 (ER)
hsa-miR-101	Down	EZH2
hsa-miR-10b	Down	HOXD10, Tiam1
hsa-miR-1226	Up	MUC1
hsa-miR-122a	Up	
hsa-miR-125	Down	HuR, ERBB2, ERBB3, BAK1, BMPR1B, CYP24, MUC1
hsa-miR-126	Down	IRS1
hsa-miR-128	Up	TGFβR1
hsa-miR-136	Up	
hsa-miR-143	Down	
hsa-miR-145	Down	MUC1, RTKN, ESR1
hsa-miR-146a		BRCA1, BRCA2
hsa-miR-146		IRAK1, TRAF6
hsa-miR-149	Up	
hsa-miR-150	Up	c-MYB
hsa-miR-155	Up	FOXO3, SOCS1, RHOA
hsa-miR-16	Down	MYB, WIP1
hsa-miR-17/92 cluster	Deleted	BRCA1, IL-8, CCND1, HBP1, AIB1, ESR1 (ER), ESR2 (ER), HIF1, STAT3
hsa-miR-185	Down	SIX1
hsa-miR-191	UP	
hsa-miR-196	UP	ANXA1
hsa-miR-200	Down	ZEB1, ZEB2, FTH1, PLCG1, BMI1, FN1, NTRK2, QKI
hsa-miR-202	Up	
hsa-miR-203	Up	
hsa-miR-204	Down	
hsa-miR-205	Down	ERBB3, ZEB1, HER3, VEGF-A

Table 8.2 Oncogenic miRNAs involved in breast cancer and their potential targets

MicroRNA	Targets identified
hsa-miR-21	Bcl-2, *PDCD4*, PTEN, *TPM1*, *TIMP3, HER2,* maspin
hsa-miR-155	Caspase 3, *SOCS1,* RhoA, FOXO3a
hsa-miR-27a	*ZBTB10*, FOXO1
hsa-miR-96	FOXO1
hsa-miR-182	FOXO1, CBS7, DOK4, NMT2, EGR1
hsa-miR-128a	TGFβR1
hsa-miR-10b	*RhoC*, HOXD10
hsa-miR-373	*CD44*
hsa-miR-520c	*CD44*
hsa-miR-221	TRPS1
hsa-miR-222	TRPS1
hsa-miR-375	RASD1
hsa-miR-224	RKIP
hsa-miR-135a	HOXA10
hsa-miR-183	CBS7, DOK4, NMT2, EGR1

ER/PR status (*miR-142-5p*, *miR-200a*, *miR-205*, and *miR-25*) have been also established [33].

Functional Studies: miRNA in Breast Cancer

The functional activity of only a few miRNAs has been practically modeled in the perspective of breast cancer, and it is clear that most of this type of work remains to be done. Potential functions of miRNAs in carcinogenesis into potential oncogenes and tumor suppressor genes have been established in regulating immune system, cell proliferation, differentiation and development, cancer and cell cycle.

Breast Cancer Oncomirs

Table 8.2 summarizes examples of miRNAs that have apparent oncogenic activity in breast cancer. Oncogenic miRNAs, commonly known as oncomirs, may act by hindering the expression of tumor suppressor genes and/or genes responsible for apoptosis and differentiation. Recent studies have identified functional oncogenic role(s) for miRNAs, their individual manipulation in breast cancer cell line models, and subsequent assessment of associated phenotypic changes. Iorio et al. showed that miRNAs aberrantly expressed in human breast cancer compared with normal breast tissue, with the most significantly downregulated miRNAs being miR-10b, miR-125b, and miR-145, whereas the most significantly upregulated miRNAs being miR-21 and miR-155 [33]. Si et al. found that the anti-miR-21-mediated cell growth inhibition associated with increased apoptosis and decreased cell proliferation [34]. Their results suggested that miR-21 functioned as an oncogene and modulated tumorigenesis through regulation of genes such as Bcl-2. miR-301 has been identified as a novel oncomir in human breast cancer, which promotes growth, proliferation, invasion, and metastases, mediated at least by FOXF2, BBC3, and PTEN genes [35]. miR-221/miR-222 activate β-catenin and contributed to estrogen-independent growth, whereas TGFβ-mediated growth inhibition was repressed by the two miRNAs [36]. miR-21, miR-210, and miR-221 expressions play a significant role in triple-negative primary breast cancers [37].

Breast Cancer Tumor Suppressor miRNAs

In contrast to oncomirs, if the expression of an miRNA is lowered in cancer cells compared to normal cells, it is regarded as a tumor suppressor (oncosuppressor). Such miRNAs are associated with tumor-suppressive activity, because they operate by inhibiting genes that promote tumorigenesis (oncogenes) and control cellular differentiation and/or apoptosis. Accordingly, the dysfunction of an oncosuppressor may ultimately lead to the development of malignant cells. Table 8.3 summarizes examples of such miRNAs, which support their role as tumor suppressors in breast cancer. The expression of *miR-125b*, *miR-145*, *miR-21*, and *miR-155* has been shown significantly reduced in breast cancer tissues [33]. Studies have shown that miR-204 exerts its function by targeting genes involved in tumorigenesis and the genomic loci encoding miR-204 are frequently lost in multiple cancers, including

Table 8.3 Tumor-suppressive miRNAs involved in breast cancer and their potential targets

microRNA	Targets identified
hsa-miR-125a	HER2, HER3, HuR
hsa-miR-125b	HER2 HER3, c-Raf
hsa-miR-205	HER2, HER3, VEGF-A
hsa-miR-27b	CYP1B1
hsa-miR-17–5p	AIB1, CCND1, E2F1
hsa-miR-17/20	Cyclin D1
hsa-miR-206	ESR1
hsa-miR-145	RTKN, ER-alpha
hsa-miR-200	ZEB1, ZEB2, PLCG1, BMI1
hsa-miR-146	NF-κB
hsa-miR-335	*SOX4, TNC*
hsa-miR-126	–
hsa-miR-206	–
hsa-miR-224	*CDC42, CXCR4*
hsa-miR-31	*FZD3*, *ITGA5*, *M-RIP*, *MMP16*, *RDX*, *RhoA*
hsa-miR-34a	*Bcl-2*, *SIRT1*, CCND1, CDK6, E2F3, MYC
hsa-miR-342	HER2Δ16
let-7	LIN28, HMGA2, H-RAS, PEBP1
hsa-miR-98	–
hsa-miR-375	MTDH
hsa-miR-203	BIRC5, LASP1
hsa-miR-30a	Vimentin
hsa-miR-7	Pak1

breast cancers, ovarian cancers, and pediatric renal tumors [38]. miR-34b is recognized as an oncosuppressor that targets cyclin D1 and Jagged-1 (JAG1) in an ER+/wild-type p53 breast cancer cell line (MCF-7), as well as in ovarian and endometrial cells, but not in ER-negative or mutant p53 breast cancer cell lines (T47D, MBA-MB-361, and MDA-MB-435) [39].

MetastamiRS Implicated in Breast Cancer Invasion and Metastasis

The current breast cancer management strategies mainly focus on early detection, tumor resection, and neoadjuvant or adjuvant treatment with radiation, chemotherapy, and/or new targeted agents. Despite advancements in the treatment of this disease, breast cancer still remains a leading cause of cancer death. Metastasis is the primary reason for high cancer death rates. Therefore, to successfully contain breast cancer, there is an urgent need to define therapeutic cocktails that could effectively target a breast tumor before it metastasizes. As discussed above, several recent research investigations have established the presence of aberrant expression of miRNAs with the potential of either promoting or suppressing tumorigenesis in breast cancer compared to normal breast tissue. The possibility that miRNAs specifically contribute to metastasis has only recently been explored. Several miRNAs have now been described as potentially promoting or suppressing metastasis (metastamiRs) and are summarized in Table 8.4.

Massimo et al. highlighted a series of recent studies that proved the involvement of miRNAs in breast cancer metastases [40]. They found (a) *miR-10b* indirectly activates the pro-metastatic gene *RhoC* by suppressing *HOXD10* and TIAM1, thus leading to tumor invasion and metastasis [41, 42]; (b) *miR-373* and *miR-520c* can also promote tumor invasion and metastasis, at least in part by regulating the gene *CD44* [43]; and (c) *miR-335, miR-206,* and *miR-126* as a suppressors of breast cancer metastasis miRNAs. The loss of *miR-335* leads to the activation of *SOX4* and *TNC* (encoding tenascin C), which are responsible for the acquisition of metastatic properties [44]. E-cadherin (CDH1) is a tumor suppressor protein that is used as a prognostic marker for breast cancer [45]. There are several studies demonstrating that the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) inhibits EMT and the initiating steps of metastasis by maintaining the epithelial phenotype of cells [46–50]. Tavazoie et al. [44] reported a set of eight miRNAs (miR-335, miR-199a, miR-122a, miR-126, miR-206, miR-203, miR-489, and miR-127) which had a lower expression in metastatic breast cancer cells as compared to their non-metastatic counterparts. Interestingly, concurrent re-expression of ITGA5, RDX, and RHOA abrogated miR-31-imposed metastasis suppression [51], indicating that these three genes were the main mediators of miR-31 effects.

In total, these significant findings are important for our understanding of malignant

Table 8.4 Role of microRNAs in breast cancer metastasis and their potential targets

miRNA	Targets identified
hsa-miR-10b	*RHOC, E-cad, HOXD10, Tiam1*
hsa-miR-373	*CD44*
hsa-miR-502c	*CD44*
hsa-miR-21	HER, TIMP3, PDCD4, TPM1, maspin PTEN, BCL-2, RHOB, MMPs
hsa-miR-200 family	ZEB1, PLCG1, BMI1, TGFβ2 FAP-1, Suz12
hsa-miR-146	NF-κB TRAF6, IRAK1, ROCK1 CXCR4, EGFR
hsa-miR-335	*SOX4, TNC, PTPRN2, MERTK*
hsa-miR-126	–
hsa-miR-206	NOTCH3, SRC-1, SRC-3, GATA-3, ER-alpha Estrogen receptor-alpha
hsa-miR-224	*CDC42*, *CXCR4*
hsa-miR-31	ITGA5, RDX, RhoA
hsa-miR-12b	STARD13
hsa-miR-30a	*Vim*
hsa-miR-34a/c	*Fra-1*
hsa-miR-9	E-cad
hsa-miR-29a	TTP
hsa-miR-103/107	Dicer
hsa-miR-210	–
hsa-miR-132	p120, Ras, GAP
hsa-miR-155	RhoA
hsa-miR-7	Pak1, EGFR
hsa-miR-17/20	Cytokines, cyclin, D1, IL-8
hsa-miR-22	CDK6, SIRT1, Sp1, ERBB3, CDC25C, EVI-1, ER-alpha
hsa-miR-126	Crk
hsa-miR-miR-127, miR-197, miR-222, miR-223	CXCL12
hsa-miR-145	IRS-1, mucin-1, c-Myc, JAM-A, fascin
hsa-miR-193b	uPA
hsa-miR-205	ZEB, VEGF, HER3
hsa-miR-448	ATB1
hsa-miR-661	MTA1, Nectin-1, StarD10
hsa-let-7	RAS, HMGA2, MYC

transformation in the breast and may have implications for the management of patients with advanced breast cancer. The use of miRNAs as anticancer therapeutic agents is promising, and such fine molecular studies definitely help in getting miRNAs closer to clinical practice.

Clinical Potential of miRNAs in Breast Cancer

miRNAs are suitable biomarkers for early cancer detection as they are present and stable in human serum or plasma. Furthermore, they appear to be differentially expressed in cancer patients compared to healthy donors. Several studies have successfully identified miRNAs linked to breast cancer subtypes and clinical–pathological features. Several miRNAs appeared to be specifically expressed in each of the clinicopathological groups. The assessment of the presence of ER, PR, and/or HER2 on breast cancer specimens is currently a routine procedure. All these markers have been independently associated with breast cancer prognosis. Actually, breast cancers presenting as triple negative (ER–/PR–/HER2–) are characterized by more aggressive behavior and poor prognosis. It is therefore of great importance to identify other factors, specially miRNAs, which play a role in receptor regulation that could be used to influence the therapeutic management of breast cancer patients. Identification of miRNAs is associated with ER and PR status, followed by others including also HER2 status evaluation and the functional role of specific miRNAs in ER, PR, or HER2 regulation.

In addition, the identification of patients who can benefit from treatment with chemotherapeutic agents in terms of quality of life and/or probability of survival is of great importance in oncology. Further ineffective chemotherapy may increase patient mortality. miRNAs have proven to be useful in predicting breast cancer cell sensitivity to chemotherapy.

MicroRNAs and Drug Response in Breast Cancer

Breast cancer patients may have a different susceptibility to anticancer drugs, due to their genetic and epigenetic background, or cancerous cells may become resistant during tumor progression. Progression of breast cancer and resistance to therapies have been attributed to the

Table 8.5 Single nucleotide variation in breast cancer microRNA genes

Genetic variation	MicroRNA	Clinical outcome
rs2910164 (G–>C)	hsa-miR-146a	Down
rs11614913 (T–>C)	hsa-miR-196-a2	Up
rs3746444 (A–>G)	hsa-miR-499	Down/up
Germ line G–>T 8nt	hsa-miR-125a	Down
rs895819 (A–>G)	hsa-miR-27a	Down
rs895819 (C/T)	hsa-miR-27a	–
ESR1 3′ UTR (target site)	hsa-miR-453	Down
SET8 3′ UTR (target site)	hsa-miR-502	Up
BMPR1B 3′ UTR (target site)	hsa-miR-125	Up

possibility of miRNAs involved in the regulation of certain signaling pathways. Several recent studies have focused on the identification of miRNAs linked to the acquisition of the resistant phenotype in breast cancer. Salter [52] and others examined the full mRNA and miRNA profile on the NCI-60 panel of cell line to identify signatures linked to sensitivity to paclitaxal, 5-fluorouracil, Adriamycin, and cyclophosphamide (TFAC): a miRNA signature linked to each drug response was identified.

Recently, several single-nucleotide polymorphisms (SNPs) have been described in precursor or mature cancer-related miRNAs or in miRNA target sites, and some of them have been linked to increased cancer risk. Table 8.5 summarizes a list of genetic alterations in miRNAs in breast cancer. Mishra et al. [53] identified SNP in human dihydrofolate reductate 3′ UTR and found it was responsible for methotrexate resistance in cancer cells due to lack of inhibition by miR-24, whose binding site is located near the polymorphism.

miRNAs differentially expressed in breast cancer not only play a key role in the regulation of apoptosis and invasion, but it appears to confer poor prognosis and drug resistance by sensitizing cells and modulating drug response. Thus, studies suggest the importance of integrating information derived from the miRNA profile with currently used markers.

Methylation of Breast Cancer miRs Genes

Epigenetics including DNA methylation plays a key role in the regulation of miRNA expression, and a number of reports explain silencing of miRNA expression linked to the aberrant DNA methylation of individual miRNA genes in breast cancer. miRNA genes can be epigenetically deactivated by aberrant DNA methylation in a manner similar to that of classical tumor genes. The involvement of epigenetic mechanisms in the regulation of miRNA gene expression in breast cancer was first addressed by Scott et al. [54] in an in vitro model system. Saito et al. [55] provide in their pioneering study evidence for the involvement of DNA methylation in the regulation of microRNA genes, suggesting epigenetic reactivation of microRNA expression as a promising novel strategy for cancer therapy.

The extensive and frequent hypermethylation of microRNA genes in human breast cancer supports the concept that epigenetic instability is an important and also an early event in human tumorigenesis. Considering the high frequency of miRNA gene hypermethylation found in breast cancer, miRNA gene methylation might serve as a sensitive marker for epigenetic instability. Given the fact that the binding specificity of an miRNA is conferred by a very short sequence, the prediction of specific targets remains a major bioinformatics challenge.

Conclusion and Future Perspective

In a nutshell, miRNAs have rewritten the rules about our understanding of molecular cancer biology since their initial discovery in 1993, followed by their association with cancer in 2002, and the subsequent identification of their presence in the systemic circulation in 2008. Both oncogenic and tumor-suppressive roles of miRNAs in breast cancers have encouraged numerous investigations regarding quantity of miRNAs that could be used as biomarkers and possibly manipulated for clinical benefit. The panorama of circulating miRNAs may be useful as diagnostic,

prognostic, and/or predictive biomarkers, some of which may also have relevance as new therapeutic targets, and this looks promising and very exciting.

Further investigations are warranted that will fully characterize miRNAs, their functional targets, and the phenotypic effects associated with their targeted manipulation, to harness the power and potential of miRNAs and translate this information to the clinic in the interest of breast cancer patients. Since their discovery, miRNAs have shown great potential, both as tumor biomarkers and potential as therapeutic targets in a comparatively short period, and are important contributors to the future management and therapeutic strategies of cancers in general and breast cancers in particular.

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9 Breast Cancer Proteomics

Ida Pucci-Minafra

Abstract

Breast cancer is still at the top of statistics for incidence and for scientific publications. This testifies the urgency for a better understanding of the biology and the molecular basis of the tumor onset and progression, in order to improve diagnostic and therapeutic strategies. From a biological point of view, the mammary gland, which undergoes cyclical morphogenesis, represents a valid model to study gene expression, epigenetic, differentiation, and cancer. A valid support to these types of research is the proteomic methodology, which over the last decades has been increasingly applied for large-scale detection of protein pathways and pathological biomarkers.

This chapter proposes the application of two complementary approaches, ex vivo and in vitro, for the identification of proteins and protein clusters, likely to be correlated with spontaneous or induced phenotypic perturbations representative of neoplastic cells and tissues.

Generally, the first goal is the establishment of a reference model for data mining through a platform of consolidated data. The proteomics methodology offers at present a large variety of protocols for gel-based and gel-free applications to the large-scale protein detection, each of them supported by the appropriate reference models.

In cases of 2D gel-based proteomics, a reference proteomic map is constructed with computerized applications of the gel-matching procedure among N-numbers of replicate maps.

In cases of cancer surgical tissues, a trustable reference is the healthy tissue surrounding the tumor. With this procedure, in our laboratory, we have identified sets of proteins preferentially expressed, or overexpressed, in the tumor tissues, after normalization with the actin content in each map, as cellularity index.

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Results from surgical explants ex vivo of ductal infiltrating carcinomas, cryo-preserved in our tissue bank, highlighted the appearance of "ubiquitous" and "sporadic" proteins. The identity of these proteins, assessed by mass spectrometry and immunologically validated, is discussed in this chapter on the light of literature data, with the aim of finding spontaneous tumor markers able to predict the cancer behavior.

The second type of approach, based on in vitro models, is utilized by a large number of researchers worldwide to study cell behavior under basal and experimental conditions.

In this chapter some examples of proteomic modulation under experimental conditions, mimicking selected aspects of the in vivo cancer progression, are presented. These aspects regarded the influences exerted by tumor-related collagens used as substrates for neoplastic cell cultures, the effects of fibroblast cocultured with the cancer cells and those exerted by transgenic decorin, a small leucine-rich proteoglycan with putative antioncogenic properties. Finally the proteomics of secreted vesicle and the responses to the biopharmacological drug trastuzumab were described. The proteins involved in each situation are commented on the basis of current literature.

Conclusively, the contribution of proteomics in recognizing hundreds of proteins involved and responsive to the internal/external tumor environment appears a winning strategy if conducted in a highly controlled way with precise reference points.

Keywords
Breast cancer • Proteomics • Surgical tissues • In vitro models • Gel-based proteomics • Proteomic modulation • Membrane vesicle sub-proteomic • Drug resistance proteomic

Introduction

Statistical Background

Breast cancer (BC) is still at the top of statistics for incidence among women worldwide. The report of Cancer Statistics, 2012 [1], reveals that over a total of 1,638,910 new cancer cases that are expected to occur in the United States in 2012, 29 % are breast cancers. An equal place in the statistics is occupied by prostate cancer, followed by lung and bronchus, colon and rectum, and thyroid, among the most representative. The number of scientific publications on "breast cancer," reported by PubMed bibliographic database, has progressively increased over the last decades, reaching an average of 12,700 in 2012 (Fig. 9.1), therefore occupying a priority position in relation to the other most common types of tumors. The extensive interest on BC studies testifies the urgency for a better understanding of the causes and the effects of tumor onset and progression, in order to improve the therapeutic strategies and the development of predictive, preventive, and personalized medicine.

Moreover, from a biological point of view, the mammary gland represents a valid model to study gene expression, epigenetics, differentiation, and cancer. Indeed, the mammary gland is one of the few organs undergoing adult cyclical morphogenesis during the fertile life span of the woman, being for large part under the influences of hormones, growth factors, neighboring cells, and molecules of the extracellular matrix (ECM) [2–4].

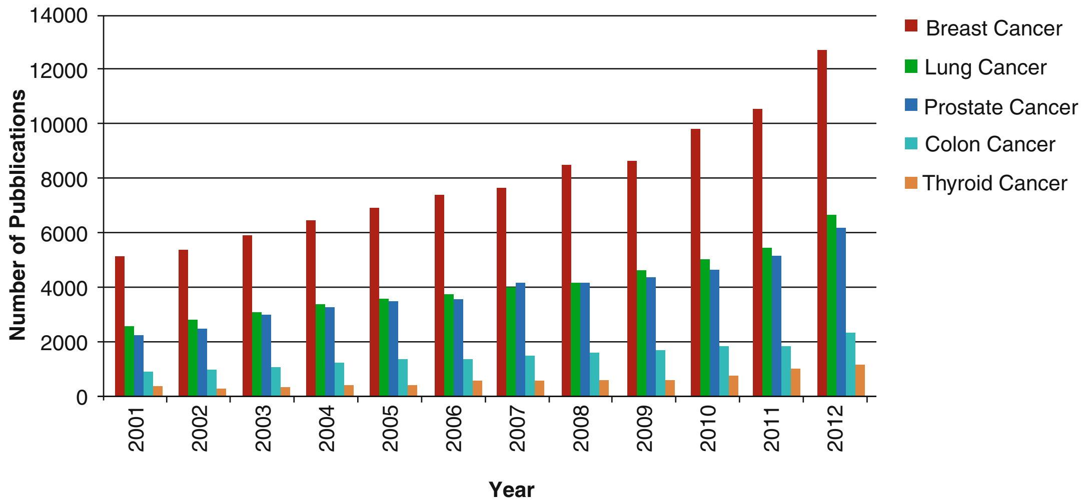

Fig. 9.1 The histogram shows the rate of increase per year in scientific publications indexed by PubMed, regarding the most frequent types of cancer: breast, lung, prostate, colon, and thyroid

The Mammary Cancer Scenery

Figure 9.2 shows a representative image of a primary breast cancer tissue, which highlights crucial aspects of its basic phenomenology. It is well known that breast cancer includes a large number of histo-types and subgroups, but all cases are characterized by some essential steps of the progressive neoplastic transformation.

In an attempt to rationalize the complexity of this figure, we can subdivide the cytohistological components into *macromodules*, *modules*, and *players*, each interacting with the other, at different levels of complexity.

The spatial organization and the cellular confines allow to outline at least the following macromodules:

1. The nuclear compartment, which includes the modules of chromatin/chromosomes, the DNA replication and the gene expression machineries, and the nuclear envelop and its accessory structures
2. The cell surface, which includes the modules of cell–cell adhesion complexes, vesiculation and membrane trafficking, antigens, and receptors
3. The cytoplasm, which incorporates the cytoskeleton and the varieties of the endoplasmic reticulum bearing a multiplicity of functional entities, including biosynthesis, metabolism, detoxification, and stress machinery
4. The secretome, which comprises extracellular enzymes and soluble factors, exosomes, and other released vesicles
5. The cell–matrix compartment that from the cell side contains ECM receptors and from the ECM side the basal lamina and other pericellular constitutive molecules. Within each module, there are an undefined number of players, namely, genes/proteins, responsible for the functional orchestration of the whole system, which can be grouped into *affinity clusters.* The protein clustering can be done according to their structural topologies or their evolutionary relationship, or by chemical, biological, or functional criteria, as needed.

The described macromodules are not to be viewed as rigid compartments; on the contrary, the functional units need to exchange activities with each other. Indeed, functional modules are dynamic entities; for instance, a protein belonging to the cytoplasm compartment, under certain circumstances, may migrate into the nucleus and play additional important functions. As a consequence, the *functional clustering* of proteins is necessarily “transverse” to the structural modules.

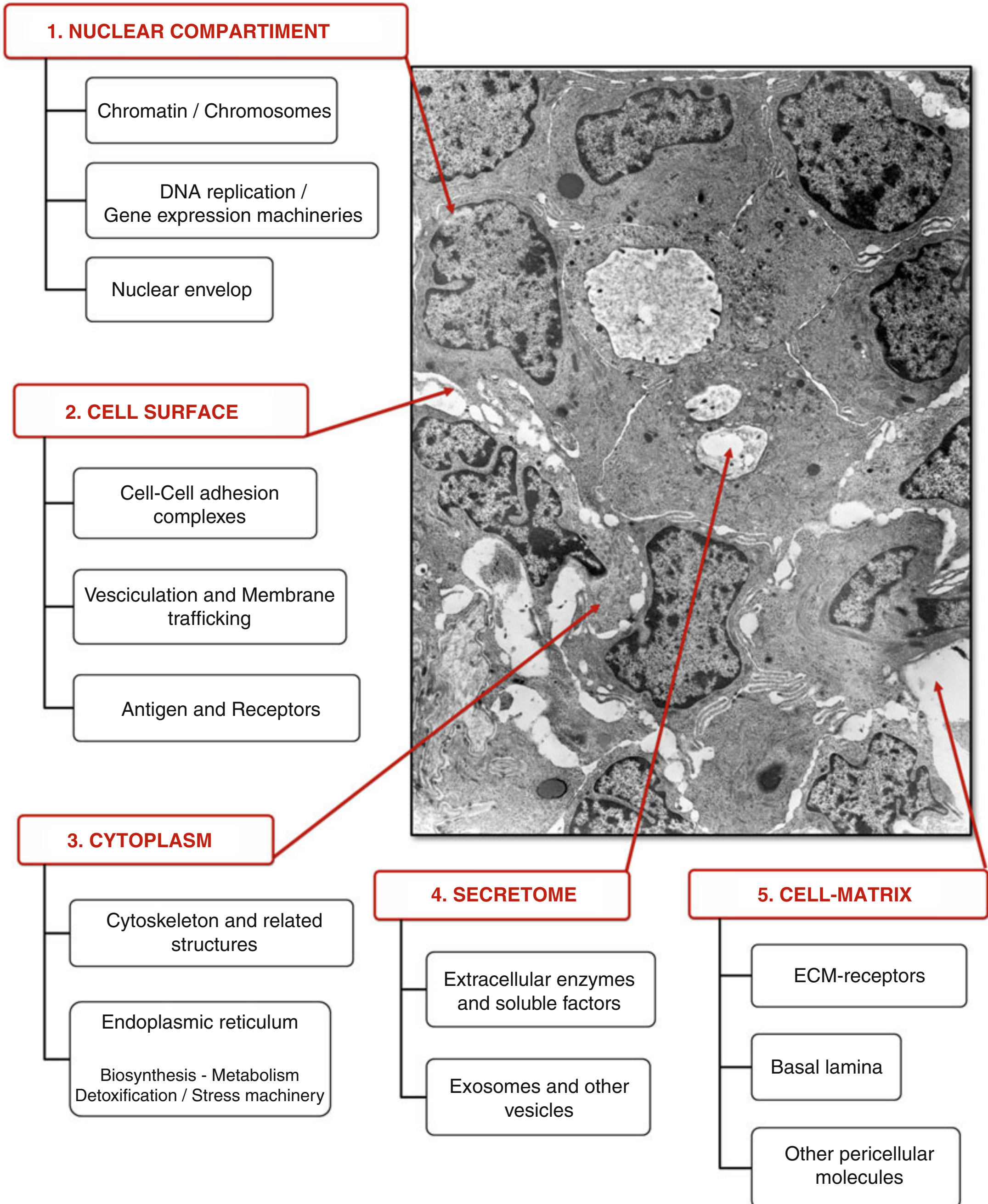

Fig. 9.2 The electron micrograph is a section of a ductal infiltrating carcinoma, where hypothetical macromodules and modules, each including the molecular players, are outlined (original magnification: 10,500×)

Returning to Fig. 9.2, it can be observed that the major cellular changes involved in the conversion of a normal breast into a malignant breast are the progressive loss of the cell–cell adhesions and of the polarized morphology, typical of the stationary epithelial phenotype.

Concurrently, once the cells detach from each other, the cell surfaces become unstable acquiring a ruffled appearance, with a tendency to release vesicles of a different nature. Fully detached cells usually acquire a mesenchymal-like phenotype, which explains well their ability to migrate into surrounding tissues. This tendency, recognized as EMT (epithelial/mesenchymal transition) and sustained by a defined set of genes, is manifested exaggeratedly in the primary culture from surgical breast cancer biopsies [5]. These alterations in gene expressions are able to drive the cancer progression, but the exhaustive number, identities, and functional network of the involved genes/proteins are still to be fully recognized.

The Proteomic Dream, the Proteomic Current Reality

The term "proteome" originally coined by Wilkins [6] was intended to describe "the total protein complement of a genome." With the beginning of the development of the proteomic research, it has become clearer and clearer that this definition could probably be applied to unicellular organisms but not equally to higher organisms, especially in humans; therefore, it is more appropriate to refer to the proteome as "the complement of protein extracted from biological samples under given conditions." The deriving information can then be used for comparative proteomics, drug responses, modulation of gene expression, etc.

The application of proteomics to surgical tissues must take into consideration several problems, some of which are inherent to the dynamic nature and the complexity of cell and tissue themselves and others related to technical implications. The former include the variable amount of extracellular components in tissue biopsies; the different turnover of proteins in a given cell, which may influence the protein concentration of the proteome; and the frequent occurrence of several posttranslational isoforms for a given primary gene product. The technical limitations are mainly related to the solubilization systems (no single procedure can be applied for the extraction of the entire set of native proteins) and to the separating systems (traditional 2D-IPG is unable to focus proteins with p*I* over 8.5). Therefore, the old dream to recognize a given "signature" or "constellation" into a single proteomic map, able to distinguish, for instance, a healthy cell from its neoplastic counterpart, has been profoundly reviewed. "Signature" and "constellation" should be deduced from the statistical evaluations of a significant number of data properly collected for specific targets. On the other hand, comparative proteomic profiling of cells and tissues, under the same extraction conditions, has provided an extraordinary amount of information.

The initial applications of the proteomic technologies were essentially based on the 2D-IPG protein separation, which, thanks to the introduction of gel strips with immobilized ampholyne systems commercially available, gave the opportunity to standardize exchangeable protocols among laboratories. An exciting result was the generation of several databases of 2D-IPG for protein identity search (e.g., SWISS-2DPAGE). The beginning of the history focuses on the method of the gel matching, based on the computerized analyses of the proteomic maps and spot detection, followed by N-sequencing identification (Edman degradation) and immunological validation of the selected spots.

Over the past 10 years, a number of additional technologies have been developed to analyze proteins on a large scale, first of all the mass spectrometry (MS) methods on digested proteins, namely, the electron spray ionization (ESI) and the matrix-assisted laser desorption/ionization (MALDI) [7].

The MS spectra are then matched with known sequences in databases (e.g., SEQUEST, MASCOT) to calculate masses, resulting in the identification of target proteins. This type of protein identification method is known as "peptide mass fingerprinting" [8].

More recently a new strategy—non-gel-based proteomics, defined as "shotgun proteomics"—emerged as a method that could offer advantages in speed, sensitivity, and automation over the gel-based techniques. Proteins are extracted from a biological sample and digested with a protease to

produce a peptide mixture [9]. The peptide mixture is then loaded directly onto a microcapillary column and the peptides are separated by hydrophobicity and charge. The charged fragments are separated in the second stage of tandem mass spectrometry. A serious limitation of non-gel-based proteomics is the low score of protein identities, due to the well-known homologies among diverse proteins. On the contrary, the 2D-based proteomics introduce two fundamental parameters useful for protein identification, which are p*I* and Mw, elevating the score for protein identification.

Additional complementary proteomic approaches, based on the differential labeling of protein extracts with stable isotopes (^{1}H and ^{2}H, ^{12}C and ^{13}C, ^{14}N and ^{15}N), have been developed to improve the evaluation of target protein expressions: the peak intensity of the differentially labeled peptide is used for quantitative evaluations. Among the isotopic labeling-based approaches, two are currently most used: the SILAC (stable isotope labeling by amino acids in cell culture) and the ICAT (isotope-coded affinity tag). The SILAC approach requires the addition of a stable isotope-labeled amino acid (i.e., ^{2}H-leucine or ^{13}C-arginine) to the cell culture. The ICAT is based on the incorporation of isotopic tags after protein extraction and is suitable for experimental conditions where metabolic labeling is not feasible (i.e., protein samples extracted from tissues); reviewed by Liang et al. [10].

All the briefly cited methods are applied to fulfill specific requirements for large-scale protein identification, and others are still in progress, such as the promising technique of the "tissue imaging using MALDI-MS," which is considered a new frontier of histopathology proteomics [11, 12].

Proteomics of Breast Cancer Tissues to Detect Putative Spontaneous Tumor Markers

The first reason of concern in detecting putative biomarkers from cancer cells and tissues is the establishment of a "normal" reference range of qualitative/quantitative protein expression. The utilization of matched tumoral and healthy tissue adjacent to the tumor is a consolidated system to identify set of proteins specifically related to the presence of neoplastic cells. In a pilot study by our group [13], we carried out a comparative proteomic profiling of paired tumoral and non-tumoral tissue counterparts extracted from 13 selected surgery specimens from ductal infiltrating carcinomas (DIC) and processed in parallel. Results provided substantial information on qualitative and quantitative differences between the two sets of tissues and allowed the constructing of a reference map of tumoral tissue to be utilized for further comparative analyses. Figure 9.3 shows a representative map of a breast cancer tissue containing 312 identified protein forms grouped into 11 categories and reported in Table 9.1. To avoid redundancy, clusterization was accomplished following the criterion of primary function of proteins. In addition, a "transverse" category is introduced in Table 9.2. This comprises proteins with diverse primary functions, all involved in the regulation of cell proliferation and cell death, according to the DAVID bioinformatics resources [14].

The proteins in the tables are indicated with their full names, accession names, and abbreviated names, according to Swiss-Prot database. The latter will be used throughout the text for reasons of brevity. The number of identified isoforms is also indicated. However, it must be noted that categories are subject to periodical remodeling, following the detection of new proteins or new functions for a known protein.

Ubiquitous and Sporadic Proteins

The extension of these analyses to a larger group of 37 selected cases of ductal infiltrating carcinomas revealed for the first time at the proteomic level the occurrence of ubiquitous and sporadically expressed proteins, unrelated to clinical parameters. To minimize the known heterogeneity of breast cancer tissues, for which proteomic profiling may be influenced by variations in cellular contents versus stromal proteins, the expression levels of individual protein spots were

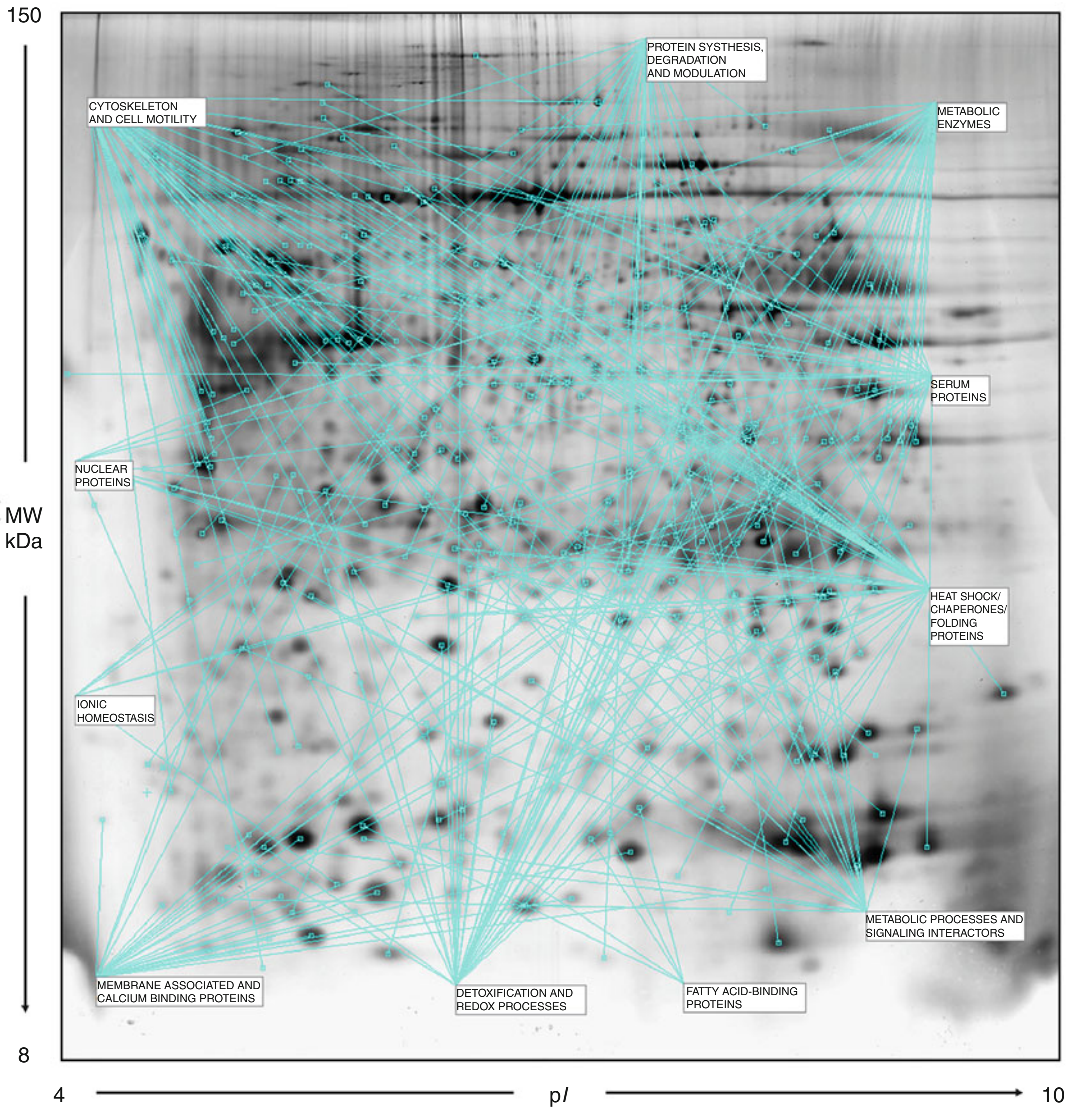

Fig. 9.3 A representative proteomic map of a breast cancer tissue (DIC) containing 312 identified protein forms grouped into 11 categories and reported in Table 9.1 [2-DE separation was performed on IPG gel strips (18 cm, 3.5–10 NL) followed by the SDS-PAGE on a vertical linear-gradient slab gel (9–16 %T). The 2D gels were analyzed using the software ImageMaster 2d Platinum]

normalized to the actin content in each tissue extract, as a "cellularity index" [15].

The Ubiquitous Proteins

Consistently, a major portion of ubiquitous proteins expressed at high levels belonged to the category of glycolytic enzymes (G3P, ENOA, PGK1, PGAM1, and TPIS). The shift of metabolism from aerobic to anaerobic has been known since the pioneering work of Otto Warburg, and subsequently this phenomenon was called the "Warburg effect" and classically attributed to a metabolic shift restricted to the cell compartment, due to the hypoxic condition of the tumor mass. More recently it has been observed that cancer cells in vitro [16] and mammospheres [17] grown under normoxic conditions also display high levels of glycolytic enzymes with respect to the

Table 9.1 Proteins identified in breast cancer tissues and clustered according to their primary function

Metabolic enzymes			
Protein name	**AC number**	**Abbreviated name**	**Protein isoform Nr**
Aconitate hydratase, mitochondrial	Q99798	ACON	2
Alpha-enolase	P06733	ENOA	7
Enoyl-CoA hydratase, mitochondrial	P30084	ECHM	
Fructose-bisphosphate aldolase A	P04075	ALDOA	4
Fumarate hydratase, mitochondrial	P07954	FUMH	
Gamma-enolase	P09104	ENOG	
Glyceraldehyde-3-phosphate dehydrogenase	P04406	G3P	5
L-lactate dehydrogenase A chain	P00338	LDHA	
L-lactate dehydrogenase B chain	P07195	LDHB	
Malate dehydrogenase, cytoplasmic	P40925	MDHC	
Malate dehydrogenase, mitochondrial	P40926	MDHM	
Neutral alpha-glucosidase AB	Q14697	GANAB	
Phosphoglycerate kinase 1	P00558	PGK 1	3
Phosphoglycerate mutase 1	P18669	PGAM1	2
Pyruvate kinase isozymes M1/M2	P14618	KPYM	3
Triosephosphate isomerase	P60174	TPIS	4
Metabolic processes and signaling interactors			
14-3-3 Protein gamma	P61981	1433G	
3-Hydroxyisobutyryl-CoA hydrolase, mitochondrial	Q6NVY1	HIBCH	
Acyl-CoA-binding protein	P07108	ACBP	
Bifunctional purine biosynthesis protein PURH	P31939	PUR9	
dCTP pyrophosphatase 1	Q9H773	DCTP1	
Glyoxalase domain-containing protein 4	Q9HC38	GLOD4	
Macrophage migration inhibitory factor	P14174	MIF	
N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	O94760	DDAH1	
N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	O95865	DDAH2	
Nucleoside diphosphate kinase A	P15531	NDKA	
Nucleoside diphosphate kinase B	P22392	NDKB	
Phosphatidylethanolamine-binding protein 1	P30086	PEBP	2
Purine nucleoside phosphorylase	P00491	PNPH	2
Pyridoxine-5′-phosphate oxidase	Q9NVS9	PNPO	
Rho GDP-dissociation inhibitor 1	P52565	GDIR1	
Rho GDP-dissociation inhibitor 2	P52566	GDIR2	
SH3 domain-binding glutamic acid-rich-like protein	O75368	SH3L1	2
SH3 domain-binding glutamic acid-rich-like protein 3	Q9H299	SH3L3	
Sialic acid synthase	Q9NR45	SIAS	3
Thiosulfate sulfurtransferase/rhodanese-like domain-containing protein 1	Q8NFU3	TSTD1	2
Thymidine phosphorylase	P19971	TYPH	
Fatty acid-binding proteins			
Fatty acid-binding protein, adipocyte	P15090	FABP4	
Fatty acid-binding protein, epidermal	Q01469	FABP5	
Fatty acid-binding protein, brain	O15540	FABP7	2
Fatty acid-binding protein, heart	P05413	FABPH	

Table 9.1 (continued)

Cytoskeleton and cell motility			
Protein name	**AC number**	**Abbreviated name**	**Protein isoform Nr**
Actin, cytoplasmic 1	P60709	ACTB	15
Actin-related protein 2/3 complex subunit 5	O15511	ARPC5	
Adenylyl cyclase-associated protein 1	Q01518	CAP1	2
Cofilin-1	P23528	COF1	4
Coronin-1A	P31146	COR1A	
F-actin-capping protein subunit alpha-1	P52907	CAZA1	
Fascin	Q16658	FSCN1	
Macrophage-capping protein	P40121	CAP G	3
Myosin light polypeptide 6	P60660	MYL6	
Programmed cell death 6-interacting protein	Q8WUM4	PDC6I	
Thymosin beta-4-like protein 3	A8MW06	TMSL3	
Tropomyosin alpha-1 chain	P09493	TPM1	
Tropomyosin alpha-4 chain	P67936	TPM4	3
Tropomyosin beta chain	P06468	TPM2	2
Tubulin alpha-1 chain	Q71U36	TBA1A	3
Tubulin beta-5 chain	P07437	TBB5	2
Vimentin	P08670	VIME	5
Vinculin	P18206	VINC	2
Membrane associated and calcium-binding proteins			
Annexin A1	P04083	ANXA1	3
Annexin A2	P07355	ANXA2	3
Annexin A4	P09525	ANXA4	
Annexin A5	P48036	ANXA5	2
Calmodulin	P62158	CALM	
Galectin-1	P09382	LEG1	2
Galectin-3	P17931	LEG3	2
Protein S100-A2	P29034	S10A2	
Protein S100-A4	P26447	S10A4	2
Protein S100-A6	P06703	S10A6	2
Protein S100-A7	P31151	S10A7	2
Protein S100-A8	P05109	S10A8	
Protein S100-A11	P31949	S10AB	3
Protein S100-A13	Q99584	S10AD	
Nuclear proteins			
Acidic leucine-rich nuclear phosphoprotein 32 family member A	P39687	AN32A	
Heterogeneous nuclear ribonucleoprotein A1	P09651	ROA1	2
Heterogeneous nuclear ribonucleoproteins A2/B1	P22626	ROA2	3
Nuclear transport factor 2	P61970	NTF2	
Nucleophosmin	P06748	NPM	
Prelamin-A/C	P02545	LMNA	2
RuvB-like 1	Q9Y265	RUVB1	
Ionic homeostasis			
Carbonic anhydrase 1	P00915	CAH1	
Inorganic pyrophosphatase	Q15181	IPYR	2

(continued)

Table 9.1 (continued)

Protein name	AC number	Abbreviated name	Protein isoform Nr
Selenium-binding protein 1	Q13228	SBP1	
V-ATPase subunit F	Q16864	VATF	
Voltage-dependent anion channel protein 2	P45880	VDAC2	

Protein synthesis, degradation and modulation			
Protein name	**AC number**	**Abbreviated name**	**Protein isoform Nr**
26S protease regulatory subunit 8	P62195	PRS8	
40S ribosomal protein SA	P08865	RSSA	
60S acidic ribosomal protein P0	P05388	RLA0	
60S acidic ribosomal protein P2	P05387	RLA2	2
Cathepsin D	P07339	CATD	3
Cystatin-A	P01040	CYTA	
Cystatin-B	P04080	CYTB	
Cytosol aminopeptidase	P28838	AMPL	
Elongation factor 1-beta	P24534	EF1B	
Elongation factor 2	P13639	EF2	3
Eukaryotic translation initiation factor 6	P56537	IF6	
Proteasome activator complex subunit 1	Q06323	PSME1	
Proteasome subunit alpha type-1	P25786	PSA1	
Proteasome subunit alpha type-5	P28066	PSA5	
Proteasome subunit alpha type-6	P60900	PSA6	
Proteasome subunit beta type-3	P49720	PSB3	
Ribosome-binding protein 1	Q9P2E9	RRBP1	
Small ubiquitin-related modifier 1	P63165	SUMO1	
U3 small nucleolar RNA-interacting protein 2	O43818	U3IP2	
Ubiquitin carboxyl-terminal hydrolase isozyme L1	P09936	UCHL1	
Ubiquitin-60S ribosomal protein L40	P62987	RL40	2
Ubiquitin-conjugating enzyme E2 N	P61088	UBE2N	
Ubiquitin-conjugating enzyme E2 variant 2	Q15819	UB2V2	
Ubiquitin-like protein Nedd8	Q15843	NEDD8	
Detoxification and redox processes			
Alcohol dehydrogenase [NADP(+)]	P14550	AK1A1	2
Aldehyde dehydrogenase, cytosolic	P00352	AL1A1	
Aldo-keto reductase family 1 member B10	O60218	AK1BA	2
Aldose reductase	P15121	ALDR	2
Chloride intracellular channel protein 1	O00299	CLIC1	2
Dihydrolipoyl dehydrogenase, mitochondrial	P09622	DLDH	
Flavin reductase (NADPH)	P30043	BLVRB	
Glutathione S-transferase P	P09211	GSTP1	
Glutathione synthetase	P48637	GSHB	
Glutathione transferase omega-1	P78417	GSTO1	2
Isocitrate dehydrogenase [NADP] cytoplasmic	O75874	IDHC	2
Peroxiredoxin 6	P30041	PRDX6	2
Peroxiredoxin-1	Q06830	PRDX1	5

Table 9.1 (continued)

Protein name	AC number	Abbreviated name	Protein isoform Nr
Peroxiredoxin-2	P32119	PRDX2	3
Peroxiredoxin-3	P30048	PRDX3	
Peroxiredoxin-4	Q13162	PRDX4	
S-formylglutathione hydrolase	P10768	ESTD	3
Superoxide dismutase [Cu-Zn]	P00441	SODC	2
Superoxide dismutase [Mn], mitochondrial	P04179	SODM	2
Thioredoxin	P10599	THIO	2

Heat-shock/chaperones/folding proteins

Protein name	AC number	Abbreviated name	Protein isoform Nr
Calreticulin	P27797	CALR	
60 kDa heat shock protein, mitochondrial	P10809	CH60	2
94 kDa glucose-regulated protein	P14625	ENPL	
Endoplasmic reticulum resident protein 29	P30040	ERP29	
Glucosidase 2 subunit beta	P14314	GLU2B	
75 kDa glucose-regulated protein	P38646	GRP75	
78 kDa glucose-regulated protein	P11021	GRP78	4
Heat shock protein HSP 90-alpha	P07900	HS90A	
Heat shock protein HSP 90-beta	P08238	HS90B	
Heat shock 70 kDa protein 1A/1B	P08107	HSP71	
Heat shock 70 kDa protein 4	P34932	HSP74	
Heat shock cognate 71 kDa protein	P11142	HSP7C	4
Heat shock protein beta-1	P04792	HSPB1	5
Parkinson disease protein 7-Oncogene DJ1	Q99497	PARK7	4
Protein disulfide isomerase	P07237	PDIA1	
Protein disulfide isomerase A3	P30101	PDIA3	4
Peptidyl-prolyl cis-trans isomerase A	P62937	PPIA	5
Peptidyl-prolyl cis-trans isomerase B	P23284	PPIB	
Ras-related protein Rab-18	Q9NP72	RAB18	
Cellular retinoic acid-binding protein 2	P29373	RABP2	2
Transitional endoplasmic reticulum ATPase	P55072	TERA	2
Stress-induced phosphoprotein 1	P31948	STIP1	2
Serum proteins			
Alpha-1-acid glycoprotein 2	P19652	A1AG2	
Alpha-1-antitrypsin	P01009	A1AT	2
Alpha-2-macroglobulin	P01023	A2MG	
Alpha-1-antichymotrypsin	P01011	AACT	
Serum albumin	P02768	ALBU	2
Apolipoprotein A1	P02647	APOA1	2
Beta-2-microglobulin	P61769	B2MG	
Complement component 1 Q	Q07021	C1QBP	
Ceruloplasmin	P00450	CERU	2
Alpha-2-HS-glycoprotein	P02765	FETUA	
Fibrinogen beta chain	P02675	FIBB	2
Ferritin light chain	P02792	FRIL	
Hemoglobin subunit alpha	P69905	HBA	2

(continued)

Table 9.1 (continued)

Protein name	AC number	Abbreviated name	Protein isoform Nr
Hemoglobin subunit beta	P68871	HBB	3
Heme-binding protein 2	Q9Y5Z4	HEBP2	
High mobility group protein B1	P09429	HMGB1	
Haptoglobin	P00738	HPT	
Ig gamma-1 chain C region	P01857	IGHG1	1
Immunoglobulin light chain	P99007	IGLC	
Serotransferrin	P02787	TFRE	
Transthyretin	P02766	TTHY	

The table reports: protein names, accession numbers (AC), and abbreviated names, corresponding to the nomenclature used in the Swiss-Prot database. The last column reports the number of isoforms for protein

Table 9.2 Proteins fulfilling the additional function of cell proliferation and cell death regulators

Protein name	AC number	Abbreviated name	Protein isoform Nr
26S protease regulatory subunit 8	P62195	PRS8	
60 kDa heat shock protein, mitochondrial	P10809	CH60	2
75 kDa glucose-regulated protein	P38646	GRP75	
78 kDa glucose-regulated protein	P11021	GRP78	4
94 kDa glucose-regulated protein	P14625	ENPL	
Annexin A1	P04083	ANXA1	3
Annexin A4	P09525	ANXA4	
Calreticulin	P27797	CALR	
Cofilin-1	P23528	COF1	4
Cystatin-B	P04080	CYTB	
Galectin-1	P09382	LEG1	2
Glutathione S-transferase P	P09211	GSTP1	
Heat shock 70 kDa protein 1A/1B	P08107	HSP71	
Heat shock protein beta-1	P04792	HSPB1	5
Macrophage migration inhibitory factor	P14174	MIF	
N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	O95865	DDAH2	
Nucleophosmin	P06748	NPM	
Nucleoside diphosphate kinase A	P15531	NDKA	
Nucleoside diphosphate kinase B	P22392	NDKB	
Peroxiredoxin-1	Q06830	PRDX1	5
Peroxiredoxin-2	P32119	PRDX2	3
Protein disulfide-isomerase A3	P30101	PDIA3	4
Rho GDP-dissociation inhibitor 1	P52565	GDIR1	
Superoxide dismutase [Cu-Zn]	P00441	SODC	2
Superoxide dismutase [Mn], mitochondrial	P04179	SODM	2
Thioredoxin-dependent peroxide reductase, mitochondrial	P30048	PRDX3	
Transitional endoplasmic reticulum ATPase	P55072	TERA	2
Tubulin beta-5 chain	P07437	TBB5	2
Ubiquitin-60S ribosomal protein L40	P62987	RL40	2

non-tumoral cells, suggesting that the increased level of glycolytic enzymes is part of a oncogenic process that cells maintain in vitro. This was recently confirmed by Altenberg et al., demonstrating that genes of glycolysis are ubiquitously overexpressed in 24 cancer classes [18].

The deregulation of glycolytic enzymes in cancer may cause cascade consequences with adverse outcomes for patients. Indeed, besides their primary metabolic role, many glycolytic enzymes may fulfill a multiplicity of other functions. Among the most remarkable examples is the G3P, which has been implicated in membrane fusion, microtubule bundling, phosphotransferase activity, and binding of nucleic acids [19]. For these reasons, G3P is now included in the possible therapeutic targets [20].

Another key glycolytic enzyme, the ENOA, in some cases, is translocated to the cell surface, where it may also act as a plasminogen receptor [21], thus mediating the activation of plasmin and consequent extracellular matrix degradation, instrumental for cancer invasion. Similarly, the PGK1, another overexpressed glycolytic enzyme, has been reported to be secreted extracellularly and plays an important role in regulating the "angiogenic switch," essential for tumor growth and metastasis [22]. In addition, PGK1 is known to interact with the E-cadherin/β-catenin complex [23], suggesting that overexpression of this protein in tumors may promote decreased cell–cell adhesion and potentiate cell migration. It has also been reported that the TPIS, an enzyme that plays a key role in the interconnection between the pentose phosphate pathway and the lipid metabolism, tends to be expressed higher in immature myeloid cells [24, 25].

Further ubiquitous protein forms with multifaceted functions include members of the folding machinery (PDA1, PPIA), heat-shock proteins (HSPA1B/HSP74, GRP94), members of redox-defense machinery (SODM, THIO, GSTP1), a master regulator of antioxidant transcriptional responses, the oncogene PARK7 [26], and some calcium-binding proteins (S10A6, CALR, ANXA2). Interestingly, CALR has been identified to exert a role in the tumor immune responses [27]. Other components, which individually or in synergy are important players of events related to tumor growth, are as follows: two regulators of the transduction machinery (GDIR and GDIS), the DDAH2, a relevant enzyme involved in the angiogenesis control [28], some calcium-binding proteins (S10A6, CALR, ANXA2), and one isoform of VINC. Finally, other ubiquitously expressed proteins were the CATD, a controversial prognostic marker for breast cancer progression [29] detected under two isoelectric variants; and the PSA5, a subunit of the multicatalytic endopeptidase complex involved in the response to DNA damages by p53 signal transduction, resulting in cell cycle arrest. Remarkably, many of the observed ubiquitous proteins are known to also be involved in the regulation of cell death (see Table 9.2).

The Sporadic Proteins

The group of sporadically expressed proteins is more heterogeneous and includes: nuclear proteins (ROA1, ROA2, NTF2), the multifunctional calcium-binding protein CALM, the enzymes LDHA and LDHB, and proteins with a role in regulating cell proliferation and metabolism (NDKA, NDKB, TERA, NEDD8, SODC, several PRDX family members). Worthy of note, PRDX2 has been identified as a breast cancer autoantigen, useful in early diagnosis of aggressive breast cancer [30], and PRDX6 has also been shown to be related to breast cancer malignancy—indeed, its overexpression leads to a more invasive phenotype and metastatic potential [31].

Some of the sporadic proteins show a high expression level when present, which suggests their possible role for patient stratification. Among them are the LEG1, the COF1, and the TAGL2. The LEG1, besides its several functions, may also act as a copromoter of MMP-2 and MMP-9 expression [32] and therefore is to be considered an enhancer of tumor invasion and metastasis. The COF1 is a key regulator of the apoptotic process [33], and it is also crucial for the actin cytoskeleton dynamics, therefore playing a role in the formation of membrane protrusions and directional spikes associated with a motile and invasive phenotype [34]. Similarly, the TAGL2, a protein able to bind actin and

tropomyosin, is also involved in cytoskeleton remodeling and in the process of membrane shedding, typical of the malignant cells. Moreover, some studies performed on renal carcinoma cell line by silencing TAGL2 showed a consequent significant inhibition of cell proliferation and invasion, suggesting an oncogenic role for TAGL2 [35].

The Growing Family of S100 Proteins

Among the sporadically expressed proteins deserving closer attention is the class of calcium-binding S100 proteins, which are considered a potentially important group of markers in cancer development and progression [36].

The S100 proteins are small, acidic calcium-binding proteins, whose name derives from the observation that they are soluble in 100 % saturated ammonium sulfate at neutral pH. The first member was identified in the nervous system by Moore in 1965 [37]. At present, at least 25 members of the S100 protein family are recognized in humans, the majority of them encoded by gene clusters located on the chromosome locus 1q21 and differentially distributed in the tissues.

In the breast cancer tissues screened among 100 DIC patients [38], we recognized 12 members (including isoforms) of the S100 protein family, all focusing within a range of pH from 4.60 to 6.80. Figure 9.4 shows the proteomic window of one of the studied cases in which all the identified S100 are represented.

These proteins may form homo- and heterodimers, or even oligomers, and may perform different functions, through a broad range of intracellular and extracellular activities [39, 40]. Intracellular functions include calcium homeostasis, regulation of phosphorylation and gene expression, cytoskeleton dynamics, and cell motility. Some of them have been shown to interact with, and to regulate, various proteins involved in cancer [41] including the p53 complex [42, 43]. Their extracellular activities are fulfilled in a cytokine-like manner through the receptor for advanced glycation end products (RAGE) [44]. Some of the secreted S100 proteins may exert chemotactic and antibacterial actions [45, 46]. Therefore, the differential occurrence and the strategic roles played by members of the S100 protein family enable them to become a powerful signature for cancer progression.

Within the sporadic forms of S100 proteins, the S100A7 was one of the most prominent, also for the high level of relative concentration reached in many cases [47]. S100A7 was originally identified as a protein overexpressed in psoriatic keratinocytes, called psoriasin [48]. Subsequently it has been found to be overexpressed in several carcinomas, including the skin [49], oral squamous cell carcinoma [50], esophagus [51], bladder [52], lung [53], stomach [54], and breast [55–58].

S100A7 was found to be secreted but also present in the cytoplasm and the nucleus of cells

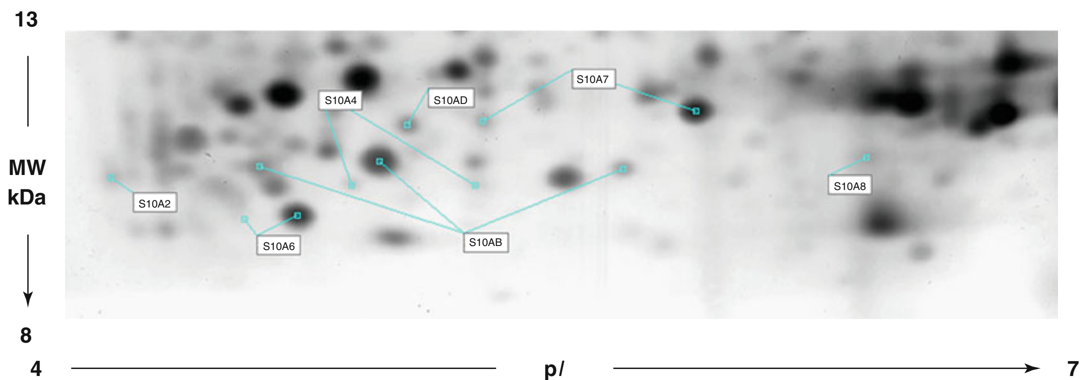

Fig. 9.4 A proteomic window of one of the studied DIC cases in which all the identified S100 proteins, and isoforms, are localized

expressing it. Intracellularly, S100A7 is also believed to be associated with several proteins such as RanBPM [59], epidermal fatty acid-binding protein, FABP5 [60], and Jab-1 [61]. The secreted form may exert a chemotactic potential [62], and, more recently, it has been implicated in inflammation with an antibacterial action [63].

The sporadic occurrence of S100A7 among patients with invasive breast cancer was confirmed in tissue arrays of breast cancer sections immunostained with a monoclonal S100A7 antibody where it was observed that, when present, the protein is primarily expressed in cancer cells, and almost absent in the unaffected glandular structures, or in the mesenchymal cells (Fig. 9.5).

In Vitro Models for the Study of Proteomic Modulation

In order to study the dynamical behavior of neoplastic cell populations, a widespread practice is the utilization of cell cultures, both primary and stabilized, able to reproduce phenotypical characteristics similar to the original tissue from where they were derived.

Figure 9.6 shows the comparative micrographs of two representative cell lines, HB2 and 8701-BC, the first immortalized from a normal human breast [64] and the second isolated from a DIC [65]. As can be observed, the modality of cell growth is emblematic: the non-tumoral cells grow in a polarized shape, tend to form ordered monolayered cellular sheets, and stop division when they reach confluence. The neoplastic cells display highly irregular cell shapes with ruffling cell surfaces and they overgrow, even under-confluence, and do not stop replication when they are over-confluent. These and other cellular models have been widely used to study proteomic responses to several experimental conditions mimicking selected aspects of the in vivo cancer progression, such as overgrowth, metabolism, vesicle releasing, cell–cell crosstalk, cell–matrix interactions, and metastatic propensity. In our laboratory a reference map was created for each cell culture under investigation. Generally, the proteomic profile of breast cancer cells revealed a large percentage of homology with the related breast cancer tissue.

Proteomic Modulation Induced by Collagen Substrates

Epigenetic influences are fundamental to sustain tumor progression. The progression from an initially benign form of localized tumor toward a more aggressive potentially metastatic stage involves several steps including, on the one hand, cell detachment from the primary mass, followed by the neoplastic cell migration into the surrounding host tissue, and, on the other hand, the severe changes of the surrounding extracellular matrix, which undergoes local degradation and new matrix deposition.

This rearrangement of the extracellular matrix includes an incremental deposition of type V collagen [66, 67] and a shift in the collagen synthesis toward an oncofetal homotrimeric collagen type composed of three α1(I) chains, also named type I trimer, and OF/LB (oncofetal laminin-binding) for its ability to bind to laminin [4].

The biological effects of these tumor-related collagen types, in parallel with the normal tissue counterparts, collagen type I and type IV, were individually investigated in the form of reconstituted monolayers used as substrates for 8701-BC neoplastic cell growth [68]. From a phenotypical point of view, the cells seeded onto type I trimer collagen overgrow and produce spikes, probably exasperating the neoplastic cell behavior in active proliferation and with motile attitude. On the contrary, the other collagen substrates induced a more differentiated stationary morphology to the cells seeded on them. The comparative analysis of substrate-induced proteomic modulation showed a partially overlapping pattern of the responsive proteins, with some preferential response. The graph of Fig. 9.7 shows an overview of the profile of the relative pixel density of protein spots, plotted as a mean of three separate experiments.

The proteins which appeared modulated preferentially, even if not exclusively, by type I trimer fall

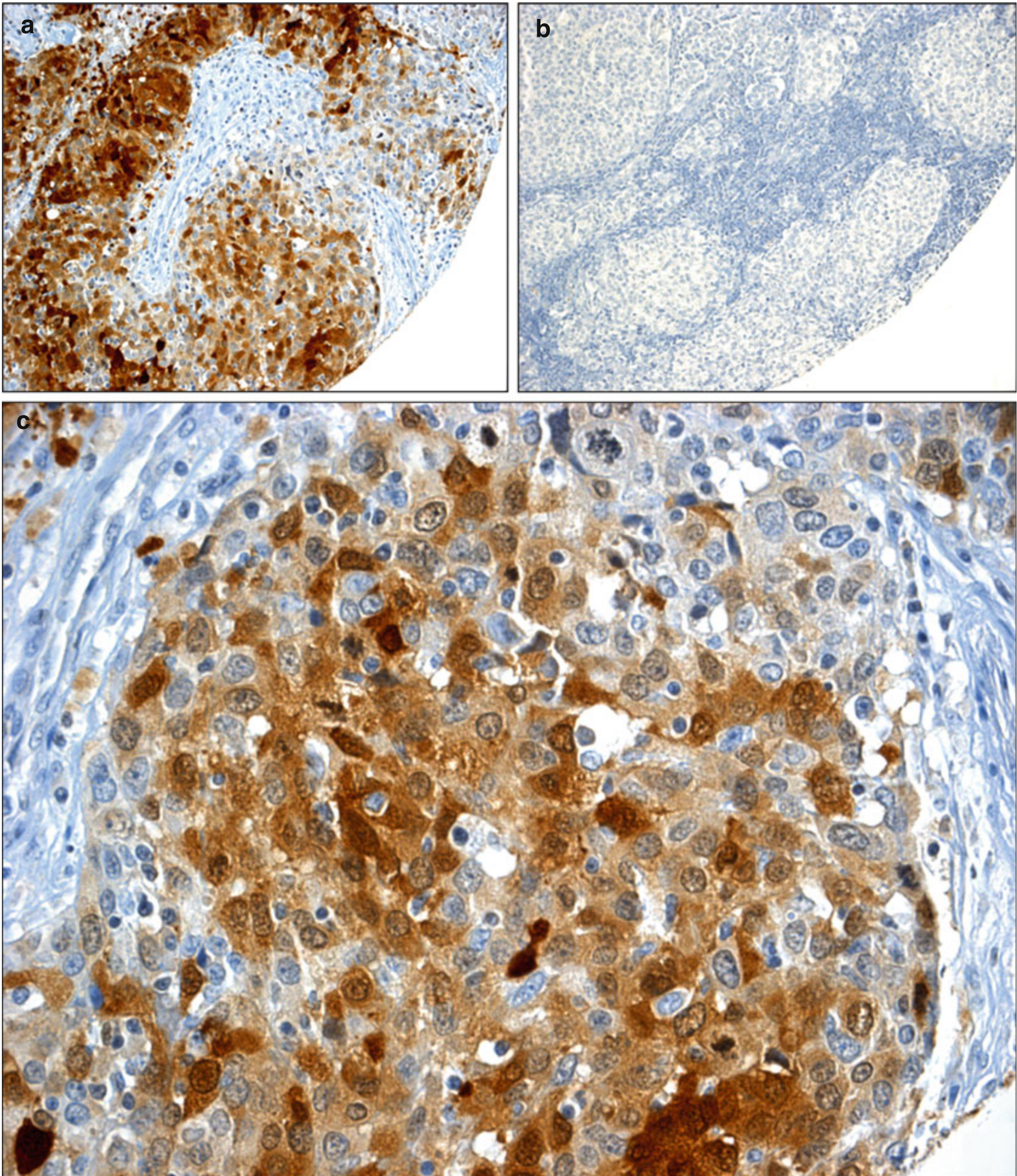

Fig. 9.5 Representative tissue microarray sections (BioMax) immunostained with a S100A7 monoclonal antibody (Santa Cruz). (**a**) An S100A7-positive DIC tumor where the neoplastic cells are selectively stained, (**b**) the same type of tumor showing complete negativity for S100A7, (**c**) an enlargement of the positive tumor section, showing the localization of the S100A7 in the neoplastic cell cytoplasm and in many nuclei of the positive cells

into three main groups, namely: with (1) the class of metabolic enzymes (one isoform of G3P, ENOA, PGK1, KPYM, ACON, MDHM), (2) the group of cytoskeleton and cell motility (TBB5, TMSL3, TPM, VIME, VINC, COF1, ALDOA), and (3) the "transverse" group of cell growth and cell death regulators (ANXA1, ANXA4, HSPB1, HSP71, NPM, TERA, SODC) in which COF1 is also

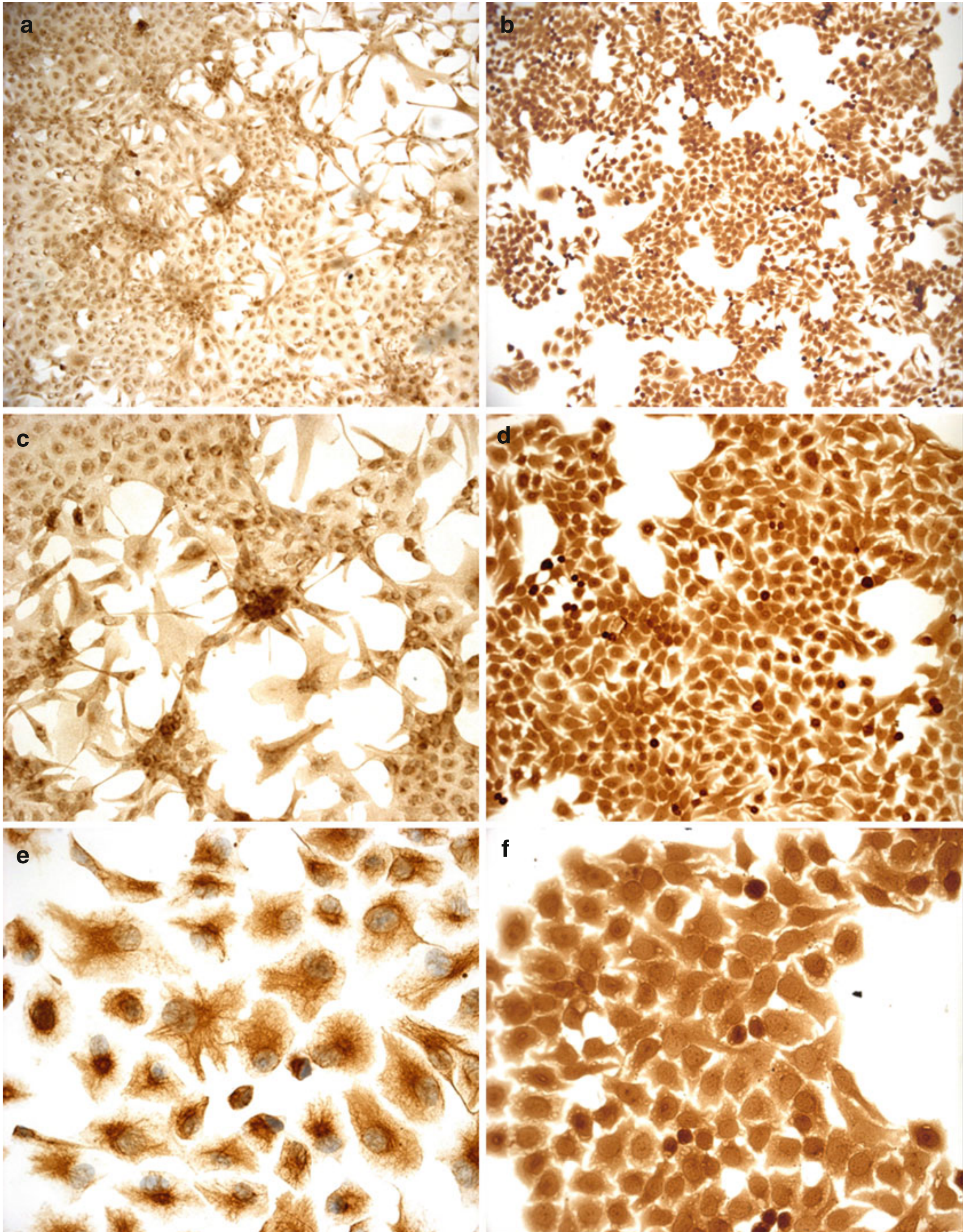

Fig. 9.6 Optical micrographs of two representative cell lines, used for the studies of in vitro proteomics, showing the different morphological features and modality of growth. (**a**, **c**, **d**) Under-confluent 8701-BC cells, isolated from a ductal infiltrating carcinoma [65]. (**b**, **d**, **e**) Under-confluent HB2 isolated from a normal human breast [64] (Original objective magnification: **a**, **b** 12.5×; **c**, **d** 25×; **e**, **f** 50×)

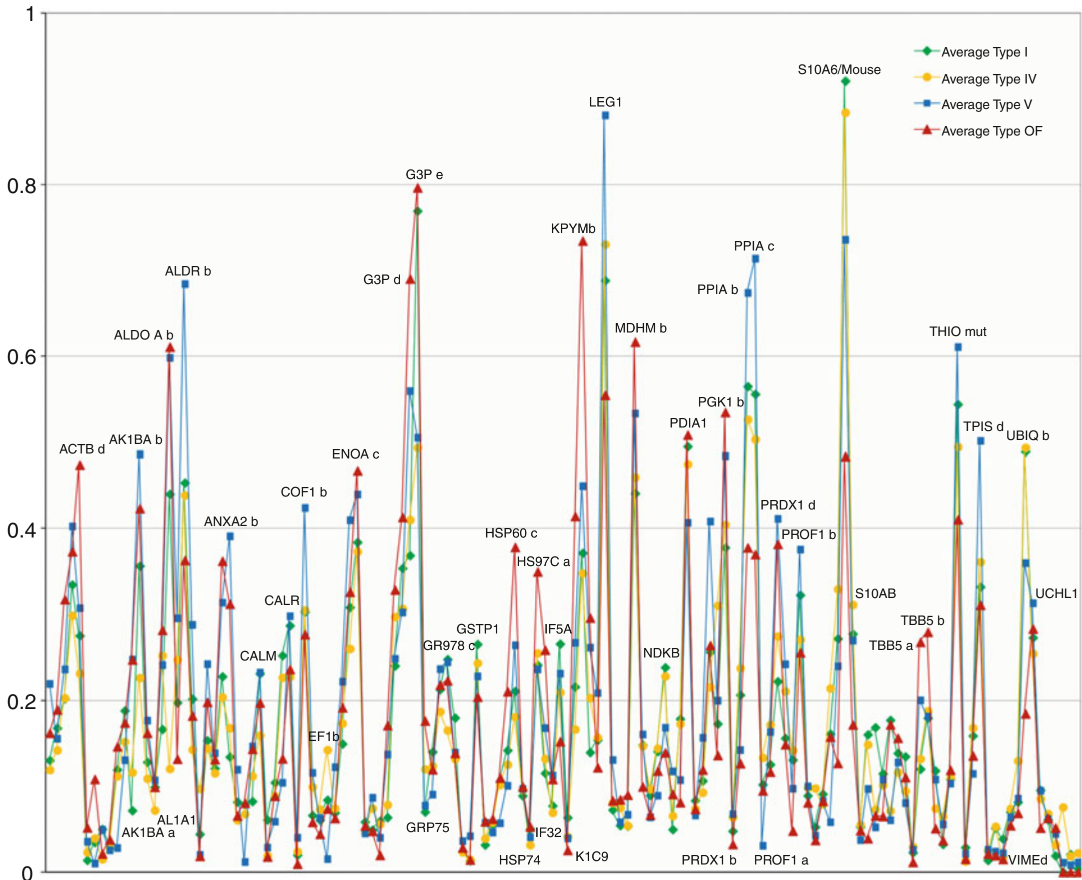

Fig. 9.7 An overview of the profile of the differential spot densities, plotted as a mean of three separate experiments, from cells cultured on different collagen types. For graphical restriction, the landmarks are limited to the higher values. In order to correct for differences in gel staining, the relative quantification of the spot density was expressed %Vol, by using Image Master software

included. All clusters converge toward the expression of a high malignant phenotype. This is suggested by the enhanced expression of four glycolytic enzymes supporting the metabolic shift of the Warburg effect, by the increasing of proteins involved in the cytoskeleton dynamics, vesiculation, and cell motility and by the relative high expression of a group of antiapoptotic proteins. It is interesting to note the expression of the vimentin considered a marker for the epithelial–mesenchymal transition, which represents a fundamental step in the process of the tumor progression.

Finally, a multivariate statistical procedure, which allows a global view of the variations induced by different experimental conditions, adopted for this study, indicated that in response to different growth substrates, members of the cell growth and cell death regulators, which include chaperons and heat-shock proteins, contributed most to the "dissimilarity" in levels of expression of the selected protein spots. Collectively, from the reported investigation, it can be assumed that when neoplastic cells cross the basal lamina and invade, the underlying stroma may come into contact with the newly deposited ECM, which in turn may convey distinct signals, and even play opposing roles in mediating cellular responses. In this context, type I trimer collagen appears to be a "permissive" substrate for neoplastic cell growth and motility, while type V collagen and, to a lesser extent, type I and type IV collagens seem to induce a more

stationary and polarized phenotype, apparently reverting the malignant traits of the neoplastic cells entering in contact with them.

Proteomic Modulation Induced by Fibroblasts

The crosstalk between cancer and host cells is known to play a crucial role in cancer progression. Fibroblasts are the major stromal cells with multiple roles in the extracellular matrix and the neighboring cell population, including neoplastic cells. In a recent article by our group, the effects of fibroblast stimulation on the breast cancer cell line (8701-BC) proteomics were investigated using a trans-well coculture system [69]. The results showed that fibroblasts induce a significant proteomic modulation on 8701-BC cells, associated with an increase of cell motility and invasion trough the reconstituted basal lamina (matrigel). These responses of the neoplastic cells to the influences of the fibroblasts appear in good agreement with the resulting proteomic modulation. In fact, the cytoskeletal proteins, including several actin-binding proteins, were the most responsive to the fibroblast media, namely, TBB5, VIME, COF1, MYL6, TPM, TMSL3, and PROF1.

In addition to these effects, the response to the fibroblast stimulation showed to induce a net increase of the expression level of three of glycolytic enzymes, namely, G3P, TPIS, and PGK1, which, as already mentioned, are able to play additional multifunctional roles besides their primary function.

By applying immune-detection assays on the cytoskeletal proteins of the neoplastic cells following fibroblast stimulation, it was observed a net decrease and fragmentation of cytokeratin 8, a typical epithelial marker with a concomitant increment of vimentin forms. These results strongly suggest the occurrence of an accentuation of epithelial–mesenchymal transition of 8701-BC cocultured with fibroblasts. Moreover, the LEG1 and ANXA2 known to be involved in several cellular processes such as cell motility, vesicle transport, and maintenance of the plasticity and dynamism of membrane-associated actin cytoskeleton [70] were also found to be increased in the cocultured cells. This also suggests a dynamic remodeling of the cytoskeleton of cocultured cells, which in turn may be responsible for the observed enhancement of migratory and invasiveness activities, according to what has also been reported in other systems [71]. On the other hand, the antiapoptotic protein, ANXA1, showed a decreased expression, probably linked to the increased growth rate of the fibroblast-stimulated neoplastic cells, concurrent with an upregulation of the c-Myc, a leader oncogene affecting diverse cellular processes involved in cell growth, cell proliferation, apoptosis, and cellular metabolism. Taken together these data suggest that fibroblasts at the front of the tumor may play a promotional effect on tumor progression.

The Challenge of Decorin, a Putative Antioncogenic Molecule

Decorin is a prototype member of the small leucine-rich proteoglycan family (SLRP), widely distributed in many connective tissues where it has been shown to perform or support a variety of functions [72] by its ability to bind collagens and other extracellular proteins [73, 74] and to interact with and downregulate several tyrosine kinase receptors [75].

To investigate the possible effects of decorin on the proteomic profile of neoplastic cells, we produced decorin-transfected clones by a transgene insertion into the breast cancer cell line 8701-BC [76]. Among the recipient clones, two were selected for the investigation: one expressing and producing the full decorin proteoglycan and the other producing only the protein core. The behavior of the two clones was largely overlapping, testifying that the molecular effects of decorin are mediated by its protein core.

From a phenotypical point of view, the clones displayed a significant growth rate reduction at 7 days of culture vs. the parental cells. Concurrently the decorin expression induced a remarkable reversion of the extensive membrane protrusions and vesiculation, typical of the parental cells.

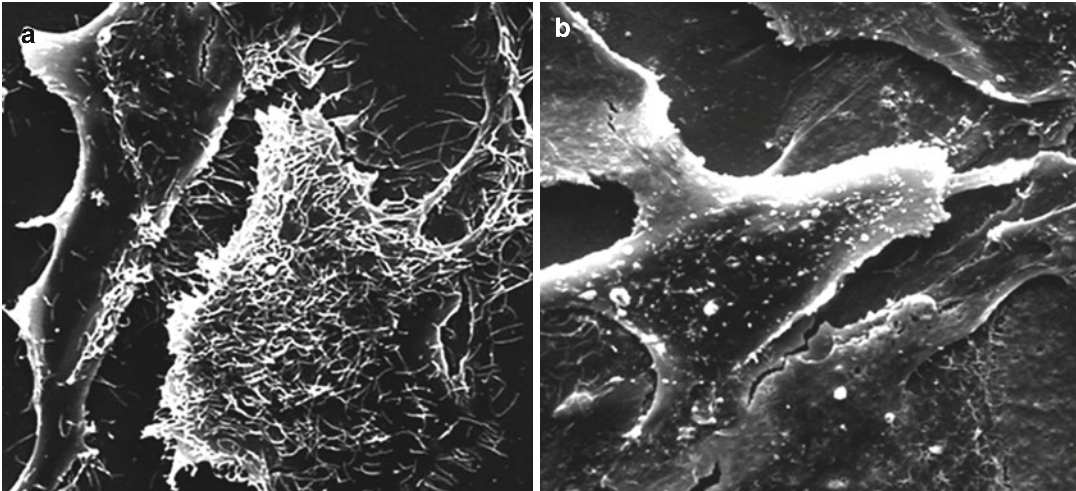

Fig. 9.8 Scanning electron micrographs of (**a**) 8701BC parental cell line, showing the extensive membrane protrusions, (**b**) transfected decorin clone showing the reversion of ruffled cell surface [76]. Original magnification 1.000 (courtesy of Dr. Martini, Bologna Unversity)

These results indicate that decorin definitely restrains these surface activities, reverting the cell morphology toward a more differentiated phenotype, well documented at the scanning electron microscope (Fig. 9.8).

The proteomic profiling of the parental cells and the transfected clones showed again that the most responsive proteins fall within the category of the metabolic enzymes. However, in this case a collective lowered expression of glycolytic enzymes and a significant increase of COX5A were detected in the clones vs. the parental cells, indicating a partial reversion of the typical Warburg effect. An elevated degree of modulation was also observed in the subgroup of actin-binding proteins, which play pivotal roles in the cytoskeleton reorganization, namely, a decrease of COF1 and PROF1 expression and an increase of TPM and TMSL, probably related to the reversion of the membrane ruffling observed at the surface of the control cells. Other significant variations concerned the proteins with roles in the apoptosis regulation, where the increment of GRP94, HSH74, PDIA1, TCTP, GRP78, PPIA, and PHB was observed together with a net decrease of MIF levels. In addition, a net decrease of S100A4 was observed, concurrently with a drastic reduction of five proteins of the redox pathway, i.e., AK1C3, AK1BA, ALDR, one isoform of THIO, and PRDX6 (a putative metastasis marker associated to the secreted vesicles). Conversely, a net increase was observed for the PSA5, for one isoform of SODM and for the SH3L1, a putative modulator of the redox function [77]. In parallel, the differential levels of transcripts for c-Myc and c-erbB2, which are frequently overexpressed in breast cancer, appeared significantly reduced in decorin-transfected clones, a result that emphasize the possible antioncogenic role postulated for decorin [75]. At the same time, the proteomic investigation, while disclosing new putative pathways for the decorin action, highlighted unexpected responses concerning a number of proteins useful to better evaluate the possible clinical application of decorin.

The Proteomic of Cancer-Released Vesicles: A Secretome Component

The phenomenon of vesicle budding is a recurring event in breast cancer cells, both inside the tumor and in vitro. An example of this phenomenon is depicted in Fig. 9.9, which shows two electron micrographs of a detail of cell surface of cancer cells shedding vesicles, (a) in tissue and (b) in vitro.

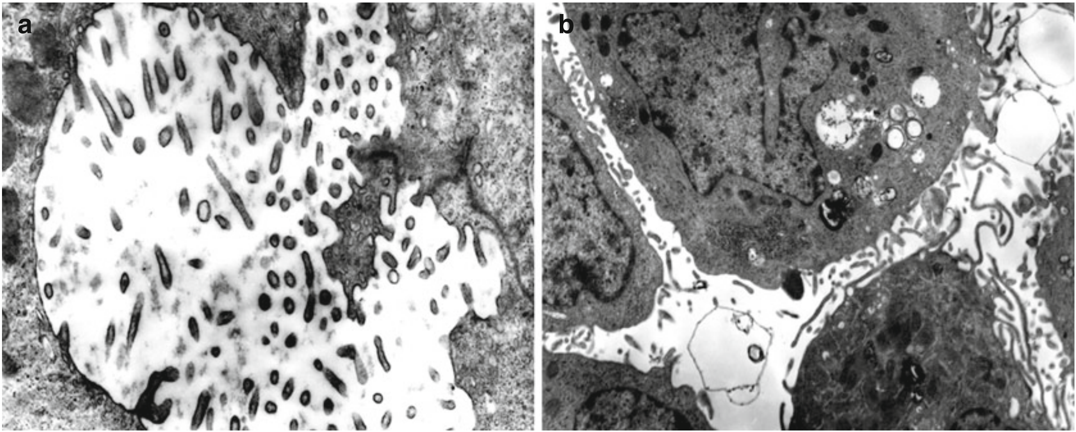

Fig. 9.9 Representative electron micrographs showing the phenomenon of vesicle releasing from the cell surface of (**a**) ductal-infiltrating carcinoma tissue, 20,000×, (**b**) cell culture of 8701-BC cells (original magnification: 10,000×)

A recent investigation was carried out by our group on the proteomic of vesicle released by MDA MB 231 breast cancer cell line [78]. Vesicles were isolated by ultracentrifugation in sucrose density gradients, evaluated under the electron microscope, monitored for appropriate markers, and submitted to the 2D-IPG for the proteomic investigation. Parallel proteomic analysis was performed on the parental cell line. The results showed a distinctive protein profile of the vesicle, in comparison with the whole cell proteome. In particular, about 20 % of proteins were found to be more abundant in the vesicle fraction in comparison with the cell lysate, as result of the protein enrichment following subcellular fractionation.

Among the most representative proteins were several protein forms of the cell–matrix compartment, namely, three isoforms of ITA3 and four of ITA6, and the laminin subunit gamma-1. The high concentration of integrin forms indicates the attitude of the released vesicle to adhere to target cells, probably addressing specific signals.

Coherent with the vesicle biogenesis [79] is the enrichment of several proteins associated with the plasma membrane (ANXA5, LG3BP-galectin-3-binding protein) and with the cytoskeleton and its regulatory proteins. The latter comprise ACTB/G, TBA1C, K1C9, two isoforms of TPM4, FSCN1, TAGL2, two isoforms of PDC6I, a protein also known as AIP1 (actin cortical patch component), or Alix (ALG-2-interacting protein X). This protein is implicated in the concentration and the sorting of cargo proteins of the multivesicular body and may inhibit cell death induced by several stimuli, through the binding to the ALG-2 (apoptosis-linked gene 2).

Interestingly, the incremented proteins within the vesicle fraction also include several proteins involved in the regulation of apoptosis, namely, the HSP71, two isoforms of PRDX2 and PRDX6, the proteasome subunits PSA6 and PSB3, the UBIQ and RL40, and the TERA. Further concentrated proteins comprise two isoforms of the adapter protein 14-3-3E, implicated in the regulation of a large spectrum of both general and specialized signaling pathways, among which cell cycle and apoptosis control [80].

Another enriched protein was found to be B2MG, the beta-chain of the major histocompatibility complex class I molecules. Since it is involved in the presentation of peptide antigens to the immune system, it could play an important role in the immune system modulation during tumor progression. The information obtained by this sub-proteomic approach confirm and stress the role attributed the vesicles as mediator of signals elaborated by the neoplastic cells and addressed to neighboring cells, probably either to the adjacent non-tumoral tissues or to the host cells, as fibroblasts and immunocompetent cells, as suggested by other authors [81–83].

Proteomic Profiling of Drug Therapy Responses

A further promising approach to the proteomics of breast cancer is the study of protein responses to the drug therapy. At present, one of the most investigated biopharmaceutical drugs is the trastuzumab, administrated to breast cancer patients overexpressing the oncogene Her-2. The overexpression of HER-2, due primarily to gene amplification, occurs in approximately 25–30 % of the invasive breast carcinomas [84], and only the patients overexpressing HER-2 are subjected to the trastuzumab therapy. Unfortunately, the initial response to the treatment is often followed by drug insensitivity, occurring within 1 year in the majority of treated patients. Although several hypotheses have been raised, the biochemical and molecular mechanisms behind the responses to trastuzumab are still unclear [85].

Trastuzumab (Herceptin®) is a recombinant humanized monoclonal antibody whose main epitope corresponds to a sequence in the extracellular domain of HER-2 adjacent to the cell membrane [86, 87]. HER-2 is an "orphan" receptor of known ligands, able to form heterodimers with other members of the HER receptor family. Its overexpression in cancer may activate multiple transduction pathways, many of which induce an increase of cell proliferation and survival, resistance to apoptosis, activation of cell motility, promotion of angiogenesis, and, ultimately, an increased tendency of the tumor to form metastasis. The trastuzumab mechanism of action in cancer therapy is likely to be accomplished through more pathways able to interfere with the consequence of HER-2 overexpression. A direct effect concerns the inhibition of HER-2 internalization and its turnover from cell membrane followed by the downregulation of cell proliferation [88, 89] and other possible implications in DNA repair processes [90]. Other indirect effects are believed to be the promotion of the antibody-dependent cytotoxicity by interaction with the Fc receptor (CD16) expressed by NK cells [91].

In one of our recent studies [92], we investigated the cancer cell responses to the trastuzumab treatment with an in vitro model system formed by the SKBR-3 parental cells, which strongly overexpress Her-2, and two derived clones, respectively, sensitive and resistant to the drug treatment. The comparative proteomic profiling of the three cultures highlighted some significant differences in the responses of the sensitive and resistant cells, both qualitative (types of proteins) and quantitative (relative expression levels). In the sensitive cells the trastuzumab treatment induced modulation of about 30 % of the identified proteins (84 out 293), while in the resistant cells only about 15 % of proteins appeared involved. The majority of responsive proteins in the sensitive cells were downregulated (69/84), contrary to the resistant cells. The proteins mainly downregulated in the sensitive cells belong to the following categories: nuclear proteins (AN32A, CCNH, PRP19), biosynthesis and degradation (PSME1, RLA0, RRBP1, RT22), metabolic enzymes (G3P, LDHB, TALDO G6PD, ACON), and heat-shock/chaperones/folding (CALR, HYOU1, PDC6I, 3 isoforms of PDIA, ENPL, HSP90A and HSP90B), some of which related to HER-2 maturation pathway. Other downregulated proteins are involved in the cytoskeletal dynamics (CAP1, CAPG, CAZA1, COR1A, EZRI), in the ionic homeostasis (ATPB, IPYR, TCTP), and in detoxification activities (CLIC1 and 3 isoforms of THIO).

The downregulation of a master glycolytic enzyme, the G3P, indicating a partial reversion of the Warburg effect, associated with the decreased expression of several proteins involved in the HER-2 maturation, represent a strong cellular response to the drug treatment. Moreover, the downregulation of EZRI and other actin-binding proteins involved in cell motility is a further indication of the partial antioncogenic effects exerted by the drug in contrasting the cancer progression and neoplastic cell dissemination. In fact, ezrin and, potentially, other members of the ERM (ezrin–radixin–moesin) family have been identified as key regulatory molecules in cancer metastasis [93].

Further investigations have observed the downregulation of some nuclear proteins. In particular the leucine-rich acidic nuclear protein

(AN32A/LANP), an ATXN1-binding inhibitor of histone acetylation, which has been found to reverse aspects of neuritic pathology [94], was not described before in breast cancer proteomic, as far as we know.

The comparative analyses between sensitive and resistant cells vs. the parental cell line highlighted a remarkable reversion of the proteomic profile to the basal protein expression values observed in the parental cells. In addition, two proteins appeared expressed de novo in the resistant cells, namely, the acidic isoform of S10AB and the more basic isoform of PRDX1. This suggests a novel role for these two proteins in the downstream pathways of the trastuzumab resistance. The resistant cells also displayed an increased expression of other HER family receptors, HER-1 and HER-4, as well as of other tyrosine kinase receptors and signal transductors, IGF-IR, TGFB-RII, AKT, p21, and p27. This suggests that these long-term responses to the drug could trigger survival and mitogenic pathways related to the evasion of pharmacological action of the trastuzumab [88, 95], in spite of HER-2 targeting by the drug.

Conclusively, the large-scale proteomic approach applied to the study of the effects induced in target cells by trastuzumab draws attention to new possibilities of actions toward the problem of its pharmacological resistance.

Some of the proteins reported above were undetected in the cancer tissue proteome presented in Fig. 9.3*, namely, transaldolase (TALDO), hypoxia upregulated protein 1 (HYOU1), 28S ribosomal protein S22 (RT22), cyclin H (CCNH), and pre-mRNA-processing factor 19 (PRP19).*

Conclusion and Future Perspective

The first conclusion that can be drawn from what we have reported is that the large-scale proteomic approach applied to the study of tumors and their metastatic propensity discloses new scenarios to be explored further. Based on large-scale detection, we proposed to distinguish two broad classes of proteins occurring in the proteomes of a group of patients with ductal infiltrating carcinoma: (1) the ubiquitous proteins, overexpressed in all tumor samples, (2) and the sporadic ones, absent or expressed very low in other tumor cases within the same study group. Ubiquitous proteins indicate that their presence is necessary for initiation and growth of the primary tumor, while the sporadic ones appear as nonessential for tumor initiation and growth; rather they may indicate a propensity to the progression of the tumors, expressing them at high levels. This is supported by the observation that many of the sporadic proteins are known to play roles in regulating cytoskeleton reorganization, cell motility, vesiculation and membrane releasing, and extracellular activities, such as some S100 proteins, galectin, and peroxiredoxin.

This collective information suggests that the leadership for tumor initiation and growth is played by protein clusters belonging to the category of growth regulation and apoptosis, as was expected, but also to the glycolytic enzymes, which appear as a new class of early-predictor tumor markers. On the other hand, the appearance of overexpressed proteins involved, for example, in cell motility and vesiculation, which are critical aspects of the neoplastic cell malignancy, is a strong indication for the propensity of the primary tumor to produce metastasis.

The second important lesson from the comparative proteomic approach performed on in vitro models, mimicking peculiar aspects of the in vivo progression, highlighted the fundamental role of the microenvironment in modulating the neoplastic cell behavior. Extracellular matrix molecule, e.g., collagens, proteoglycans, and soluble factors deriving from the tumor–host crosstalk, radiates diversified epigenetic influences which can modify, at least in part, the behavior of neoplastic cells. The cellular responses concern mainly the proliferation rate, the extent of adhesion to the substrate, and metabolic adaptation. Therefore, it can be hypothesized that the tumor microenvironment may produce distinct sets of signals, which in turn generate either "permissive" pathways for cell proliferation, migration, and metabolic adaptation or "restrictive" ones to limit cell proliferation, or to promote apoptosis, and to favor cell

adhesion and a more stationary state for neoplastic cells. A major finding is that neoplastic cells, even at an advanced stage of malignancy, are still able to capture and respond to a number of extracellular signals, which in principle could even revert their potential aggressive phenotypes toward a more "benign" state.

In this context, the contribution of proteomics in recognizing hundreds of proteins involved and responsive to the internal/external tumor environment is a winning strategy if conducted in a highly controlled way, and when precise reference points are established.

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Metabolomics in Breast Cancer

10

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Abstract

Breast cancer is a clinically heterogeneous disease, which necessitates a variety of treatments and leads to different outcomes; in fact, only some women will benefit from chemotherapy. Identifying patients who will respond to chemotherapy and thereby improve their long-term survival has important implications to treatment protocols and outcomes, while identifying nonresponders may enable these patients to avail themselves of other investigational approaches or other potentially effective treatments.

Furthermore, prognostic tools in early breast cancer are inadequate. The evolving field of metabolomics may allow more accurate identification of patients with residual micrometastases.

Metabolomics is a new, rapidly expanding field dedicated to the global study of metabolites in biological systems. Many of the studies have focused on identifying altered metabolic levels in breast cancer cells or tissues and relating these changes to their associated metabolic pathways. Metabolomics provides a strong link between genotype and phenotype and may provide some insight into oncogenesis.

The relatively new approach using metabolomics has just begun to enter the mainstream of cancer diagnostics and therapeutics. As this field advances, metabolomics will take its well-deserved place next to genomics, transcriptomics, and proteomics in both clinical and basic research in oncology.

Results of these investigations show promise for larger studies that could result in more personalized treatment protocols for breast cancer patients.

Keywords

Breast cancer • Metabolomics • Therapy response • Prognosis

Introduction

Breast cancer, although histologically similar, is clinically a very heterogeneous and phenotypically diverse disease, which results in a range of

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treatment effectiveness and outcomes [1]. It is composed of several biological subtypes that have distinct behavior and response to therapy. This heterogeneity was first noted over 100 years ago with the identification that simple removal of the ovaries was therapeutic in some breast cancer patients, but not others. Breast cancer characterization (profiling) has significantly advanced since the turn of the millennium due to the development of sophisticated technologies, such as gene expression arrays, which permit simultaneous measurement of thousands of genes to create a molecular portrait of the tumor.

As an alternative approach for biomarker discovery, metabolomics (or metabolite profiling) enables identification of small-molecule metabolites in biofluids and tissues that are sensitive to altered pathology [2–4]. High-throughput analytical techniques of nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) combined with multivariate statistical analyses provide information on a large number of metabolites, including those that have altered levels between healthy subjects and patients with various diseases, including cancer [5–7].

So far, the metabolomic-based approaches have been used in a large variety of applications, including early disease detection, drug response, toxicity and nutritional studies, and basic systems biology [8–11]. Compared with other biomarker discovery approaches for breast cancer, metabolomics provides a strong link between genotype and phenotype and may provide some insight into oncogenesis. Also, once established, tests based on metabolic profiles are relatively inexpensive and rapid and can be automated [12].

A growing number of metabolomic studies are contributing toward an improved understanding of breast cancer, and these advances have been reviewed [9, 13, 14]. Many of the studies have focused on identifying altered metabolic levels in breast cancer cells or tissues and relating these changes to their associated metabolic pathways [15–18]. A very recent study using metabolic profiling of numerous human cancer cell lines found a high correlation between breast cancer (and other cancer) proliferation and the glycine biosynthetic pathway [19]. Previously, differences between normal and metastatic mammary epithelial cell lines—including upregulation of fatty acid synthesis and alterations in glycolysis, the TCA cycle, and others—were detected using ^{13}C stable isotopic label tracing by 2D NMR and GCeMS methods [18]. Breast cancer tumors could be separated from non-involved tissues based on intensities from spectra generated by high-resolution magic angle spinning (HR-MAS) NMR spectroscopy with a sensitivity of 83 % and a specificity of 100 %. Some metabolites, such as choline and glycine, were found to be significantly upregulated in tumors larger than 2 cm [20].

In another NMR study, a multivariate statistical model based on 67 urinary metabolites successfully identified all the breast cancer patients with high specificity (93 %) [21].

Breast cancer prognostic factors, such as estrogen and progesterone receptor status, could be predicted by HR-MAS NMR-based metabolomics on tissue samples [22].

Metastatic breast cancer patients could be differentiated from early-stage patients with 72 % prediction accuracy using serum samples detected by NMR-based metabolomics [14].

For identifying breast cancer recurrence, a predictive model built on 11 biomarkers detected by combining NMR and two-dimensional gas chromatography mass spectrometry (GC/MS) provided 86 % sensitivity and 84 % specificity [23].

For predicting the response to chemotherapy in the neoadjuvant setting, a metabolomic approach is used. Four metabolites that were identified from NMR and MS methods are well correlated with a pathological complete response (pCR). A statistical model built based on these metabolites predicts pCR with high sensitivity and specificity [24].

Predicting Response to Neoadjuvant Chemotherapy

Neoadjuvant chemotherapy can significantly benefit breast cancer patients; however, the varied response to such therapy means that a significant

proportion of the patient population is subjected to ineffective treatment while at the same time being exposed to the therapy's toxicities [25]. Pathological complete response (pCR), which is defined as the disappearance of the invasive cancer cells in the breast after chemotherapy, is used to evaluate patient response and is strongly associated with improved long-term survival rates [26–28]. Unfortunately, less than 30 % of patients overall show complete response to neoadjuvant chemotherapy [29]. An ability to predict response to chemotherapeutic agents should enable development of personalized treatment protocols, improving survival rates and reducing unnecessary exposure of patients to toxic drugs.

Research focused on finding useful molecular or clinical predictors of pCR to neoadjuvant chemotherapy in breast cancer is relatively sparse. Imaging studies, such as magnetic resonance imaging (MRI) [30] and scintimammography [31, 32], were proposed to predict pathological responses to neoadjuvant chemotherapy, but they are somewhat limited by low sensitivity combined with high costs.

High levels of MUC-1 antigen (CA 15.3) in pretreatment serum and its fall after chemotherapy can predict responses as well [33], but many patients do not exhibit elevation of this marker before treatment, and hence it is not helpful for such patients [34]. Approaches using genomics and immunohistochemistry have been explored to find serum and tissue biomarkers [26, 35–37]. It has been shown that gene signatures such as HER2 overexpression/amplification and lack of ER expression were associated with pCR and certain neoadjuvant chemotherapy regimens [38–40].

Other molecular markers such as tumor RNA [41], glucose-regulated protein (GRP78) [42], and hormone receptors [18, 43] have also been identified as potential predictors of pCR. However, suboptimal performance is a major issue that limits their wide applicability. Circulating tumor cells (CTC) have also been established as providing outcome predictions from particular therapies; however, CTCs can be detected in less than 30 % of early-stage breast cancer patients, which limits their clinical applicability [44].

Study and Results

In this study, a metabolomic approach is used to predict the response to chemotherapy in the neoadjuvant setting. Serum samples from 28 patients obtained before preoperative chemotherapy have been studied using a combination of NMR, liquid chromatography mass spectrometry (LC-MS), and multivariate statistics methods. Four metabolites that were identified from NMR and MS methods are well correlated with pCR. A statistical model built based on these metabolites predicts pCR with high sensitivity and specificity.

Comparison of the NMR data between different groups of patients using the Student's *t*-test showed four metabolites to be statistically significant ($p<0.05$) (Table 10.1). These *p*-values indicate that levels of three metabolites, isoleucine, threonine, and glutamine, were significantly different between pCR and stable disease (SD) groups and the levels of two metabolites, threonine and glutamine, were different between PR and SD. Only one metabolite, histidine, differed significantly between pCR and partial response (PR). The LC-MS data showed that the most statistically differentiating compounds found were long-chain lipids or fatty acids. The most interesting of these, linolenic acid, was validated using a pure, commercially obtained compound. This metabolite separated pCR from SD samples

Table 10.1 Summary of NMR metabolites having low *p*-values

Chemical shift	Multiplicity	Assignment	*p*-value (pCR vs. SD)	*p*-value (pCR vs. PR)	*p*-value (PR vs. SD)
4.24	m	Threonine	0.04	0.28	0.30
1.00	s	Isoleucine	0.04	0.01	0.02
2.09	m	Glutamine	0.01	0.10	0.01
7.07	s	Histidine	0.29	0.20	0.54

perfectly. Statistical analysis shows linolenic acid to be significantly different between pCR and SD groups ($p < 0.01$). The concentration distribution for all the metabolites except histidine showed a consistent trend from pCR to PR to SD; while threonine, glutamine, and linolenic acid increased, isoleucine decreased.

Further analysis focused on evaluating the performance of the metabolites in combination. Combining three NMR-derived markers (threonine, glutamine, and isoleucine) with LC-MS detected linolenic acid. The model provides 100 % selectivity and 80 % sensitivity for the prediction of pCR vs. SD with an AUROC of 0.95.

The results suggest that metabolites in the serum of breast cancer patients are indicators of tumor/host metabolism and that they can predict both sensitivity and resistance to chemotherapy a priori.

A prediction model for the outcome of breast cancer neoadjuvant chemotherapy based on metabolic profiling studies is presented. It combines NMR and LC-MS methods. A combination of four metabolites, three detected by NMR: threonine, glutamine, and isoleucine, and one by MS, linolenic acid, distinguishes groups of patients with no, partial, or complete response.

It clearly indicates that several blood-based metabolite markers are sensitive to response and that the approach is promising for predicting the response to chemotherapy. In addition, considering the strong performance as a biomarker, linolenic acid and possibly other fatty acids might be of particular interest for further validation studies.

Potential Early Diagnosis

For breast cancer, screening mammography is considered the gold standard for early detection; however, the sensitivity of this test is between 54 and 77 %, depending on the type of mammography [45]. Furthermore, mammography is uncomfortable for many patients and exposes them to radiation. As a result, many women do not obtain yearly mammograms. There is a need to find a general screening test for all cancers that would ideally be noninvasive and have high sensitivity and specificity.

Monitoring of blood or urine for glucose and creatinine continues to be an integral part of diagnostic tests run today. Although these one- or two-component chemical tests provide a quick and inexpensive way to monitor health, what distinguishes metabolomics from clinical chemistry is that metabolomics measures tens to hundreds and potentially thousands of metabolites at once, rather than just one or two. Through urinary measurement, it has the potential to become a general screening test because it is convenient, easy to obtain, and noninvasive. In this study, metabolomics is applied to study urine from women with breast cancer.

Study and Results

Comparison of 67 metabolite concentrations from healthy subjects (*n*:62) and subjects with breast cancer (*n*:38) revealed significant differences. Application of multivariate statistical data analysis (OPLS-DA) to this dataset resulted in distinction between individuals with breast cancer and those without. Five of the healthy individuals overlapped with the breast cancer category. The model parameters and validation of the PLS-DA (multivariate statistical data analysis) suggested a good model. OPLS-DA class prediction was performed as for the EOC subjects, on a total of 20 subjects, 10 each of breast cancer and healthy. As may be observed, all breast cancer and healthy test subjects were correctly classified [21].

Analysis of urinary metabolite changes revealed that many metabolites decreased in relative concentration with a cancer phenotype when compared with healthy. That the majority of urinary metabolites appeared to decrease in concentration in cancer patients is a similar result to what has been seen in colon cancer tissue metabolomics. Interestingly, some metabolites that were shown to increase in cancer tissue (such as some of the amino acids) were lower in the urine of cancer patients. Concentrations of many amino

acids decrease in cancer patients relative to healthy. Decreases in tricarboxylic acid (TCA) cycle intermediates are suggestive of a suppressed TCA cycle. In a study of urinary markers of colorectal cancer, it was observed that several TCA cycle intermediates decrease in those with colorectal cancer as compared with those without [46]. The biological reason behind the metabolite changes is largely speculative at this point but likely involves a shift in energy production, as tumors rely primarily on glycolysis as their main source of energy. This phenomenon is known as the Warburg effect [47], and decreases in TCA cycle intermediates and glucose in the urine could be indicative of this phenomenon. Clearly, lower glucose concentrations were observed in women with ovarian cancer as compared with breast cancer. This could be because of the fact that more of the women with ovarian cancer were in an advanced stage of the disease. Furthermore, the use of amino acids by tumors requires the upregulation of amino acid transporters, [48] pulling these metabolites from the blood. Decreases in circulating glucose and amino acids could subsequently result in an overall decrease in energy metabolism elsewhere in the body, diminishing other metabolic pathways such as the urea cycle, resulting in lower concentrations of urea and creatine, and potentially affecting gut microbial population and/or metabolism.

So, it is suggested that a urine test is faster, easier to administer, less costly, and noninvasive and could be used as a prescreen to other forms of more invasive or uncomfortable screening.

Prediction of Prognostic Factors

There are few predictive and prognostic markers in breast cancer, but some specific markers are routinely being used for treatment planning and evaluating prognosis [49]. Estrogen receptor (ER) and progesterone receptor (PgR) status predict a possible endocrine responsive tumor, whereas human epidermal growth factor receptor 2 (HER-2)-positive tumors may be suitable for trastuzumab treatment. ER, PgR, and axillary lymph node status, together with tumor size and lymphovascular invasion, are important for predicting the clinical outcome of breast cancer patients [49–51].

High-resolution magic angle spinning magnetic resonance spectroscopy (HR-MAS MRS) can be used to describe the metabolic profile of intact tissue samples. Metabolic profiles have been shown to correlate with characteristics of several malignant diseases such as breast [15, 17, 20], brain [52], colon [53], and cervical cancer [54]. More than 30 metabolites have been described by HR-MAS MRS analysis of breast cancer tissue [20].

The study of the metabolic profile of certain cell or tissue types in combination with multivariate and analytical statistics is referred to as metabolomics. In a study, Bathen et al. showed that hormone receptor and axillary lymph node status, as well as histological grade, could be predicted by MR metabolomics [17]. The study by Bathen et al. was, however, performed using spectra from a restricted number of patients (*n*:77) and verified on a small amount of blind samples (*n*:12).

The purpose of a recent study [22] was to further explore the potential of MR metabolomics to provide clinically useful prognostic factors for breast cancer patients. The use of HR-MAS MRS and chemometrics as tools for determining prognostic and predictive factors of breast cancer was evaluated. Several multivariate classification techniques exist, and in this study, partial least squares discriminant analysis (PLS-DA), probabilistic neural networks (PNNs), and Bayesian belief networks (BBNs) were used. The relationship between the metabolic profiles of breast cancer tissue and the status of ER, PgR, and axillary lymph nodes was examined, and blind samples were predicted for verification.

Study and Results

ER and PgR status were best predicted by PLS-DA (Tables 10.2 and 10.3). For ER status, the number of correctly classified blind samples were 44/50 and 42/50 for Kennard-Stone and SPXY sample selection, respectively, while PgR

Table 10.2 Results from prediction of ER status[a]

	PLS-DA (1 LVs)	BBN	PNN
Kennard-stone			
Correct classification	**44/50**	39/50	40/50
Sensitivity (%)	**90**	95	82
Specificity (%)	**82**	18	73
SPXY			
Correct classification	42/50	41/50	42/50
Sensitivity (%)	87	97	90
Specificity (%)	73	38	64

Correct classification: number of samples in the test set predicted to have the correct ER status. Sensitivity: the proportion of ER-positive samples correctly classified. Specificity: the proportion of ER-negative samples correctly classified

[a]The best predictions are emphasized in bold

Table 10.3 Results from prediction of PgR status[a]

	PLS-DA (1 LVs)	BBN	PNN
Kennard-Stone			
Correct classification	**39/50**	35/50	35/50
Sensitivity (%)	**81**	77	71
Specificity (%)	**74**	58	68
SPXY			
Correct classification	36/50	36/50	36/49[b]
Sensitivity (%)	77	84	80
Specificity (%)	63	53	63

Correct classification: number of samples in the test set predicted to have the correct PgR status. Sensitivity: the number of PgR-positive samples correctly classified. Specificity: the number of PgR-negative samples correctly classified

[a]The best predictions are emphasized in bold

[b]One row not classified

status had a correct blind sample classification of 39/50 for the Kennard-Stone test set and 36/50 for SPXY. Similar results for both Kennard-Stone and SPXY sample selection indicate robust classification by PLS-DA. The sensitivity and specificity of classification were approximately equal; this is in contrast to the results of PNN and BBN where the sensitivity was higher than the specificity. The higher sensitivity may be due to the fact that, especially for ER status, there are more positive than negative samples. This could lead to networks that are more specialized in recognizing positive than negative samples. Since the probability of a sample being positive is much higher than the probability of it being negative, the network achieves a greater number of total correct classified samples by classifying most of the samples as positives. In PNNs, this can be partly overcome by the customized fitness function, allowing the user to insert a penalty whenever a negative sample is classified incorrectly. In this study, the same penalty was used for both the Kennard-Stone and the SPXY training and test sets. Although this improved the classification ability of the networks compared to networks without penalty, the classification error was still higher than that achieved by PLS-DA.

A PLS-DA model of the whole dataset with three latent variables (LVs) explains 43.8 % of the *X*-variance and 42.7 % of the *Y*-variance. The score values for ER+and ER- samples are significantly different for all three LVs (*t*-test, $p<0.001$), and it is possible to discriminate between ER+and ER- samples in a score plot of LV1, LV2, and LV3. ER+and ER- samples are mainly separated on the first LV that represents 70 % of the *Y*-variance explained by the model, and ER- samples have higher score for LV1 than ER+samples. The loading profile for LV1 reveals that samples with higher score for LV1 have more of the metabolites glycine (Gly), glycerophosphocholine (GPC), choline (Cho), and alanine (Ala) and less ascorbate (Asc), creatine (Cr), taurine (Tau), and phosphocholine (PC) than samples with lower LV1 scores. The regression vector of the PLS-DA model gives an indication of the overall influence of the variables based on all three LVs. The regression vector of ER- samples appears similar to LV1 and shows the same metabolic patterns. In addition, lactate (Lac) appears to be more expressed in ER- samples.

Axillary lymph node status was best predicted by BBN with 34 of 50 blind samples correctly classified. However, this was only true for the samples chosen by SPXY sample selection, and the same number of correctly classified samples was not achieved using Kennard-Stone sample selection. PLS-DA and BBN gave similar results, and overall, all three methods gave unacceptably high classification errors. However, the number

of correctly classified samples was better than expected by chance for all methods. This indicates that there is a difference between the MR spectra of lymph node-positive and lymph node-negative patients and that the metabolic profile is altered in patients with lymphatic spread compared to patients without spread.

In conclusion, ER and PgR status were successfully predicted by MR metabolomics. There is also a relationship between metabolic profile and lymph node status, although prediction of lymph node status based on MR spectra did not reach a reliable level of correctly classified samples. By combining MR spectroscopy with multivariate modeling, the biological differences between different metabolic profiles could be revealed. Here hormone receptor-negative patients appear to have more of the metabolites glycine (Gly), glycerophosphocholine (GPC), and choline (Cho) than receptor-positive patients. The data also indicate different metabolic profiles between ER status and PgR status. Thus, this study has shown that MR profiles contain prognostic information that may be of benefit in treatment planning and patient follow-up, and MR metabolomics may become an important tool for clinical decision-making in breast cancer patients.

Identification of the Presence of Micrometastasis

Current approaches, using traditional clinicopathological features or gene profiling, assess the primary tumor and estimate the risk of recurrence based on the presumption of micrometastatic disease. These tools have limitations. Consequently, an individual's risk may be over- or underestimated.

The 21-gene Oncotype Dx assay was assessed in 355 placebo-treated patients from the NSABP-B14 trial in node-negative ER-positive disease. Ten-year distant recurrence-free survival for these patients treated with surgery alone was 86, 62, and 69 % for low, intermediate, and high recurrence scores, respectively [1]. The 70-gene MammaPrint applied to 151 lymph node-negative patients, only ten of whom received any adjuvant therapy, showed differential 10-year distant metastases-free survival between good and poor prognosis signatures at 87 and 44 %, respectively [55]. A striking feature of these studies is that some individuals, despite apparent high-risk disease, clearly have excellent long-term outcomes. This reflects heterogeneity of disease, host, and risk and highlights overestimation of risk by current prognostic tools.

An alternative to presuming residual disease is actual measurement of micrometastases. Studies of micrometastatic disease are intriguing, particularly those of isolated tumor cells (ITC) in the bone marrow and circulating tumor cells (CTC) [56–58]. Of particular interest is that not all patients with ITC or CTC develop clinically detectable metastatic disease. Thus, tumor survival depends on both favorable tumor and host characteristics. Indeed, assessment of this dynamic multifactorial interaction is a strength of the evolving field of metabolomics.

Transformed human cells exhibit profound metabolic shifts, particularly reflecting the induction of cell membrane phospholipids biosynthesis and breakdown, and preferential use of glucose through non-oxidative pathways. Metabolomic analyses of patient serum and urine samples have been shown to delineate between healthy, benign, and malignant conditions. Specifically with breast cancer, there is cell line evidence of metabolomic distinction between normal and malignant and, even more specifically, identification of malignant breast cell lines with greater metastatic potential. With breast tissue, metabolomic analyses distinguish normal tissue, benign disease, carcinoma in situ, and invasive carcinoma. The subsequent challenge is to capture the malignant metabolomic signal among the complex serum metabolomic fingerprint for an individual [59].

Information on the metabolite pattern alterations that can be significantly associated to the pathology is directly obtained through statistical analysis of the NMR profiles. A metabolomic fingerprint may exist for micrometastatic disease. More specifically, a fingerprint may exist which identifies the interaction between host and any residual disease.

Metabolomic analyses in breast cancer patients with early and metastatic disease have been carried out and compared. Prognostic ability of the fingerprint has been explored by comparison with 10-year mortality rates determined by the current prognostic tool Adjuvantionline. The pilot model, developed in 44 early breast cancer patients, was then validated in a second cohort of 45 early breast cancer patients.

Study and Results

The appeal of metabolomics is concurrent assessment of tumor and host. Indeed, survival of a specific tumor in a specific host relies on a dynamic interaction, with evasion of normal host immunity and favorable stromal environment for metastatic deposits as key factors. In this recent study, metastatic subjects were characterized by higher values of phenylalanine, glucose, proline, lysine, and N-acetyl cysteine and lower values of lipids, when compared to the spectra of both post- and preoperative patients [60].

A strength of metabolomics, as compared with current prognostic tools, may be confirmation rather than assumption of micrometastatic disease. Results reveal differential metabolomic fingerprints for most early and metastatic breast cancer patients. Among the normal noise of the metabolomic fingerprint, most patients were distinguished based on metabolomic analysis of one serum sample [60].

Metabolomic analysis assigns more patients to low risk than are assigned by Adjuvantionline. Similarly, when compared with conventional clinical and pathological factors, prognostic gene expression signatures generally identify more patients of low risk. The 21-gene Oncotype Dx shows direct concordance of 36 % in relapse risk stratification compared with an adjusted Adjuvantionline [61]. The 70-gene MammaPrint, when compared with Adjuvantionline, had stronger predictive power and provided lower-risk estimates for more patients [62]. These low-risk patients may be spared or receive less intensive adjuvant treatment.

In conclusion, the benefit of metabolomics is the incorporation of both a specific tumor profile with metastatic features and a specific host profile conducive to tumor growth. A preliminary exploration in a limited number of patients of a potential role for the evolving field of metabolomics in assessment of micrometastatic disease in early breast cancer has been presented. Clearly, this approach requires refinement and validation, but the distinction identified between early and late disease and the prognostic role of the metabolomic fingerprint provide an exciting platform for further work.

Early Detection of Recurrence

Common methods of routine surveillance for recurrent breast cancer include periodic mammography, self- or physician-performed physical examination, and blood tests. The performance of such tests is lacking and extensive investigations for surveillance have not proven effective [63]. Often, mammography misses small local recurrences or leads to false positives, resulting in suboptimal sensitivity and specificity and unnecessary biopsies. In view of the unmet need for more sensitive and earlier detection methods, the last decade or so has witnessed the development of a number of new approaches for detecting recurrent breast cancer and monitoring disease progression using blood-based tumor markers or genetic profiles. The in vitro diagnostic (IVD) markers include carcinoembryonic antigen (CEA), cancer antigen (CA 15–3, CA 27.29), tissue polypeptide antigen (TPA), and tissue polypeptide specific antigen (TPS). Such molecular markers are thought to be promising since the outcome of the diagnosis based on these markers is independent of expertise and experience of the clinician, and their use potentially avoids sampling errors commonly associated with conventional pathological tests such as histopathology. However, currently, these markers lack the desired sensitivity and/or specificity, and often respond late to recurrence, underscoring the need for alternative approaches [64].

A new approach is to use metabolite profiling (or metabolomics), which can detect disease based on a panel of small molecules derived from the global or targeted analysis of metabolic profiles of samples such as blood and urine, and this approach is increasingly gaining interest. Metabolite profiling utilizes high-resolution analytical methods such as nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS) for the quantitative analysis of hundreds of small molecules (less than 1,000 Da) present in biological samples. Owing to the complexity of the metabolic profile, multivariate statistical methods are extensively used for data analysis. The high sensitivity of metabolite profiles to even subtle stimuli can provide the means to detect the early onset of various biological perturbations in real time. Metabolite profiling has applications in a growing number of areas, including early disease diagnosis, investigation of metabolic pathways, pharmaceutical development, toxicology, and nutritional studies. Moreover, the ability to link the metabolome, which constitutes the downstream products of cellular functions, to genotype and phenotype can provide a better understanding of complex biological states that promises routes to new therapy development.

Metabolite profiling gg methods are applied to investigate blood serum metabolites that are sensitive to recurrent breast cancer. We utilize a combination of NMR and two-dimensional gas chromatography resolved MS (GCxGC-MS) methods to build and verify a model for early breast cancer recurrence detection based on a set of 257 retrospective serial samples. Performance of the derived 11-metabolite biomarker model is compared with that of the currently used molecular marker, CA 27.29, in particular, for providing a sensitive test for follow-up surveillance of treated breast cancer patients.

This is the first metabolomic study that combines the information-rich analytical methods of NMR and MS to derive a sensitive and specific model for the early detection of recurrent breast cancer. The results indicate that such an approach may provide a new window for earlier treatment and its benefits.

Study and Results

The development of a metabolomic-based profile for the early detection of breast cancer recurrence is presented in a recent study [23]. The investigation makes use of a combination of analytical techniques, NMR and MS, and advanced statistics to identify a group of metabolites that are sensitive to the recurrence of breast cancer.

The new method distinguishes recurrence from no evidence of disease (NED) patients with significantly improved sensitivity compared to CA 27.29. Using the predictive model, the recurrence in over 55 % of the patients was detected as early as 13 months before the recurrence was diagnosed based on the conventional methods.

Breast cancer recurs in over 20 % of patients after treatment. Up to nearly 50 % improvement in the relative survival of patients can be achieved by detecting at least local recurrence at asymptomatic phase, underscoring the need to develop reliable markers indicative of secondary tumor cell proliferation [65]. Currently, a number of rapid and noninvasive tests based on circulating tumor markers such as carcinoembryonic antigen and cancer antigens are commercially available. However, the performance of these markers may be too poor to be of significant value for improving early detection because the levels of these markers are also elevated in numerous other malignant and nonmalignant conditions unconnected with breast cancer. Considering such limitations, the American Society of Clinical Oncologists (ASCO) guidelines recommend the use of these markers only for monitoring patients with metastatic disease during active therapy in conjunction with numerous other examinations and investigations [66]. The results presented in a recent study [23] based on the detection of multiple metabolites in the patients' blood provide a new approach for earlier detection.

Although perturbation in the metabolite levels were detected for nearly all the 40 metabolites that were used in the initial analysis (Table 10.4), the use of smaller numbers of metabolites provided improved models. Particularly, the group of 11 metabolites (7 from NMR and 4 from GC;

Table 10.4 Summary of clinical and demographic characteristics of the patients used in the Asiago et al. study

	Control		Recurrence	
Clinical diagnosis	Samples	(Patients)	Samples	(Patients)
No evidence of disease (NED)	141	(35)		
Pre-recurrence (pre)	–		67	(20)
Within recurrence (within)	–		18	(18)
Post-recurrence (post)	–		31	(20)
Age mean (range)	53	(37–75)	55	(36–69)
Breast cancer stage				
Stage I	47	(11)	7	(1)
Stage II	53	(16)	21	(5)
Stage III	10	(3)	34	(6)
Unknown	25	(6)	54	(8)
Estrogen receptor status				
Positive	65	(15)	67	(11)
Negative	64	(18)	33	(7)
Unknown	12	(3)	16	(2)
Progesterone receptor status				
Positive	52	(13)	71	(11)
Negative	77	(20)	29	(7)
Unknown	12	(3)	16	(2)
CA27.29	140	(36)	92	(19)
Site of recurrence				
Bone	–		37	(6)
Breast	–		13	(2)
Liver	–		11	(2)
Lung	–		10	(2)
Skin	–		6	(2)
Brain	–		15	(2)
Lymph	–		6	(1)
Multiple sites	–		18	(3)

Table 10.5) contributed significantly to distinguishing recurrence from NED. Further, the predictive model derived from these 11 metabolites performed significantly better in terms of both sensitivity and specificity when compared to those derived using individual metabolites or a group of metabolites derived from a single analytical method, NMR or MS, alone. Evaluation of other models with fewer metabolites indicated that they could also provide useful profiles. The AUROC for an 8-metabolite profile (4 detected by NMR and 4 by GC-MS) was 0.86, while a 7-marker model detected by NMR alone had an AUROC of 0.80. Nevertheless, the model based on 11 metabolites had the best performance and clearly outperformed the accepted monitoring assay CA 27.29 currently used for monitoring patients. These results promise a significant improvement for early detection and potentially better treatment options for recurring patients.

A number of studies to date have used NMR or MS methods to detect altered metabolic profiles in different types of malignancy owing to the ability of the analytical techniques to analyze a large number of metabolites in a single experiment. In particular, several investigations have focused on establishing breast cancer biomarkers using a metabolomic approach, and numerous metabolites including glucose, lactate, lipids, choline, and amino acids are shown to correlate with breast cancer [20, 67]. A sensitivity of 100 % and specificity of 82 % in the classification of tumor and non-involved tissues was achieved from the analysis of NMR data [20].

Table 10.5 Smaller numbers of metabolites provided improved models

Metabolites	Within and post vs. NED *p*-value	Pre-recurrence vs. NED *p*-value
1. Formate	0.0022	0.2
2. Histidine	0.000041	0.18
3. Proline	0.018	0.9
4. Choline	0.000022	0.77
5. Tyrosine	0.25	0.1
6. 3-hydroxybutyrate	0.86	0.96
7. Lactate	0.96	0.54
8. Glutamic acid	0.000018	0.74
9. N-acetylglycine	0.01	0.96
10. 3-hydroxy-2-methyl-butanoic acid	0.00004	0.35
11. Nonanedioic acid	0.4	0.089

p-values for 11 markers, 7 NMR (numbers 1–7) and 4 GCxGC-MS markers (numbers 8–11) for different groups using all samples; within and post-recurrence vs. NED, pre-recurrence vs. NED as determined from the univariate Student's *t*-test

NED no evidence of disease, *Within* within recurrence, *Post* post-recurrence

A majority of these investigations focused on either breast cancer tumors or cell lines and all used NMR methods alone, except for a recent study that utilized a combination of NMR and MS methods [18].

The 11-serum metabolites represent some of the changes in metabolic activity of several pathways associated with breast cancer, including amino acids metabolism (glutamic acid, histidine, proline, and tyrosine), glycolysis (lactate), phospholipid metabolism (choline), and fatty acid metabolism (nonanedioic acid). Choline is one of the most prominent metabolites in cell biology and is invariably associated with increased activity of tumor cell proliferation in breast cancer. Increased lactate is one of the early findings of metabolic changes reported for breast tumors. Similarly, association of a number of amino acids, fatty acids, and organic acids with breast cancer has been established earlier. Correlation of the metabolites with clinical parameters, such as the cancer stage and estrogen and progesterone receptor status, contributes to the extent by which the disease can be detected early. Recently, a link between tumor metabolites and estrogen and progesterone receptor status was shown with a prediction accuracy of 88 and 78 %, respectively, indicating the metabolic profile does vary with estrogen and progesterone receptor status of the patient [22]. These results support our observations and suggest that inclusion of such parameters may help advance further development of early-detection metabolite profiles.

Therefore, the development of a new tool for the surveillance of breast cancer recurrence based on the metabolic profiling of blood samples from patients obtained serially is recently showed.

The performance of the model was optimal when metabolites detected by both NMR and MS were combined. This multiple metabolite model outperforms the current diagnostic methods employed for breast cancer patients, including the tumor marker CA 27.29, for which comparison data on the same samples was available for direct comparison. Metabolic profiling of blood serum by NMR and mass spectroscopy can detect breast cancer relapse before it occurs, opening a window of opportunity for patients and oncologists to improve treatment.

Conclusion and Future Perspective

The study of all metabolites produced in the body, called metabolomics, which often includes flora and drug metabolites, is the omics approach that can be considered most closely related to a patient's phenotype. Metabolomics has a great and largely untapped potential in the field of oncology, and the analysis of the cancer metabolome to identify biofluid markers and novel druggable targets can now be undertaken in many research laboratories

The cancer metabolome has been used to identify and begin to evaluate potential biomarkers and therapeutic targets in a variety of malignancies, including breast, prostate, and kidney cancer. We discuss the several standard techniques for metabolite separation, identification, and usefulness in breast cancer, with their potential problems and drawbacks. Validation of biomarkers and targets may entail intensive use of labor and technology and generally requires a

large number of study participants as well as laboratory validation studies. The field of pharmacometabolomics, in which specific therapies are chosen on the basis of a patient's metabolomic profile, has shown some promise in the translation of metabolomics into the arena of personalized medicine.

The relatively new approach to using metabolomics has just begun to enter the mainstream of cancer diagnostics and therapeutics. As this field advances, metabolomics will take its well-deserved place next to genomics, transcriptomics, and proteomics in both clinical and basic research in oncology.

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11 Lipidomics in Breast Cancer

Alvin Kamili and Jennifer A. Byrne

Abstract

The prevalence of breast cancer within industrialized populations and the accessibility of tissue for research have meant that genomic, transcriptomic, and proteomic studies have often focused on breast cancer. This has led to major advances in the understanding of this disease and to findings that have been translated to many other types of cancer. Like other common cancers, breast cancer shows a lipogenic phenotype, meaning that significant quantities of lipids are synthesized and stored within breast cancer cells. As the importance of this lipogenic phenotype is becoming better appreciated, studies are beginning to focus upon how lipogenesis is regulated in breast cancer and the critical genes and pathways involved. Lipidomic studies have also begun to characterize lipid profiles in breast cancer cells and tissues and to study the biological consequences of these altered profiles. This chapter will provide an overview of lipid biology in human breast cancer, focusing upon our current understanding of breast cancer lipogenesis, how this contributes to tumor formation and progression, what is understood of its molecular basis, and how the techniques of lipidomics are beginning to be applied to this disease.

Keywords

Lipidomics • Lipid/fatty acid metabolism • Mass spectrometry • Obesity • Breast cancer

Introduction

Breast cancer is the most common cancer affecting women and a major cause of death from cancer. It is thus both an important clinical problem and a tractable disease to explore. Primary tumors are almost always surgically excised, leading to availability of primary material for study in different tissue forms. Furthermore, many breast cancer cell lines have been derived, are readily cultured in vitro, and have been characterized in extensive

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molecular detail [1, 2]. The combined availability of both primary tissue and cell lines has meant that breast cancer researchers have acted as “early adopters” of profiling technologies, and breast cancer has often served as a test case for new technology implementation. Some of the earliest gene expression profiling studies were conducted in breast cancer [3, 4], which then paved the way for analyses of less common cancer types. More recently, next-generation sequencing analyses of very large breast cancer cohorts [5, 6] have permitted a level of molecular characterization of the breast cancer genome that would have been difficult to foresee even 10 years ago. The significance of these approaches, both to our understanding of breast cancer as a disease and to our ability to interrogate and understand other cancer types and biological systems, cannot be overstated.

Like other common cancers, breast cancer shows a lipogenic phenotype, meaning that significant quantities of lipids are synthesized and stored within breast cancer cells. Lipids are of unique importance to mammary gland biology, as lipids are a major and important constituent of milk and drive the rapid postnatal growth and development of mammalian infants. As the significance of the lipogenic phenotype within cancer cells is becoming more broadly appreciated, studies are increasingly focusing upon how lipogenesis is regulated in breast cancer and the critical genes and pathways involved. Lipidomic studies have also begun to characterize lipid profiles in breast cancer cells and tissues and to study the biological consequences of these altered profiles. The present chapter will therefore provide an overview of lipid biology in human breast cancer, focusing upon our current understanding of breast cancer lipogenesis, how this contributes to tumor formation and progression and what is understood of its molecular basis. We will also describe dietary influences on the risk of developing breast cancer, and overweight and obesity as causes of breast cancer, before discussing lipidomics methods and how these are beginning to be applied to the study of breast cancer.

Lipid Requirements of Mammalian Cells

Actively proliferating cells must generate biomass in order to build new cells and hence require a variety of lipids to build new membranes, lipid cofactors, and lipid-modified proteins [7, 8]. Examples of the structures of some of the major classes of biological lipids are shown in Fig. 11.1. Lipids build the extensive networks of intracellular and pericellular membranes that define and partition cel-

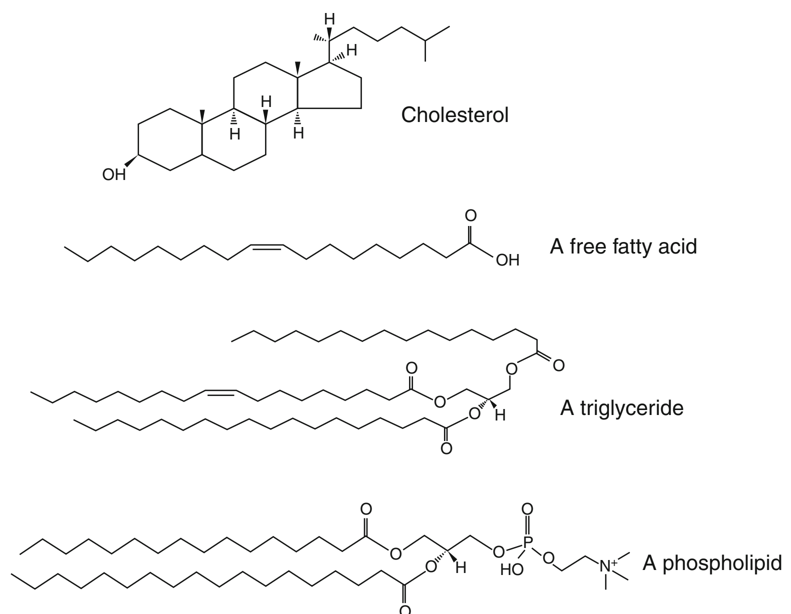

Fig. 11.1 Lipid structure representations. Examples of structures from the major classes of biological lipids (cholesterol, free fatty acid, triglyceride, phospholipid) are shown

lular organelles and their functions. In mammalian cells, most membrane lipids consist of glycerolphospholipids (PLs), such as phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, sterols (mainly cholesterol), and sphingolipids (mainly sphingomyelin) [9]. Fatty acids in general, and the 16-carbon saturated fatty acid palmitate in particular, can also be used for other functions. For example, palmitate and other fatty acids are added by enzymatic processes to increase the hydrophobic nature of proteins to facilitate membrane-associated signalling [10, 11]. Among the proteins that are modified by palmitate are Ras, Wnt, hedgehog, and small protein GTPases [10, 11], each of which is implicated in a variety of cancers.

Living cells acquire fatty acids for their metabolic demands from two major sources, exogenous dietary and de novo endogenous synthesis (Fig. 11.2). Proliferative embryonic cells actively use de novo synthesized fatty acids, whereas most adult normal cells (with the exception of the liver and lactating mammary gland) preferentially use exogenous fatty acids. Fatty acids are present in the diet as triglycerides, and following dietary intake, these are packaged in the intestinal epithelium into chylomicrons (Fig. 11.3). These chylomicrons are then secreted into the lymphatic system and enter the circulation via the thoracic duct. Lipolysis of these particles, initially in tissues such as the heart and lungs by the enzyme lipoprotein lipase (LPL), produces free fatty acids (FFA) that quickly associate with serum albumin. Remnant particles return to the liver where their triglycerides are assembled with apolipoprotein B 100 (apoB) for secretion as very low-density lipoprotein (VLDL) particles. In the circulation, lipolysis of VLDL produces LDL, which in turn is taken up by peripheral tissues, whereas FFA uptake is mediated by fatty acid translocase/CD36 (Figs. 11.2 and 11.3).

In tissues capable of de novo lipogenesis, FFA are also synthesized from the precursor acetyl-CoA by multiple enzymes including acetyl-CoA carboxylase (ACACA) and fatty acid synthase (FASN) (Fig. 11.2). Cholesterol can also be synthesized from acetyl-CoA, as well as taken up through the LDL receptor (LDL-R). FFA are used either for energy production via β-oxidation or for the synthesis of complex lipids such as

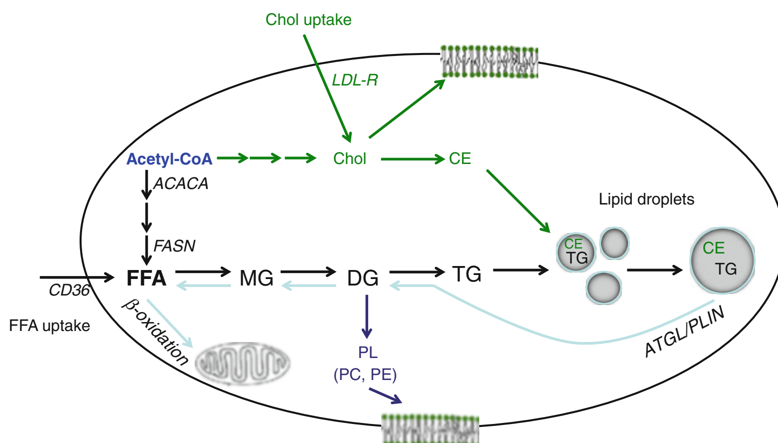

Fig. 11.2 Summary of cellular lipogenesis using endogenous and exogenous substrates. Free fatty acid (*FFA*) is obtained from two pathways, de novo synthesis and FFA uptake. Fatty acid is synthesized from acetyl-CoA by multiple enzymes including acetyl-CoA carboxylase (*ACACA*) and fatty acid synthase (*FASN*). FFA uptake is mediated by fatty acid translocase/CD36. FFA is used for either energy production via β-oxidation or complex lipid synthesis such as monoglyceride (*MG*), diglyceride (*DG*), and phospholipids (*PL*), primarily phosphatidylcholine (*PC*) and phosphatidylethanolamine (*PE*). To avoid lipotoxicity, excess FFA must be converted to triglyceride (*TG*), which is then incorporated into lipid droplets. Cholesterol (*Chol*) is derived from both acetyl-CoA and uptake through the LDL receptor (*LDL-R*). Cholesterol forms part of the plasma membrane, but excess cholesterol needs to be esterified into cholesterol ester (*CE*), which is then incorporated into lipid droplets (shaded circles). When cells require energy generation from reserved lipid stores, these can be released through lipolysis. This process is regulated by adipose triglyceride lipase (*ATGL*) and the PAT protein perilipin (*PLIN*)

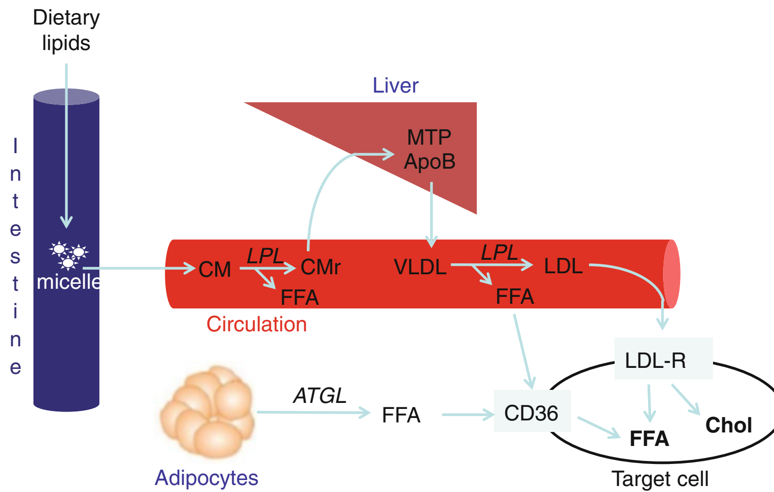

Fig. 11.3 Uptake of dietary lipids. Following dietary intake, lipids are packaged in the intestinal epithelium into chylomicrons (*CM*), which enter the circulation. Lipoprotein lipase (*LPL*) converts CM into chylomicron remnants (*CMr*), releasing free fatty acid (*FFA*). CMr are cleared by the liver where their triglycerides are assembled with apolipoprotein B 100 (*apoB*) by microsomal triglyceride transfer protein (*MTP*) for secretion as very low-density lipoproteins (*VLDL*) particles. In the circulation, lipolysis of VLDL by LPL produces FFA and low-density lipoproteins (*LDL*), which in turn are taken up by peripheral tissues via fatty acid translocase/CD36 and the LDL receptor (*LDL-R*), respectively. LDL is rich in FFA and cholesterol (*Chol*); hence, an uptake by LDL-R increases both cellular FFA and cholesterol. Additionally, FFA can be obtained from lipolysis of adipocyte lipid stores, facilitated by adipose triglyceride lipase (*ATGL*), which are then mobilized to target cells for uptake by CD36

monoglycerides, diglycerides, and phospholipids (Fig. 11.2). As FFA are toxic to cells, excess FFA must be converted to triglyceride, which is then incorporated into lipid storage organelles, known as lipid droplets (Fig. 11.2). Similarly, excess cholesterol must be esterified into cholesterol ester (CE), which is also incorporated into lipid droplets. Lipid droplets commonly consist of a core of neutral lipids surrounded by phospholipid monolayer and associated proteins and vary greatly in size [8, 12]. Small lipid droplets represent reservoirs that can be rapidly accessed. Conversely, it is more efficient to store lipid in large versus small lipid droplets, and hence, adipose cells contain a single unilocular lipid droplet for maximum storage efficiency [12]. When cells require energy generation from reserved lipid stores, these can be released through lipolysis. This process is regulated by adipose triglyceride lipase (ATGL) and the PAT protein perilipin (PLIN) (Fig. 11.2).

Lipids in Normal Breast Biology

Mammalian infants are typically born after long gestation periods, yet remain highly reliant on their mothers after birth. As lipids represent the most dense source of energy, lipids in milk are vital to drive rapid neonatal growth and are particularly required for postnatal brain development. Milk lipid composition is the most variable attribute of milk and is affected by animal genetics, physiology, and the environment [13]. Lipid-rich milk therefore promotes neonate growth, progressively reducing neonate dependency and promoting survival. Breast-feeding human infants is known to avert serious health problems in neonates, children, and adults, leading to huge savings in medical costs [14]. The regulation of milk fat composition in ruminants is also of major economic significance, both in terms of livestock breeding and in improving the quality of milk available to consumers [13].

The mammary gland largely develops postnatally, undergoing proliferation under the influence of ovarian hormones during puberty, but then remaining largely quiescent until pregnancy. During this time, the epithelial ductal tree expands into the mammary fat pad and undergoes further branching. Alveolae develop and begin to produce milk during the late stage of pregnancy. Lipids are both taken up from the circulation and synthesized within breast epithelial cells, packaged into lipid droplets, and then released into the alveolar lumen as bilayer membrane-coated structures called milk fat globules [15]. Despite the medical and economic importance of milk in human and other species, milk composition and its regulation remain incompletely understood [14].

Lipids in Breast Cancer

As breast epithelial cells take up, synthesize, and secrete lipids during late pregnancy and lactation, it is not surprising that breast cancer is one of many cancers characterized by a lipogenic phenotype. Similar to embryonic cells, the breast and other types of cancer cells endogenously synthesize 95 % of fatty acids, despite the abundance of extracellular fatty acids available to them [16–18]. Cancer cells are highly dependent on de novo lipogenesis for their proliferation, and the lipogenic pathway is activated at a relatively early stage in various types of tumors [19]. The majority of newly synthesized fatty acids in cancer cells are converted predominantly to phospholipids and then incorporated into membrane lipids by proliferating cancer cells. It has been recently suggested that activation of de novo lipid synthesis in cancer cells leads to increased incorporation of saturated fatty acids into cell membranes, which in turn protects cells from both endogenous and exogenous damage [20]. Altered membrane properties occurring in response to de novo lipogenesis may also influence the uptake and activity of chemotherapeutic drugs in cancer cells [20].

Early Studies Demonstrating a Lipogenic Phenotype in Breast Cancer

Efforts to study biochemical alteration of breast cancer were initiated over 40 years ago. In 1966, Rees et al. investigated the lipid composition of mammary glands and mammary carcinomas from rats in various hormonal states using thin-layer chromatography (TLC) and gas–liquid chromatography [21]. Although they could identify triglyceride and phospholipid profiles in the tissues investigated, quantification of lipid species was limited to percentages of total lipids [21]. The limitations of TLC also challenged Hilf et al. in 1970 when comparing lipids in human breast cancer and normal breast tissue [22]. Although they were able to identify differences in cholesterol, FFA, triglycerides, and cholesterol esters in infiltrating ductal carcinomas compared to normal breast tissue, it was unclear which species of lipids were uniquely altered [22]. Nevertheless, they found that cholesterol, FFA, and cholesterol esters were increased in breast cancer, while triglyceride levels were decreased [22]. Sakai et al. reported similar findings, with additional data on the fatty acid composition of phospholipids and triglycerides [23]. The fatty acid compositions of phospholipids were significantly different between human breast cancer and noncancerous excised breast tissues [23]. Specifically, the proportion of monounsaturated (oleate 18:1) and polyunsaturated (docohexanoate 22:6n-3) fatty acids in the major phospholipids was significantly higher in cancer compared to noncancerous tissues [23].

Molecular Basis of Lipogenesis in Breast Cancer Cells

Lipids can either be obtained through the diet or synthesized within cells (Fig. 11.2). The processes of lipid uptake, synthesis, and subsequent metabolism are regulated by numerous transporters and enzymes (Fig. 11.2), the discussion of

most of which is beyond the scope of this review. Since the expression of a vast number of proteins is deregulated in cancer cells through genetic, transcriptional, and posttranscriptional mechanisms, it would not be unexpected if some regulators of lipid uptake, synthesis, and metabolism would be thus affected, if only by chance. However, if these deregulated processes provide an advantage to the cancer cell, they will be selected for within the highly competitive environment of cancer tissue. It is now recognized that metabolic deregulation is a hallmark of cancer [24] and that changes in the expression and function of key lipogenic enzymes is actively selected for during tumorigenesis.

In cancer cells, increased glucose uptake results in increased conversion of pyruvate to acetyl-CoA in the mitochondria. Acetyl-CoA is then incorporated into the tricarboxylic acid cycle, which produces citrate in the presence of ATP. Accumulated citrate is exported to the cytoplasm where it is converted by ATP-citrate lyase (ACLY) to generate cytosolic acetyl-CoA, the precursor for FFA synthesis (Fig. 11.2). Acetyl-CoA is then carboxylated by acetyl-CoA carboxylase (ACACA) to synthesize malonyl-CoA, which is then converted to palmitate by fatty acid synthase (FASN) [19]. The unbiased analysis of large numbers of genes and proteins through genomics or proteomics approaches, respectively, has made it increasingly apparent that ACACA, ACLY, and FASN play key roles in tumor progression (Fig. 11.2). Of these three proteins, the expression of FASN and its role in mediating tumor growth has been most heavily investigated, as will be discussed in the following section.

Fatty Acid Synthase (FASN)

Increased FASN expression, relative to normal tissue, has been documented in tumors of the prostate, breast, colon, ovary, endometrium, bladder, and lung [25]. Additionally, FASN overexpression has been noted in melanoma, retinoblastoma, and soft tissue sarcoma [25–27]. FASN overexpression is primarily regulated at the transcriptional level in tumors following oncogene activation, tumor suppressor loss, or growth factor stimulation [28]. FASN levels can also be modulated by posttranslational modification and gene duplication [29, 30]. The expression levels of FASN are highest in metastatic tumors, correlate with decreased survival, and are predictive of poor outcome and disease recurrence in several tumor types [31–34]. These data suggest that FASN not only provides a metabolic advantage that may drive tumor cell survival and proliferation but may also promote a more aggressive tumor phenotype.

In normal physiology, fatty acid synthesis is crucial for development, as mice with the homozygous deletion of *Fasn* display an embryonic lethal phenotype [35]. On the other hand, with the exception of the liver, adipose tissue, and lactating mammary gland, FASN is expressed at low or undetectable levels in most normal adult tissues [25]. Therefore, unlike in cancer cells, fatty acid synthesis does not seem to be required for normal adult tissue maintenance. Accordingly, mice harboring liver-specific deletions of *Fasn* display normal liver function and no obvious phenotype, as long as they are maintained on a normal diet [36].

Coincident with the differences in FASN expression between normal and tumor tissues, there also seem to be mechanistic differences in how fatty acids are used in normal and tumor cells. In the liver and adipose tissue, fatty acids are synthesized in response to excess caloric intake. These fatty acids primarily partition toward triglyceride synthesis for fat storage. In contrast, tumor FASN-derived fatty acids preferentially partition into phospholipids that segregate into the plasma membrane or lipid rafts [37]. Additionally, it has been hypothesized that FASN also contributes to the redox status of tumor cells through oxidation of NADPH during the fatty acid synthesis cycle [38]. When all factors are taken into account, it is likely that FASN and fatty acid synthesis provide substrates to affect multiple cellular functions which support a proliferative phenotype.

ERBB2 Signalling and Lipogenesis

ERBB2 (HER2/neu) is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases that regulates biological

functions ranging from cellular proliferation to transformation, differentiation, motility, and apoptosis. ERBB2 expression levels must be tightly controlled to ensure normal cellular function [39]. In vitro and in vivo studies clearly demonstrate that deregulated ERBB2 expression and activity play a pivotal role in oncogenic transformation, tumorigenesis, and metastasis [40–44]. In breast cancer, amplification of the *ERBB2* gene is associated with poor prognosis, shorter relapse time, and low survival rate [40–44].

Aberrant expression of *ERBB2* can trigger the activation of multiple downstream signalling pathways, including the phosphatidylinositol 3′-kinase (PI3K)/PTEN/AKT pathway and the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway. These pathways induce cell proliferation and differentiation, decrease apoptosis, and/or enhance tumor cell motility and angiogenesis. Despite the recognized association of ERBB2 and these signalling pathways, less has been known about the specific effectors regulated by ERBB2 that ultimately contribute to its oncogenic effects. The use of transcriptomic analyses to identify genes that were differentially expressed in response to exogenous ERBB2 expression in breast epithelial cells identified increased *FASN* transcript and protein levels [45]. Similarly, in a panel of human breast cancer cell lines endogenously expressing different levels of ERBB2 and FASN, high levels of both FASN protein expression and FASN enzymatic activity were found to positively correlate with both *ERBB2* amplification and ERBB2 protein overexpression [46]. A proteomic study further revealed that proteins involved in glycolysis and de novo lipogenesis pathways were highly expressed in ERBB2-positive breast carcinomas [47], supporting the notion that ERBB2-driven oncogenesis depends upon the lipogenic phenotype [19]. Additionally, mouse NIH-3T3 fibroblasts and human breast epithelial MCF10A cells engineered to overexpress ERBB2 exhibited a significant upregulation of *FASN* transcript and protein levels [48]. Increased FASN protein levels were also reported to be significantly higher in ERBB2-positive invasive breast tumors examined in tissue microarray format [49].

Control of endogenous FASN levels occurs through modulation of the expression and/or maturation status of the transcription factor sterol regulatory element-binding protein-1c (SREBP-1c). In ERBB2-overexpressing tumor cells, SREBP-1c expression and activation is driven by constitutive activation of the P13K/AKT and/or MAPK/ERK1/2 pathways [19]. Supporting this notion, pharmacological inhibitors of PI3K and MAPK downregulate SREBP-1c and decrease *FASN* transcription, ultimately reducing lipogenesis in ERBB2-overexpressing cancer cells [50]. FASN overexpression by ERBB2-mediated oncogenic stimuli can also be abrogated by deletion of the major SREBP-binding site from the *FASN* promoter [51].

An alternative mechanism for ERBB2-FASN induction has also been proposed by Yoon et al [52]. They reported that the induction of FASN in ERBB2-overexpressing breast cancer cells was neither accompanied by changes in *FASN* transcript levels nor was mediated by the activation of SREBP-1c. Rather, the 5′- and 3′-untranslated regions of *FASN* mRNAs appeared to be involved in selective FASN translational induction that was mediated by the mammalian target of rapamycin (m-TOR)-regulated signal transduction. In this translational mechanism of FASN regulation, the activation of mTOR significantly increased the synthetic rate of FASN, whereas ERBB2-induced upregulation of FASN protein expression was inhibited by both the PI3K inhibitor LY294002 and the mTOR inhibitor rapamycin [52]. These observations suggest that ERBB2-driven FASN overexpression can be regulated at multiple levels.

Gene Amplification of Lipogenic Genes

Gene amplification is a frequently employed mechanism which increases the expression of targeted genes. Classical cytogenetics approaches first identified genomic regions which were subjected to increased copy number, and then the advent of comparative genomic hybridization (CGH) considerably facilitated genomic copy number studies [53]. Array-based CGH and copy number analyses using single-nucleotide polymorphism profiling have largely

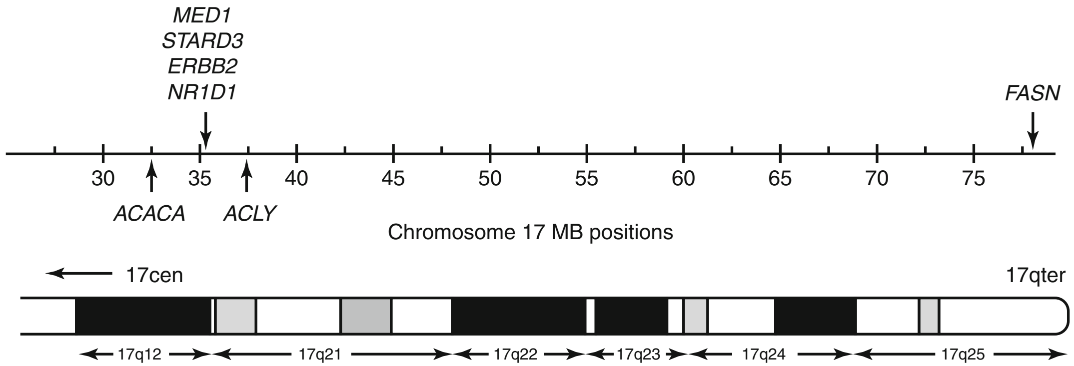

Fig. 11.4 Positions of chromosome 17q genes with known roles in lipogenesis (shown using hg 18 chromosome 17 coordinates, in MB), with the corresponding cytogenetic bands indicated on the lower ideogram. Approximate positions of genes are shown using vertical arrows: *ACACA*, 32.7 MB; *ACLY*, 37.3 MB; *MED1* (previously known as *PPAR γ-binding protein*), 34.8 MB; *STARD3* and *ERBB2*, 35.1 MB; *N1RD1*, 35.5 MB; and *FASN*, 77.6 MB. All genes except *FASN* map within 4.6 MB and could be commonly affected by genomic events leading to increased copy number in breast and other cancers

superseded classical CGH, and next-generation sequencing is playing an increasingly significant role in identifying, quantifying, and physically mapping copy number changes in cancer and other cell types [54, 55]. Whereas many genes may be affected by copy number changes, only a proportion of these are likely to contribute to the cancer phenotype and represent gene amplification targets.

Genomic profiling and other approaches have shown that genes encoding key enzymes within the lipogenic pathway are increased in copy number and/or overexpressed in breast cancer. As described above, the oncogene *ERBB2* located at chromosome 17q (35.1 MB) is amplified in approximately 15 % of breast cancer cases [56] and increases lipogenesis within cancer cells, at least in part by regulating FASN expression and function (see the section "ERBB2 Signalling and Lipogenes").

It is striking that genes coding for three key enzymes of the fatty acid biosynthetic pathway also reside on human chromosome 17q, namely, *FASN* (77.6 MB), *ACACA* (32.7 MB), and *ACLY* (37.3 MB) [51, 57–59]. A number of these and other lipogenic genes cluster at chromosome 17q12-q21 within 5 MB of each other (Fig. 11.4) and could be commonly affected by copy number increases [60]. In contrast, *FASN* lies toward the telomeric end of chromosome 17q and does not form part of the lipogenic gene cluster around *ERBB2* (see Fig. 11.4). To date, only one study has evaluated the correlation of FASN expression with gene copy number alterations in cancer cells. Using fluorescence in situ hybridization analysis in paraffin-embedded tissue microarrays, a significant increase in *FASN* copy number was found in a proportion of prostate adenocarcinomas and metastases, which was associated with increased FASN protein detection [30]. It is as yet unclear whether increased *FASN* copy number plays a significant role in driving increased FASN levels in breast cancer cells.

Experimental evidence has begun to support the concept that increased copy number at the *ERBB2* amplicon allows cancer cells to produce high levels of intracellular lipid, while concomitantly promoting the conversion of FFA to triglycerides to avoid lipotoxicity [61, 62]. It has been proposed that the co-amplification of other lipogenic genes with *ERBB2* further increases the reliance of such tumors on lipogenesis [62]. Two genes that have been identified to be important for ERBB2-positive breast cancer cell survival, but not that of other breast cancer cells or normal mammary epithelial cells, are *mediator complex subunit 1* (*MED1*, previously known as *peroxisome proliferator-activated receptor (PPAR) γ-binding protein* or *PBP*) and the nuclear receptor *NR1D1* (*nuclear receptor subfamily 1, group D, member 1*), a PPARγ target protein [62]. The *MED1* and

NR1D1 genes within the *ERBB2* amplicon (see Fig. 11.4) not only positively affect transcriptional rates of the lipogenic genes *FASN*, *ACLY*, and *ACACA* but also further regulate lipid storage during adipocyte differentiation [63–68]. More recent experimental evidence supports the notion that co-amplification of *MED1* and *NR1D1* synergistically enhances FFA to triglyceride conversion in ERBB2-positive cells in order to avoid lipotoxicity [39, 62].

Lipogenic amplification target genes have also been identified at other genomic loci beyond chromosome 17q. For example, the "*Spot 14*" (*S14* or *THRSP*) gene encodes a nuclear protein that is associated with fatty acid synthesis and is located at chromosome 11q13 [69]. High Spot 14 levels as detected by immunohistochemistry were significantly associated with tumor recurrence in breast cancer, but were not associated with either hormone receptor or ERBB2 status in the cohort examined [70].

Overweight and Obesity as Causes of Breast Cancer

Since the 1980s, the percentages of overweight and obese adults and children have risen markedly in the Western world, leading to an impending global health crisis of unprecedented proportion. This has been attributed to a combination of ready access to calorie-rich foods and reduced rates of activity. Overweight and obesity also represent a major environmental cause of cancer [71], which may overtake tobacco use as the leading such cause of cancer as smoking rates decline. The manner in which obesity predisposes individuals to cancer is still a subject of debate, and the causal role that obesity plays is likely to be different in the case of different cancer types. It may be difficult to separate, for example, the effects of obesity from the effects of lack of exercise, or from increased or reduced intakes of particular dietary components, which may have effects beyond contributing to the overweight or obese state.

Increased circulating levels of estrogen serve to drive the proliferation of estrogen receptor-positive breast cancers, and to date, the significance of obesity in relation to breast cancer incidence and risk has been proposed to lie primarily in adipose tissue representing the major site for estrogen synthesis in postmenopausal women [72]. In this tissue, estrogen is synthesized from androgens by aromatase, which is a major drug target in postmenopausal women with estrogen receptor-positive disease. Women with large breasts were reported to have a higher incidence of breast cancer relative to women with average-sized breasts, which could reflect amounts of increased glandular tissue from which tumors can derive and/or higher local estrogen levels generated from increased adipose tissue [73]. Obesity is also known to result in adipose tissue becoming increasingly dysfunctional, leading to the secretion of a variety of factors termed adipokines, which may promote tumor initiation or progression [74]. However, it is possible that a high-fat diet leading to obesity may also promote breast cancer through other mechanisms, as will be discussed below.

Diet and Breast Cancer

A large body of evidence substantiates an important role for de novo lipogenesis in cancer. Given the fact that breast cancer derives from cells with the ability to both synthesize lipid and take up lipid from the circulation, it is important to consider possibly dietary influences on breast cancer risk and development. To date, the role of dietary saturated fat in contributing to breast cancer risk is somewhat controversial. Positive associations between saturated fat or animal fat consumption and cancer have been reported in cohort studies [75] and in studies investigating cancer incidence in 20 countries [76]. Dietary intake of palmitic acid has also been significantly associated with increased breast cancer risk [77]. In general, inaccuracies in reporting dietary intake and difficulties in conducting mechanistic studies on human populations have hampered investigations on the role of dietary saturated fat in cancer development. However, measuring fatty acid composition of adipose tissue using lipidomics techniques may provide a composite measure of dietary fat intake over several years, due to the low turnover rate of stored lipids within adipose tissue [78].

Uptake of Dietary Lipids by Breast Cancer Cells: Part of the Picture?

While there are difficulties in conducting epidemiological dietary studies, some molecular studies have indicated the possible involvement of fatty acid uptake (in particular, saturated fatty acid uptake) in fuelling cancer cells. Studies have shown that LDL receptors are upregulated in tumor cells [79]; therefore, the LDL receptor-mediated pathway is a possible route for fatty acid delivery to peripheral tissues, especially tumor cells. Kuemmerle et al. also reported that cancer cells could also uptake released fatty acid from the lipolysis process through fatty acid translocase CD36 [80]. Immunohistochemical analysis confirmed the presence of LPL and CD36 in breast liposarcoma and prostate cancer tissues [80].

Excessive intake of dietary lipids is a well-known cause for obesity, which is in turn a risk factor for breast cancer [81, 82]. In the mammary gland, a large percentage of the cells are adipocyte or adipocyte precursor cells [83]. The abdominal fatty tissue known as the omentum has been described as a preferred metastasis location for ovarian cancer. Nieman et al. reported that adipocyte-ovarian cancer coculture led to the direct transfer of lipids from adipocytes to ovarian cancer cells and promoted in vitro and in vivo tumor growth [84]. Furthermore, coculture induced lipolysis in adipocytes and β-oxidation in cancer cells, suggesting that lipids stored in adipocytes can act as an energy source for the cancer cells [84]. Considering that the breast is an organ rich in adipose tissue, the transfer of fatty acids between breast cancer cells and breast adipocytes could also occur.

Therapeutic Targeting of Lipogenesis in Breast Cancer

A number of approaches have either been tested or may be applied to target lipogenesis in breast cancer. However, despite overwhelming evidence of the importance of lipogenesis in cancer, and in breast cancer in particular, progress in targeting this pathway has been described as modest at best [85]. Limiting factors have been described as the previous lack of crystallographic structures for relevant targets that has impeded drug design and in establishing structure–antitumor relationships [85]. The most heavily investigated target to date is FASN. Numerous FASN inhibitors have been reported and tested in the context of breast cancer, but their application has been limited in some cases by anorexic side effects [86]. Researchers are continuing to develop alternative inhibitors without these side effects [87]. Other key metabolic enzymes that could represent therapeutic targets in cancer cells are ACACA and ACLY. These targets are of great interest for the treatment of diabetes and obesity but have been explored to a limited extent in the context of cancer [85].

Due to the health and economic impact of the obesity pandemic, novel therapies are being aggressively developed against a variety of targets [88]. With rapidly improving knowledge of the significance of altered lipid synthesis and possibly uptake by cancer cells, such agents show increasing possibility of being adopted for cancer use. Redeploying approved drugs has advantages over the development of novel agents, in that there are preexisting pharmacokinetic, toxicity, and side effects data. For example, agents targeting fatty acid-binding proteins are being developed in the context of insulin resistance and other conditions [89] but could conceptually be applied to cancers where fatty acid-binding proteins are known to be overexpressed. The PAT protein family, which regulates lipid storage in lipid droplets, is also viewed as potential drug targets in the treatment of obesity [88] and are expressed in some lipogenic cancers [90]. The eventual targeting of lipid droplet-associated proteins could be applied to treat lipogenic cancers characterized by increased expression of these targets, where the overexpression of lipogenic genes may represent predictive biomarkers.

Characterizing Lipogenesis in Breast Cancer Cells

Lipid Detection Methods

Thin-layer chromatography (TLC), gas chromatography (GC), and high-performance liquid chromatography (HPLC) have been used in lipid research for many years. In 1966, Rees et al. studied the influence of hormonal status on lipid composition of rat mammary carcinomas, mammary glands, and related tissue [21]. In this study, they used TLC and GC to identify the levels of glycerolipids, sterols, and phospholipids relative to the percentage of total lipids. Following its emergence, TLC became widely accepted as a conventional analysis method for lipids in the 1960s [91, 92], with the advantages of being fast, simple, and inexpensive. However, the major limitation of TLC is its restricted resolution, which significantly hinders its application.

Since most lipids are not volatile and some lipids are easily degraded under high temperature, GC is not a very widely used method in lipidomics, due to the complexity of derivatization required before separation [93]. The derivatization may eliminate much structural information about lipid molecular species, especially polar lipids. Therefore, when using GC to analyze different categories of lipids, complex pre-separation is absolutely necessary [94]. These problems result in the much less frequent application of GC than liquid chromatography. Nevertheless, GC technology is appropriate for the analysis of fatty acids, because the resolution capacity of GC is much higher than that of liquid chromatography. The separation of cis/trans isomers, which is rarely achieved with other lipid detection methods, can be achieved using conventional GC–MS methods.

HPLC is the most widely used separation technique in lipidomics. In contrast to other separation techniques, HPLC has good reproducibility and high resolution and can separate almost all lipid molecular species. HPLC systems are relatively isolated from the environment, limiting the contact between samples and air and thus avoiding self-oxidation and degradation of lipids. In recent years, lipid separation by liquid chromatography and detection by mass spectrometry has become one of the core techniques for the growing field of lipidomics (see the section "Lipidomic Approaches").

Other methods to detect lipids in biological systems include nuclear magnetic resonance (NMR) spectroscopy and biochemical approaches. NMR spectroscopy is an excellent tool to study molecular structures of purified lipids (^{1}H-NMR and ^{13}C-NMR) and for investigating the structure and dynamics of lipid membranes (^{1}H, ^{2}H, and ^{31}P high-resolution and solid-state NMR) [95]. For the analysis of phospholipid mixtures, ^{31}P-NMR is by far the most appropriate approach. The linear response and relatively high speed of ^{31}P-NMR allow for accurate and selective analysis with high sample throughput [96]. One disadvantage is that NMR techniques have only moderate sensitivity compared with mass spectrometry. Many lipids can also be detected using biochemical approaches (e.g., optical/colorimetric assays). This type of measurement is highly quantitative, but often experimentally challenging in that optimization of conditions can require significant effort.

Lipidomic Approaches

The term "lipidome" describes the complete lipid profile within a cell, tissue, or organism and is a subset of the "metabolome," which also includes the three other major classes of biological molecules, namely amino acids, sugars, and nucleic acids [97]. Efforts to characterize lipids in cells are relatively recent and have been driven by some spectacular advances in mass spectrometry instrumentation and applications. The dramatic increase in lipidomic research over the past decade has been triggered by impressive developments in analytical technologies, initiated by the

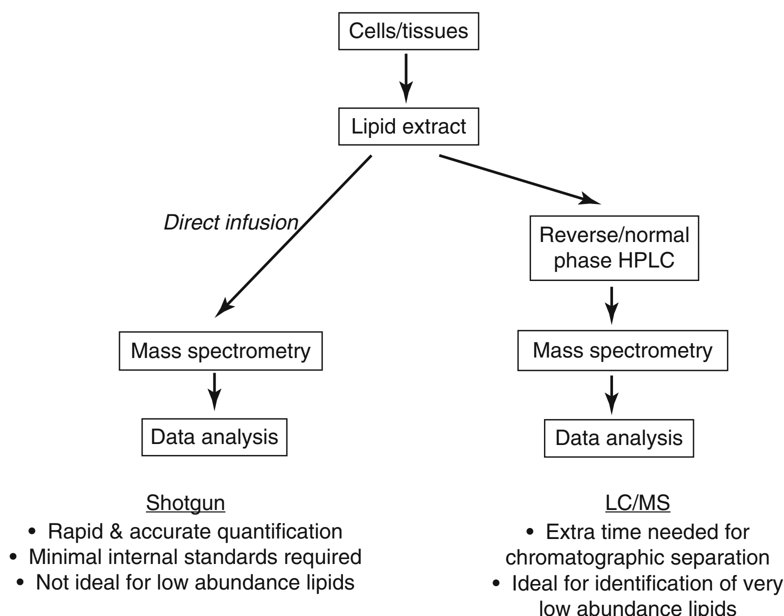

Fig. 11.5 Lipidomic analysis by ESI-MS can either be coupled to liquid chromatography (*LC-MS*) or performed as shotgun lipidomics. The advantages and disadvantages of both methods are shown

application of electrospray ionization mass spectrometry (ESI-MS) to the characterization of membrane phospholipids [98–101]. Technical developments include very high sensitivity and specificity mass and chromatographic resolutions and the increased availability of authentic synthetic lipid standards. These, coupled with impressive developments in data and bioinformatics analysis, have facilitated the detailed molecular analysis of a wide diversity of lipids, ranging from phospholipids and triglycerides to sterols and glycolipids.

Lipidomic analysis by ESI-MS can be categorized in two broad groups, either coupled to liquid chromatography (LC-MS) or shotgun lipidomics (Fig. 11.5), in which the specificity of analysis of different lipid classes in a directly infused sample is provided by diagnostic tandem MS/MS scans. Shotgun lipidomics is an excellent technique for identifying the major pools of phospholipids. Using this method, lipid class (head group) identification is accomplished using precursor ion scans (PIS) and neutral loss scans (NLS) in positive-ion modes and/or negative-ion modes. PIS and NLS are full scan methods mainly offered by triple quadrupole or quadrupole time of flight (Q-TOF) MS devices [102]. The fatty acid content of individual lipids is then identified by PIS analysis in negative-ion mode. For example, phosphatidylinositol species PI 38:4 (PI 18:0/20:4) would be identified by a precursor ion scan of 241 *m/z* in negative-ion mode and associated fatty acid side chains would be identified as 283 *m/z* (C18:0) and 303 *m/z* (C20:4) [103]. Overall, shotgun lipidomics analyses are prone to ion suppression of detection of minor components by molecules that become preferentially ionized but are rapid and accurate for quantification using a limited number of internal standards.

For the analysis of lipid classes using LC-MS, ion suppression is less of an issue and this approach enables resolution of isobaric molecular species of identical molecular mass, but different molecular structures. LC-MS in lipidomics is characterized by an additional layer of separation preceding *m/z* analysis. A chromatographic separation step substantially increases the number of detectable lipids due to reduced suppression effects in the ion source [104, 105]. In this manner, the identification of very low abundance lipids is possible without any manual intervention in the analysis process [106]. Normal phase and reversed phase, as two different modes of HPLC, have both been used for different purposes in lipidomics analysis. The normal-phase

method is used to separate different classes of lipids based on polar head groups and the reverse-phase method is often used to separate different molecular species in one class based on their different fatty acyl chains [93]. In addition to *m/z* values, LC-MS also offers retention time values for identification purposes. LC-MS is, however, more time-consuming, and as different periods of gradient liquid chromatography elution will have different ionization capacity, multiple standards are required for accurate quantification.

Similar to other "omics" technologies, lipidomics generates large sets of data. The diversity of lipid chemical structures presents a challenge both from experimental and informatics standpoints. So far, although there is a general consensus in the lipidomics community to adopt the lipid classification introduced by Lipid Maps [97], there is no similar consistency for data analysis programs to interrogate lipidomics mass spectrometry results. The need for a robust, scalable bioinformatics infrastructure is high at a number of different levels: (a) establishment of a globally accepted classification system; (b) creation of databases of lipid structures, lipid-related genes and proteins; (c) efficient analysis of experimental data; (d) efficient management of metadata and protocols; (e) integration of experimental data and existing knowledge into metabolic and signalling pathways; and (f) development of informatics software for efficient searching, display, and analysis of lipidomics data [97]. These requirements need to be addressed by collaborative efforts between researchers working in biology, chemistry, and bioinformatics.

Technical Developments in Lipidomics Relevant to Breast Cancer Research

In 2008, Haynes et al. described a method for quantitation of subpicomole amounts of long-chain and very-long-chain fatty acyl-CoA by reverse-phase liquid chromatography combined with electrospray ionization tandem mass spectrometry in positive-ion mode with odd-chain length fatty acyl-CoAs as internal standards [107]. RAW264.7 macrophage cells and human breast cancer MCF7 cells were used as examples in this optimization, and their analysis revealed large differences in fatty acyl amounts and sub-species distributions [107]. The amounts of very-long-chain fatty acyl (>C20) and long-chain fatty acyl (<C20) were similar in cancer cells, whereas in noncancerous cells, the majority of fatty acyls were long chain [107]. Further lipidomics studies in breast cancer cell lines were performed using positive and negative modes on electrospray linear ion trap and electrospray triple quadrupole mass spectrometry [108, 109]. These instruments combine sensitivity, specificity, selectivity, and speed for accurate analysis of phospholipids [110]. Comparing three different breast cell lines (nonmalignant mammary epithelial MCF10A cells, nonmetastatic breast cancer T-47D cells, and metastatic breast cancer MDA-MB-231 cells), they reported that phosphatidylcholines and phosphatidylinositols were decreased in nonmalignant cells relative to cancer cells [109]. Furthermore, the MDA-MB-231 cell line possessed the highest levels of phosphatidic acids, phosphatidylcholines, and phosphatidylinositols [109]. Advanced mass spectrometry has also been applied to characterize lipid profiles directly in breast cancer patients, with palmitic acid, stearic acid, linoleic acid, and total fatty acid being emphasized as having the greatest potential to act as biomarkers of breast cancer [111].

Integration of Lipidomics, Genomics, and Proteomics in Breast Cancer Research

The application of genomics, transcriptomics, and proteomics to breast cancer has generated huge amounts of information regarding the molecular changes that occur in breast cancer tissues and cell lines. In comparison to genomics, transcriptomics, and proteomics, lipidomics is a relatively new approach [99]. As such, the number of publications including the term "lipidomics" (1,475 publications, October, 2012, identified using the full-text search function of Highwire) is far exceeded by those including the

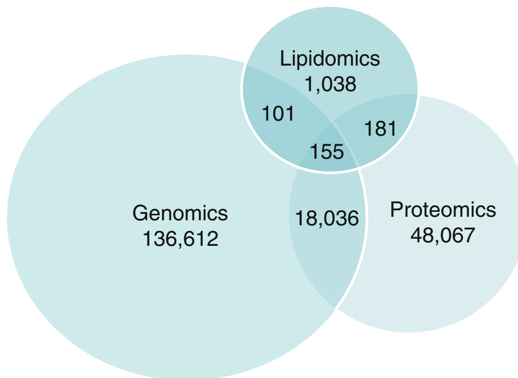

Fig. 11.6 Venn diagram showing the numbers of articles including the search terms "genomics" ($n=154{,}904$), "proteomics" ($n=66{,}439$), and/or "lipidomics" ($n=1{,}475$) or any combination thereof. Relatively high proportions of lipidomics articles included either "genomics" ($n=256$, 17.4 %), "proteomics" ($n=336$, 22.8 %), or both terms ($n=155$, 10.5 %), reflecting the status of lipidomics being a relatively new field, which is open to integration with other "omics" disciplines. Article numbers were generated using the full-text search function of the Highwire literature search engine on 12 October 2012. The Venn diagram is shown for illustration and is not drawn to scale

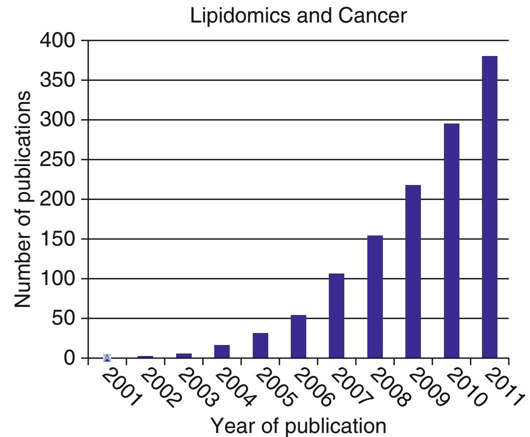

Fig. 11.7 Cumulative numbers of scientific publications that include the terms "lipidomics" and "cancer," published by the end of each calendar year from 2001 (zero articles) to 2012 (470 articles in total). Article numbers were generated using the full-text search function of the Highwire literature search engine on 12 October 2012

term "genomics" (>154,000) or "proteomics" (>66,000) (Fig. 11.6). Nonetheless, the overall number of publications including both the terms "cancer" and "lipidomics" is rising rapidly (Fig. 11.7).

Many studies have performed genomic and transcriptomic analyses of breast cancer, in order to identify genes with significant relationships between gene copy number and transcript level and hence genes which may represent amplification targets or tumor suppressor genes [112–114]. Predictions from transcriptomic studies are then frequently validated using protein detection techniques, to identify genes that are reproducibly differentially expressed at both the transcript and protein levels [115]. These integrative approaches have highlighted ways in which lipid metabolism and profiles are altered in tumors. For example, associations between gene copy number and expression identified both *ACACA* (chromosome 17, 32.7 MB) and *NR1D1* (chromosome 17, 35.5 MB) (Fig. 11.4) as being potentially druggable amplification targets in breast cancer [116].

In contrast, very few studies have attempted to integrate lipidomic and other -omic profiles in any biological context [117]. However, the ability of lipidomics to illuminate molecular mechanisms of disease when combined with transcriptomics data has been recently demonstrated in the context of breast cancer. Lipidomics analysis of a large cohort of human breast tissues revealed increased incorporation of de novo synthesized fatty acids into membrane phospholipids in tumors versus normal breast tissues [118]. *In silico* transcriptomics data [119] were then interrogated to identify candidate proteins possibly underpinning these changes. Candidate proteins were investigated using immunohistochemistry, revealing that breast cancers with high levels of de novo synthesized fatty acids also demonstrated high FASN and ACACA levels in cancer cells in situ [118]. A similar approach was employed by Brockmöller et al. to investigate the expression of glycerol-3-phosphate acyltransferase (GPAM) in breast cancer tissue and to describe associations between GPAM immunohistochemical staining and metabolomic profiles [120].

While few breast cancer studies have integrated lipidomics with other high-throughput approaches, the importance of integrating lipidomics

with other "omics" technologies is clearly understood by the research community. This is indicated by the fact that of all publications including the term "lipidomics" ($n = 1{,}475$), 17 and 23 % also mentioned "genomics" or "proteomics," respectively, and 11 % included all three terms (Fig. 11.6). The slightly higher co-use of the terms "lipidomics" and "proteomics" could reflect the fact that proteomics and lipidomics employ similar platforms, and it has been proposed that many proteomics groups could undertake lipidomics projects [99]. Thus, in the short term, we might expect more frequent integration of proteomics and lipidomics approaches in cancer.

Conclusion and Future Perspective

Breast cancer is both an important clinical problem and a tractable disease to explore. The combined availability of both primary tissue and cell lines has meant that breast cancer researchers have acted as "early adopters" of profiling technologies, and breast cancer has often served as a test case for new technology implementation. A number of factors are now leading to increased use of lipidomics techniques in the study of breast cancer, beyond technological developments within the lipidomics field itself. It is now clear that obesity is a major environmental cause of cancer, which contributes both directly and indirectly to breast cancer incidence. The importance of obesity in driving common cancers is leading to increased recognition of the fact that lipid metabolism is also greatly altered in cancer relative to normal cells, although whether and how these phenomena are linked need further investigation. Alterations in cancer lipid metabolism have been shown through direct investigations and indirectly through genomics, transcriptomics, and proteomics approaches, which highlight alterations in gene copy number, expression, or protein levels of key regulators of lipid metabolism. While molecular therapies for cancer continue to represent a major area of drug development, there is increased recognition that drugs developed for metabolic conditions such as obesity may also be applied for cancer therapy [85].

Lipidomics faces some particular challenges not shared by other "omics" fields, such as genomics, transcriptomics, and proteomics. Sequencing the human genome deduced the gene set available to build both transcripts and proteins and therefore defined the theoretical boundaries of molecules relevant to these fields. In contrast, the number of biological lipids has not been defined and at present, cannot be predicted [99]. The full identification of all lipid species is rendered further challenging as some are likely to be present at low abundance [99], and if these cannot be predicted, they are less likely to be identified. Furthermore, lipids exert their functions through interactions with proteins, RNA, and other molecules within cells, and these interactions are only beginning to be analyzed and defined [121].

Despite such challenges, the lipidome is likely to present a wealth of opportunities in terms of cancer diagnosis and treatment. Identification of lipid classes and their structures opens new possibilities for exploration of lipid alterations in cancer, providing novel biomarkers and the basis to develop novel therapeutics strategies. The metabolome has been described as the amplified output of a biological system, with small changes in individual enzymes potentially leading to large outputs that can be robustly quantified [122]. The immense structural diversity of lipids, while currently a major challenge, also provides opportunities to define highly specific biomarkers in disease states such as cancer. Just as enzymatic regulators of lipid metabolism have been proposed as therapeutic targets in breast and other cancers, disease-restricted lipids themselves may prove to be therapeutic targets, which may be less susceptible to the development of drug resistance through individual gene mutations. Given the immense biological and clinical relevance of lipids to many human diseases, ongoing efforts to identify and classify biological lipids, and more frequent integration of lipidomics with other experimental approaches, we may see lipidomics grow to rival other more well-established "omics" fields within the next 10 years.

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12 Breast Cancer Stem Cells and Cellomics

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Abstract

"Omics" technologies are powerful high-throughput analytic tools to get inside whole-system level alterations of cancer cells. Since cancer stem cells are groups of vital cells in heterogeneous tumor populations, their omics analysis will enable better understanding in most controversial issues. These issues are mostly treatment resistance and metastatic ability of tumors. The relation between breast cancer patients' survival rates and stem cells in breast tumor has revealed the significance of breast cancer stem cells. The high tumor-forming capacity of these stem cells makes it necessary to get more comprehensive insight about their biology. Genomics, proteomics, and epigenomics are helpful tools for this purpose. The general step of these high-throughput methods is the isolation of breast cancer stem cells based on specific markers. Another shared feature of these omics approaches is to use a large set of interested genes, transcripts, or proteins. In addition to these two common key features, the remaining experimental setups may change from one to another omics analysis. The outcome of these approaches can yield some signatures, which will be mostly critical for later therapeutic strategies. Taken altogether, omics approaches not only reveal comprehensive understanding about breast cancer stem cells but also open the doors to more effective targeted therapies.

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Introduction

Stem Cells and Cancer Stem Cells

The concept of stem cells giving rise to cancers was proposed almost 150 years back [1]; however, the hypothesis of cancer stem cells (or tumor-initiating cells) is a relatively new one proposed about 15 years ago [2]. Cancer stem cells share important characteristics of stem cells, with some specific modifications. There are two basic features of stem cells: self-renewal ability, in which stem cells go to asymmetric division in highly regulated fashion and produce their exact copies, and differentiation capability to other mature cells forming tissue and organs. Self-renewal of cancer stem cells is not highly regulated and also their differentiation products are not normal organ or tissue; rather, they form tumor tissue. Similar to normal stem cells, cancer stem cells are resistant to apoptosis and their life span is longer than other differentiated cells in tumor. In addition, stem cells have the capability to migrate to other tissues; similarly, cancer stem cells can metastasize to other tissues [3]. These shared characteristic features give a clue about the origin of cancer stem cells. The most well-supported hypothesis asserts that they originate from normal stem cells or progenitor cells in related tissue. Another opinion supported by some researchers is that cancer stem cells might be derived from normal cells. This second theory requires more genetic and cellular changes, so cancer stem cells are most likely derived from normal stem cells or from their less mature forms, progenitor cells [4]. However, to date, it has not been proved which hypothesis for the origin of cancer stem cells holds true.

According to cancer stem cell hypothesis, a tumor consists of heterogeneous cells and a small number of cells in a tumor exist as cancer stem cells, which are responsible for propagation and invasiveness of the tumor. Specific markers play critical roles in the identification of these cells from whole tumor cell population. These are generally "cluster of differentiation" molecules abbreviated as CD markers. Each tumor type has its own cancer stem cell markers.

Cancer stem cells are critical in therapies of tumors, as they are observed to be resistant to most of the available chemotherapeutics and radiotherapy. Hence, even though the prescribed therapies kill the differentiated cells of the tumor, the cancer stem cells cause a relapse of the tumor in the patient. In the new therapeutic approach, cancer stem cells are targeted together with debulking agents (chemotherapeutic agents or radiotherapy). This new therapeutic paradigm is most likely more curative than conventional therapy and recurrence of tumor does not occur [5]. Several strategies targeting cancer stem cells in brain tumors revealed successful results [6]. Treatment strategies targeting stem cells in breast tumors also have been used (e.g., dendritic cell [DC]-based immunotherapy) [7].

Breast Cancer Stem Cells

Similar to other tumor types, breast tumors include a small number of cancer stem cells. These cells have high tumor-forming capacity compared to other non-stem cells in breast tumors. The tumorigenicity of breast cancer stem cells (BCSC) is understood via injection into mice. Only 100 BCSC have the ability to form tumors, but tens of thousands of non-BCSC cannot form tumors in a xenograft model [8]. The tumorigenicity potential of BCSC is most likely the reason of metastasis (approximately 40 % of breast cancer patients), which may occur after 7–10 years from treatment [7]. In terms of increasing treatment efficiency, one of the important issues is isolation

of these highly tumorigenic tumor stem cells. Marker identification is a critical step in defining BCSC.

Identification of Breast Cancer Stem Cells

Adhesion molecule CD44 expression and non-expression (or low expression) of CD24 are used together as important markers for identification of BCSC. It has been observed that a fewer number of injected $CD44^{+}/CD24^{-/low}$ cells into mice caused tumor formation as compared to a higher number of heterogeneous cells, which indicates that these cells are more invasive than the others because other cells cannot form tumors despite their high number [8]. In addition to the $CD44^{+}/CD24^{-/low}$ profile, stem cells also show epithelial surface antigen (ESA) positivity. These three cell surface markers are critical for BCSC characterization from either cell line or primary sample [9].

Similar to neurospheres formed by brain tumor stem cells, BCSC also form spheroid cell clusters in cell culture, which are called "mammospheres." The passage number in which mammosphere is formed increases when chemotherapy-treated breast cancer patient samples are used. Untreated patient samples form mammospheres up to three generations, but treated patient samples can have mammospheres up to eight to ten generations. This situation implies that breast cancer stem cell resistance mechanisms are most likely activated against chemotherapy, so the capacity of mammosphere generation increases in chemotherapy-treated patient samples [10]. Moreover, knockdown of α6-integrin/ITGA6 in breast cancer stemlike cells caused reduction in the mammosphere formation capacity and tumorigenicity abrogation [11]. Thus, α6-integrin/ITGA6 is an important marker of breast cancer stem cells.

Increased level of aldehyde dehydrogenase activity (ALDH) in both normal and cancer human mammary epithelial cells is an indicator of stem/progenitor cells. ALDH1 has been found to be a marker of poor prognosis after examination of 577 breast cancer samples [12]. Aldehyde dehydrogenase family members A1 and A3 (ALDH1A1, ALDH1A3) are used together as markers of BCSC [13]. Breast cancer cells sorted according to BCSC markers ALDH1 (+), CD44 (+), and CD24 (−) are capable of generating tumors only with 20 cells [14].

General Identification Techniques

The side population (SP) technique uses specific dyes, such as Hoechst 33342 or Rhodamine 123. These dyes are excluded from breast cancer stem cells but not from differentiated cells. SP cells isolated from breast cancer cell line MCF7 represent 2 % of the total cell line. This is most likely the tumorigenic part of the whole cell line [10]. However in some cases, in vitro-obtained SP cells may not correlate with in vivo tumor formation capability [15]. Therefore, SP technique can be used with other BCSC markers/techniques to be able to identify BCSC more reliably.

ALDEFLUOR assay is based on enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) oxidizing retinol to retinoic acid. ALDEFLUOR positivity as a marker of breast cancer stem cells can be used along with other markers to identify breast cancer stem cells more consistently. For example, ALDEFLUOR-positive breast cancer cell population with $CD44^{+}$ and $CD24^{-}$ displayed high tumorigenic potential as expected, but another group of ALDEFLUOR-positive breast cancer cells with additional $CD133^{+}$ is also shown to have tumorigenic and metastatic ability. These two identified groups are most likely breast cancer stem cells [16]. In addition, sometimes, ALDH1 may not be detectable in breast cancer cell lines, which indicates the necessity of other BCSC marker usage [15].

Nonadherent mammosphere formation in cell culture is another important and frequently used method to identify BCSC. Differentiation capability of these mammospheres implies their stem/progenitor potential. This mammosphere formation is based on BCSC capacity to be able to survive in serum-free conditions. When these mammospheres are analyzed, their SP population and $CD44^{+}/CD24^{-}$ cells have been found to be greater than heterogeneous breast cancer cell

line. Mammosphere formation in cell culture is suitable to develop further therapeutic approaches specifically for BCSC [17].

Other newer strategies have been reported recently by a group of researchers to improve the identification and isolation of breast CSCs [18–20]. The method utilizes the fluorescent dye PKH26, which labels the cell membrane followed by culturing the mammary cells to form mammospheres in suspension cultures. The slow cycling cells retain the dye after about 7–10 days and can be sorted on the basis of the fluorescence intensity of PKH26. About 0.2–0.4 % of the total cell population are comprised of the $PKH26^{hi}$ cells, which can form secondary mammospheres, divide asymmetrically, and express pluripotency markers as well as reconstitute the mammary epithelium after transplantation into NOD/SCID mice [20].

Other than these methodologies, omics approaches have become more popular in recent years. Some examples to omics approaches are genomics, epigenomics, and proteomics. These will be further explained in the next section, "Omics Approaches for Breast Cancer Stem Cells."

Omics Approaches for Breast Cancer Stem Cells

Genomics

Genomics studies include analysis of whole-genome genetic alterations such as somatic mutations and epigenetic alterations (specifically, it may be called epigenomics). Sequencing and gene expression profiling of cancer genomes are basic experimental approaches of genomics studies. Although cancer stem cells form a very small fraction of the whole tumor, they carry vital importance for breast cancer patients. Decreased survival has been demonstrated for patients whose genetic profile is similar to stem cells [21]. However, the gene expression profile of the bulk of breast tumor cells can sometimes cause nonclear identification of breast cancer stem cell expression profile [22]. For this reason, it is more advantageous to isolate breast cancer stem cells (BCSC) before getting detailed genomic analysis of BCSC. Purification of BCSC is generally based on markers, especially CD44 positivity. There are additional markers such as CD24. However, CD24 can be negative- or low-expressed in BCSC [8]. CD24 negativity together with CD45 negativity has been used in magnetic cell-sorting method as additional markers to CD44 positivity [22]. In another study, $CD24^+$ and $CD44^+$ cells have been asserted as not always the same group of cells, but they are certainly clonally related to each other [21]. In contrast to CD24, CD44 positivity is a common feature of BCSC, which is used as a marker for identification and isolation of stem cells.

For a better understanding of BCSC genomics, next-generation sequencing and then gene expression profiling based on microarray technology have been performed after isolation of BCSC by Hardt's group [22]. Whole-genome microarray results correlated with next-generation sequencing results. In next-generation sequencing, RNAs of 500 $CD44^+/CD24^-/CD45^-$ breast cancer cells (or simply breast cancer stem cells) and 500 bulk tumor cells are used. Since RNAs of cells are the sequencing materials, this is also called "transcriptome sequencing." A set of differentially expressed genes is found in the group of breast cancer stem cells via both sequencing and microarray methods. Cancer stem cell markers CD44, ALDH1A3 (ALDH1 isoform), CD34, CD133, ITGB1, and PROCR have significantly higher expression levels compared to control group. Important pathways involved in cancer stem cell maintenance are also examined. Canonical Wnt signaling is one of those pathways [23]. Wnt pathway genes such as WNT2, WNT3, WNT11, FZD4, and TCF4 are found upregulated by both methods, sequencing and microarray. One of the other important pathways is Notch, which was previously shown to affect self-renewal of BCSC [24]. JAG1 and NOTCH3 are upregulated, whereas JAG2 is downregulated in BCSC. Epithelial to mesenchymal transition genes, slug, integrin α6/β1, α-SMA, fibronectin-1, and cadherin 11, are all upregulated in BCSC. On the other hand, keratin

and claudin-1, which are typical epithelial genes, are downregulated.

In addition to genomic investigation of previously known BCSC-related genes, some pathways, which are involved in unfavorable breast cancer prognosis, are examined. Epithelial growth factor (EGF/EGFR), platelet-derived growth factor (PDGF/PDGFR), and hepatocyte growth factor (HGF/MET) pathways are found upregulated in BCSC. This finding may open further doors about BCSC impact on unfavorable prognosis of breast cancer. Other prognostic breast cancer markers which are mostly associated with bad outcomes, such as protease inhibitors PAI-1, TIMP1, TIMP2, and TIMP3, also have higher expression levels. Furthermore, BCSC demonstrate higher PI3K activity compared to control bulk cells, which might be the reason for drug resistance in BCSC [25]. Similar to PI3K hyperactivity, TGF-β pathway is also upregulated in BCSC population [22]. To sum up, the overall genomic expression profile of BCSC implies the critical role of stem cells in breast cancer. General breast cancer genomic studies are performed more than BCSC studies [26–30]. However, genomic analysis focusing on stem cells in breast tumors might be more beneficial in terms of developing therapeutic approaches and understanding BCSC molecular biology.

In Shipitsin's study, breast cancer stem cell genomic analysis was performed after purification of stem cells according to marker CD44 and a CD44$^+$ cell-specific gene PROCR, which encodes a cell surface receptor [21]. CD24 is not used together with CD44; rather, CD24$^+$ group is distinguished from CD44$^+$ breast cancer cells at some points such as expression of self-renewal-associated pathway Hedgehog expression level. Gli1 and Gli2 have higher expression levels in CD44$^+$ group compared to CD24$^+$ cells. Previously, CD44$^+$/(CD24$^+$ or low-expressed) cells were defined as BCSC [8]. However, it is likely that there can be difference between these two marker groups in terms of self-renewal potential. Therefore, they mostly analyze CD44$^+$ cells as BCSC. After purification, they have used SAGE (serial analysis of gene expression) genomic approach for analysis of BCSC, which is a comprehensive analysis methodology first described in 1995 [31]. SAGE is almost similar to DNA microarray. However, in SAGE, there is no probe usage and it is more quantitative (so, it may be asserted to be more exact) than microarray. Functional analysis of high-throughput data from SAGE library has been performed via MetaCore data-mining technology. One of the most important findings from canonical pathway analysis is TGF-β pathway (e.g., TGF-β1 and TGFBR2) upregulation in CD44$^+$ cells. TGF-β pathway activity is not only involved in stem cell self-renewal regulation but also in cancer progression and metastasis [32, 33]. Thus, it most likely has a critical role in genomic regulation of stem cells in breast cancer. This finding has therapeutic implications; for example, using TGFBR kinase inhibitor leads to mesenchymal to epithelial transition of CD44$^+$ cells, which indicates positive therapeutic effect.

Gene Signatures

Gene signatures are basically formed from gene expression profiling of cells. Generally, a high number of patient data sets are used for isolation of breast tumors. Then, it is necessary to purify a highly tumorigenic subgroup from the whole tumor. After purification of stemlike cells from breast tumors, RNA amplification and then microarray analysis are performed. Normalized microarray data reveal differentially expressed genes in breast cancer stem cells. This type of high-throughput analysis is beneficial in order to group investigated genes. Gene signatures are useful for genomic understanding of patient survival (metastasis-free/shorter distant metastasis-free/longer distant metastasis-free/overall survival).

Gene Signature A and B

Shipitsin's group analyzed three cohorts of breast cancer patients via microarray [21]. Two of these investigated patient data sets (first test data set, 286 patients; second test data set, 125 patients; there is also third group of patients which is used as test data set) are used as training sets to identify gene signatures, which are named signature "A" and signature "B" genes (Table 12.1), and

Table 12.1 Some genes included in signatures A and B

Gene	Description	Function(s)	Signature type and importance
Annexin A5 (ANXA5)	Gene encodes indirect inhibitor of the thromboplastin-specific complex involved in the blood coagulation cascade	Calcium ion binding, calcium-dependent phospholipid binding, phospholipase inhibitor activity, phospholipid binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Actin, gamma 1 (ACTG1)	Gene encodes a cytoplasmic actin found in nonmuscle cells	ATP binding, identical protein binding, protein binding, structural constituent of cytoskeleton	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
ADP ribosylation factor 3 (ARF3)	The protein encoded by this gene belongs to the ARF-like (ADP ribosylation factor-like) subfamily of the ARF family of GTP-binding proteins which are involved in regulation of intracellular traffic	GTP binding, metal ion binding, phospholipid binding, protein binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
ATPase, Na+/K+transporting, beta 3 polypeptide (ATP1B3)	Family of gene product is Na+/K+−ATPase which is an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of Na and K ions across the plasma membrane	Sodium/potassium-exchanging ATPase activity	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
HLA-B associated transcript 3 (BAT3) *Other name*: BAG6 (BCL2-associated athanogene 6)	Gene encodes a nuclear protein that is cleaved by caspase 3 and is implicated in the control of apoptosis	Hsp70 protein binding, polyubiquitin binding, proteasome binding, protein binding, ribosome binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Caldesmon 1 (CALD1)	Gene encodes a calmodulin- and actin-binding protein that plays an essential role in the regulation of smooth muscle and nonmuscle contraction	Actin binding, calmodulin binding, myosin binding, tropomyosin binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Centaurin, delta 2 (CENTD2) *Other name*: ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 1 (ARAP1)	Gene encodes a protein that associates with the Golgi, and the ARF-GAP activity mediates changes in the Golgi and the formation of filopodia	Zinc ion binding, protein binding, phosphatidylinositol-3,4,5-trisphosphate binding, Rho GTPase activator activity, ARF GTPase activator activity	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Chloride intracellular channel 1 (CLIC1)	Chloride intracellular channel 1 is a member of the p64 family; the protein localizes principally to the cell nucleus and exhibits both nuclear and plasma membrane chloride ion-channel activity	Chloride channel activity, protein binding, voltage-gated chloride channel	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Chitobiase di-N-acetyl (CTBS)	Chitobiase is a lysosomal glycosidase involved in degradation of asparagine-linked oligosaccharides on glycoproteins	Chitinase activity	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients

Table 12.1 (continued)

Gene	Description	Function(s)	Signature type and importance
Dihydropyrimidinase-like 3 (DPYSL3)	DHPases are a family of enzymes that catalyze the reversible hydrolytic ring opening of the amide bond in five- or six-membered cyclic diamides	SH3 domain binding, chondroitin sulfate binding, hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in cyclic amides, phosphoprotein binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Dishevelled, dsh homolog 3 (DVL3)	Gene encodes a cytoplasmic phosphoprotein that regulates cell proliferation	Beta-catenin binding, protease binding, receptor binding, signal transducer activity	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Exostoses 1 (EXT1)	Gene encodes an endoplasmic reticulum-resident type II transmembrane glycosyltransferase involved in the chain elongation step of heparan sulfate biosynthesis	Heparan sulfate N-acetylglucosaminyltransferase activity, protein heterodimerization activity, protein homodimerization activity, transferase activity, transferring glycosyl groups	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Fibroblast growth factor receptor 1 (FGFR1)	Protein encoded by this gene binds both acidic and basic fibroblast growth factors and is involved in limb induction	Heparin binding, protein tyrosine kinase activity, ATP binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Ferritin, light polypeptide (FTL)	Gene encodes the light subunit of the ferritin protein which is the major intracellular iron storage protein in prokaryotes and eukaryotes	Ferric iron binding, iron ion binding, oxidoreductase activity	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Guanine nucleotide binding protein: GNB2L1	WD40 domain encoded by gene is found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction	Protein complex scaffold, protein kinase C, protein tyrosine kinase activity, receptor binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Hypoxia inducible factor 1, alpha subunit (HIF1A)	HIF-1 functions as a master regulator of cellular and systemic homeostatic response to hypoxia	Signal transducer activity, DNA binding transcription factor activity, protein kinase binding, histone deacetylase binding, enzyme binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Interleukin 13 receptor, alpha 1 (IL13RA1)	Gene encodes a subunit of the interleukin 13 receptor. This subunit forms a receptor complex with IL4 receptor alpha, a subunit shared by IL13 and IL4 receptors	Cytokine receptor activity, protein binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Lipin 2 (LPIN2)	According to mouse experiments, gene functions during normal adipose tissue development and may play a role in human triglyceride metabolism	Phosphatidate phosphatase activity, transcription coactivator activity	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients

(continued)

Table 12.1 (continued)

Gene	Description	Function(s)	Signature type and importance
Staufen, RNA binding protein (STAU1)	Staufen is a member of the family of double-stranded RNA (dsRNA)-binding proteins involved in the transport and/or localization of mRNAs to different subcellular compartments and/or organelles	Double-stranded RNA binding, protein binding	*Signature A*[1] implying shorter distant metastasis-free survival of breast cancer patients
Activity-dependent neuroprotector (ADNP)	This gene encodes a protein that is upregulated by vasoactive intestinal peptide and may be involved in its stimulatory effect on certain tumor cells	Chromatin binding, metal ion binding, sequence specific DNA binding, protein binding	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients
ATPase, H+transporting, lysosomal accessory protein 1 (ATP6AP1)	This gene encodes a component of a multisubunit enzyme that mediates acidification of eukaryotic intracellular organelles	ATP binding proton-transporting ATP synthase activity-rotational mechanism, proton-transporting ATPase activity-rotational mechanism, transporter activity	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients
Basic leucine zipper transcription factor, ATF-like (BATF)	Gene encodes nuclear basic leucine zipper protein that belongs to the AP-1/ATF superfamily of transcription factors	Protein binding, sequence specific DNA binding	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients
Clathrin, heavy polypeptide (CLTC)	Clathrin is a major protein component of the cytoplasmic face of intracellular organelles	Protein binding, structural molecule activity	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients
Coiled-coil and C2 domain containing 1A (CC2D1A)	This gene encodes a transcriptional repressor that binds to a conserved 14-bp 5′-repressor element and regulates expression of the 5-hydroxytryptamine (serotonin) receptor 1A gene in neuronal cells	Signal transducer activity, RNA polymerase II core promoter proximal region sequence specific DNA binding	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients
Interferon regulatory factor 1 (IRF1)	IRF1 serves as an activator of interferons alpha and beta transcription	Sequence specific DNA binding transcription factor activity, RNA polymerase II core promoter proximal region sequence specific DNA binding	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients
Plexin A3 (PLXNA3)	This gene encodes a class 3 semaphorin receptor and may be involved in cytoskeletal remodeling and apoptosis	Semaphoring receptor activity, transmembrane signaling receptor activity	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients
Sorting nexin 5 (SNX5)	This gene encodes a member of the sorting nexin family	Phosphatidylinositol binding	*Signature B*[1] implying longer distant metastasis-free survival of breast cancer patients

Adapted from Shipitsin et al. [21]
The full list of signature genes is available in the referenced article

make a correlation between gene signatures and patient outcomes. Signature A-B gene groups are composed of upregulated-downregulated breast cancer genes in $CD44^+$ and $PROCR^+$ cell SAGE libraries. It is possible to name this group as breast cancer stem cells, which have higher self-renewal capability compared to $CD24^+$ cell SAGE libraries, respectively. High expression of signature A corresponds to shorter distant metastasis-free survival of patients, and high expression of signature B corresponds to longer distant metastasis-free survival of patients. Patient survival analysis is made via Kaplan-Meier analysis after getting signature data as a heat map from microarray results.

Invasiveness Gene Signature

Liu's group has formed another gene signature called as "invasiveness gene signature (IGS)" (Table 12.2) including differentially expressed 186 genes in $CD44^+CD24^{-/low}$ breast cancer stem cells compared to normal breast epithelial cells [34]. IGS is used for association between gene expression levels and patient survival and risk of death. This association is made upon some bioinformatic analysis. Pearson correlation coefficients positive (>0) and negative (≤0) are found for patients in data set. These two coefficients imply two patient groups: one is positively related with IGS, which corresponds to positive correlation coefficient, and the other group of patients has negative correlation coefficient and negatively related with IGS. Positive correlation coefficient means higher risk of metastasis and reduced overall survival, and negative correlation coefficient has the opposite meaning for patients. Analysis of 295 breast cancer patients revealed that 62 % of patients, whose gene expression levels show similarity with IGS (correlation coefficient, >0), could survive in estimated 10 years of overall survival. The metastasis-free survival ratio is 54 % for those patients with positive correlation coefficient. The survival picture is better for patients whose gene profiling is negatively related to IGS (correlation coefficient, ≤0). Overall survival ratio of these patients is 98 % and metastasis-free survival is 82 % in estimated 10 years. From these results, it is possible to conclude that invasiveness gene signature has prognostic significance for breast cancer patients.

Table 12.2 Some genes included in invasiveness gene signature (IGS)

Gene	Description	Function(s)	Gene functional subgroup in all other IGS genes
Zinc and double PHD fingers family 2 (DPF2)	The protein encoded by this gene functions as a transcription factor which is necessary for the apoptotic response following deprivation of survival factors	Zinc ion binding, apoptotic process	Apoptosis[a]
B cell CLL/lymphoma 2 (BCL2)	This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some cells such as lymphocytes	B cell and T cell homeostasis, apoptosis, axon regeneration, cell aging, developmental growth, cell-cell adhesion	Apoptosis[a]
Secretagogin, EF-hand calcium-binding protein (SCGN)	The encoded protein is a secreted calcium-binding protein which is found in the cytoplasm	Calcium ion binding	Calcium ion binding[a]
alkB, alkylation repair homolog 1 (ALKBH1)	This gene encodes a homolog to the *E. coli* alkB gene product, which is part of the adaptive response mechanism of DNA alkylation damage repair	DNA demethylation, DNA repair, neuron migration, developmental growth, ferrous iron binding	Cell cycle[a]

(continued)

Table 12.2 (continued)

Gene	Description	Function(s)	Gene functional subgroup in all other IGS genes
CD59 molecule, complement regulatory protein (CD59)	This gene encodes a cell surface glycoprotein that regulates complement-mediated cell lysis, and it is involved in lymphocyte signal transduction	Cell activation, blood coagulation, innate immune response, complement binding	Cell surface receptor[a]
Mitogen-activated protein kinase 14 (MAPK14)	The protein encoded by this gene is a member of the MAP kinase family, which acts as an integration point for multiple biochemical signals, and is involved in a wide variety of cellular processes	Angiogenesis, blood coagulation, cell morphogenesis, chemotaxis, MAP kinase activity	Chemotaxis[a]
Matrix metallopeptidase 7 (MMP7)	The enzyme encoded by this gene degrades proteoglycans, fibronectin, and elastin	Proteolysis, antibacterial peptide secretion, regulation of cell proliferation	Collagen metabolism[a]
Matrix Gla protein (MGP)	The protein encoded by this gene is secreted and likely acts as an inhibitor of bone formation	Cell differentiation, calcium ion binding, cartilage condensation	Differentiation[a]
Sodium channel modifier 1 (SCNM1)	SCNM1 is a zinc finger protein and putative splicing factor	Metal ion binding, RNA splicing	Ion-channel activity[a]
Toll-like receptor adaptor molecule 2 (TICAM 2)	TICAM 2 is a Toll/interleukin-1 receptor	Inflammatory response, innate immune response, toll-like receptor signaling pathway, signal transducer activity	Membrane protein[a]
Glucosamine-6-phosphate deaminase 1 (GNPDA1)	Gene encodes an allosteric enzyme that catalyzes the reversible conversion of D-glucosamine-6-phosphate into D-fructose-6-phosphate and ammonium	Hydrolase activity, single fertilization, glucosamine catabolic process	Metabolism[a]
DNA (cytosine-5-)-methyltransferase 3 alpha (DNMT3A)	This gene encodes a DNA methyltransferase that is thought to function in de novo methylation, rather than maintenance methylation	DNA methylation, spermatogenesis, cellular response to amino acid stimulus	Methyltransferase[a]
Actin-related protein 2/3 complex, subunit 5 (ARPC5)	This gene encodes one of seven subunits of the human Arp2/3 protein complex, which controls of actin polymerization in cells	Actin binding, innate immune response, actin cytoskeleton organization	Morphology[a]
Nucleolar protein 8 (NOL8)	NOL8 binds Ras-related GTP-binding proteins	RNA binding, DNA replication, positive regulation of cell growth	Nucleotide binding[a]
Dual specificity phosphatase 10 (DUSP10)	Gene product inactivates their target kinases by dephosphorylating both the phosphoserine/threonine and phosphotyrosine residues	Protein dephosphorylation, response to stress, inactivation of MAPK activity, negative regulation of JNK cascade	Phosphatase[a]
v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 4 (ERBB4)	Gene encodes a member of the Tyr protein kinase family and the epidermal growth factor receptor subfamily	Cell migration, cell proliferation, lactation, innate immune response, heart development	Proliferation[a]

Table 12.2 (continued)

Gene	Description	Function(s)	Gene functional subgroup in all other IGS genes
Cleavage stimulation factor, 3′ pre-RNA, subunit 1 (CSTF1)	This gene encodes one of three subunits, which combine to form cleavage stimulation factor (CSTF). CSTF is involved in the polyadenylation and 3′ end cleavage of pre-mRNAs	RNA binding, splicing, mRNA polyadenylation, gene expression	Protein binding[a]
p21 protein (Cdc42/Rac)-activated kinase 2 (PAK2)	The p21-activated kinases (PAK) are critical effectors that link Rho GTPases to cytoskeleton reorganization and nuclear signaling	Apoptotic process, axon guidance, innate immune response, phosphorylation, viral process, ATP binding, protein kinase activity, T cell costimulation	Protein kinase[a]
Nucleoporin 37 kDa (NUP37)	Nuclear pore complexes are composed of subcomplexes, and NUP37 is part of one such subcomplex	Mitosis, cell division, glucose transport, protein transport, transmembrane transport	Protein transport[a]
Stanniocalcin 2 (STC2)	The encoded protein may play a role in the regulation of renal and intestinal calcium and phosphate transport, cell metabolism, or cellular calcium/phosphate homeostasis	Hormone activity, response to cellular stress, cell-cell signaling, embryo implantation	Signal transduction[a]
Ataxin 3 (ATXN3)	The protein encoded by this gene contains (CAG)n repeats in the coding region, and the expansion of these repeats from the normal 13–36 to 68–79 is one cause of Machado-Joseph disease	Cell death, cellular response to heat, nucleotide excision repair	Transcription factor[a]
Heparan sulfate 2-O-sulfotransferase 1 (HS2ST1)	This gene encodes a member of the heparan sulfate biosynthetic enzyme family that transfers sulfate to the 2 position of the iduronic acid residue of heparan sulfate	Sulfotransferase activity, carbohydrate metabolic process	Transferase[a]
Membrane-associated ring finger (C3HC4) 8, E3 ubiquitin protein ligase (MARCH8)	MARCH8 is a member of the MARCH family of membrane-bound E3 ubiquitin ligases	Zinc ion binding, protein polyubiquitination, MHC class II protein binding	Ubiquitination[a]

Adapted from Liu et al. [34]

The full list of signature genes for each subpart (indicated as [a]) is available in an article. This table includes some of the IGS genes, whose number is in total 186. For a full list of genes, please see the referenced article

Human Normal Mammary Stem Cell (hNMSC) Signature

hNMSC signature includes 1,090 upregulated genes and 1,217 downregulated genes in PKH26$^+$ cells. (Since the gene signature involves a long list of genes, it will not be presented here as a table.) Then, gene profiles of different graded breast tumors (G3, less differentiated; G1, highly differentiated) are examined via gene set enrichment analysis (GSEA) and compared with hNMSC signature. G3 tumors' gene profiling demonstrates high hNMSC signature identity, but G1 tumors' gene profile is not so similar. This implies that G3 tumors have more breast cancer stem cells. In other words, they are less differentiated, which is a previously known feature of tumor. Therefore, hNMSC signature has been found as a useful tool to predict breast tumor grade [20].

hNMSC signature is different from other breast cancer stem cell-related gene signatures because it is formed from healthy individual normal mammary stem cells. However, it is possible to use it for prediction of breast tumor grades. Also, hNMSC signature and breast tumor grade-3 correlation (but noncorrelation between this signature and G1 tumors) show that different breast tumor grades have different breast cancer stem cell contents. Therefore, in the future, hNMSC signature may play important roles in breast cancer therapy.

The 31-Gene Signature of Breast Cancer Stem Cell Subgroups

Microarray analysis of two distinct subsets of breast cancer stem cells ($CD44^{+}/CD24^{-}$ and $CD44^{+}/CD24^{-}$ cells) revealed 1,777 upregulated genes and 1,883 downregulated genes [35]. $CD44^{+}/CD24^{-}$ cells showed more than twofold significance for 599 of these genes compared to the $CD44^{+}/CD24^{-}$ group. Higher cancer stem cell capacity of $CD44^{+}/CD24^{-}$ cells is found compared to the other group. A total of 198 untreated breast cancer patients are examined; 64 ER (negative) group patients are used as a training set. The aim of forming a training set is that some of 599 genes can be used in the prediction of patients' distant metastasis situations. Twenty-five of 64 patients demonstrated distant metastasis and K-nearest neighbor algorithm as classifier is used to identify a 31-gene signature (Table 12.3). Also, this signature has been validated in another test group. Sensitivity of the signature is 70 % and specificity is 97 %. Patients who will not experience distant metastasis can be predicted more accurately because of high specificity.

Table 12.3 Some important genes from 31-gene signature

Gene	Description	Function(s)
Suppression of tumorigenicity 14 (ST14)	The protein encoded by this gene is an epithelial-derived, integral membrane serine protease	Serine-type peptidase activity, proteolysis
Annexin A6 (ANXA6)	Annexin VI belongs to a family of calcium-dependent membrane and phospholipid binding proteins	Calcium ion binding, calcium ion transport
Polypeptide N-acetylgalactosaminyltransferase3 (GALNT3)	This gene encodes UDP-GalNAc transferase 3, a member of the GalNAc-transferases family	Calcium ion binding, manganese ion binding, cellular protein metabolic process, carbohydrate metabolic process
Occludin (OCLN)	This gene encodes an integral membrane protein that is required for cytokine-induced regulation of the tight junction paracellular permeability barrier	Apoptotic process, cell-cell junction organization, structural molecule activity, protein complex assembly
Peptidylarginine deiminase, type IV (PADI4)	This gene is a member of a gene family which encodes enzymes responsible for the conversion of arginine residues to citrulline residues	Calcium ion binding, chromatin modification, cellular protein modification process
Cadherin 2, type 1, N-cadherin (CDH2)	This gene is a classical cadherin from the cadherin superfamily. The encoded protein is a calcium-dependent cell-cell adhesion glycoprotein comprised of five extracellular cadherin repeats, a transmembrane region, and a highly conserved cytoplasmic tail	Cell adhesion, cell migration, alpha catenin binding, protein phosphatase binding
Keratin 13 (KRT13)	The protein encoded by this gene is a member of the keratin gene family; most of the type I cytokeratins consist of acidic proteins which are arranged in pairs of heterotypic keratin chains	Structural molecule activity, response to radiation, epidermis development

Table 12.3 (continued)

Gene	Description	Function(s)
Microtubule-associated protein (MAP1B)	The product of this gene is a precursor polypeptide that presumably undergoes proteolytic processing to generate the final MAP1B heavy chain and LC1 light chain	Hydrolase activity, microtubule binding, cellular process, dendrite development, axon extension
Serine peptidase inhibitor (SPINT1)	The protein encoded by this gene is a member of the Kunitz family of serine protease inhibitors	Serine-type endopeptidase inhibitor activity, extracellular matrix organization
Poliovirus receptor-related 3 (PVRL3)	This gene encodes a member of the nectin family of proteins, which function as adhesion molecules at adherens junctions	Cell adhesion molecule binding, protein binding, cell junction assembly, homophilic cell adhesion

Adapted from Leth-Larsen et al. [35]
This table includes some explanations for 10 of 31 genes; for the complete list, please see the referenced article

Table 12.4 Significantly upregulated proteins in breast cancer stem cells (possible proteomic signature of breast cancer stem cells)

cAMP-response element binding protein: CREB	Bad
Cyclic AMP-dependent transcription factor 1	Phosphorylated-CREB
Mesothelin	Phosphorylated-protein kinase C (PKC)
Thyroid transcription factor 1	Wee1
Phosphorylated focal adhesion kinase	Cell division cycle 42
p38	Twist

These 12 proteins are significantly upregulated in breast cancer stem cells

Proteomics

Proteomics, which is the large-scale analysis of proteins in cells, is a useful omics approach to enlarge the database obtained from genomics techniques. Protein posttranslational modifications such as acetylations, glycosylations, protein cleavages, and phosphorylations cannot be detected via genomic approaches because these events do not occur at the level of mRNA. These modifications are significant for determination of protein localization, stability, and functions. Also, gene profiling relies on mRNA level of genes but all synthesized mRNAs are not necessarily translated into proteins. Thus, it is possible to assert that proteomic profile of cells reflects more than genomic profile. From a therapeutic perspective, proteins can be more realistic targets than mRNAs because most mRNAs are degraded in limited times [36].

Kanojia's group performed breast cancer stem cell proteomics analysis via liquid chromatography and mass spectrometer methods after isolation of stem cells from Her2/Neu transgenic mice breast tumor [37]. Human epidermal growth factor receptor 2 (Her2) is a receptor tyrosine kinase and encoded by a known proto-oncogene, ERBB2. Her2 is overexpressed in approximately 30 % of breast cancer patients. Its elevated level is associated with mammary tumorigenesis and it is involved in metastasis of breast tumors. The relation between Her2 and breast tumor aggressiveness might be due to breast cancer stem cells (BCSC) because Her2 overexpression causes stem cell population increase in breast tumors [38].

Differentially expressed proteins are detected in breast cancer stem cells (Table 12.4) compared to non-stem cells. In silico analysis of differentially expressed proteins in breast cancer, stem cells reveals the corresponding eight genes: ferritin heavy chain 1 (FTH-1), prostaglandin-endoperoxide synthase-1 (PTGS-1), prostaglandin-endoperoxide synthase-2 (PTGS-2), prothymosin alpha (PTMA), S100 calcium-binding protein A4 (S100A4),

S100 calcium-binding protein A6 (S100A6), thioredoxin reductase 1 (TXNRD1), and keratin 14 (KRT14). These genes can be assigned as another signature of breast cancer stem cells, which is found via proteomics method LC-MS/MS. Specifically, prognostic effects of PTGS-2, TXNRD1, and KRT14 genes in breast cancer have been previously defined [39–41]. Also, upregulation of FTH1, which is involved in iron metabolism, inhibits TNF-α- induced apoptosis [42].

Epigenomics

Breast cancer cells not only have genetic- or protein-level alterations but also epigenetic changes. Histone modifications, DNA methylation, and nucleosomal remodeling are some types of epigenetic alterations that affect breast cancer progression [43]. These changes eventually modulate gene expression or silencing. There are different methods for genome-wide detection of DNA methylation such as restriction landmark genomic scanning (RLGS), differential methylation hybridization (DMH) with CpG island array, and microarray analysis in the presence/absence of demethylating drug. Also, chromatin immunoprecipitation (ChIP) combined with DNA arrays (ChIP-on-chip) can be used to profile histone modifications [44].

Epigenetic reprogramming has critical importance for the breast cancer stem cell phenotype. For this reason, Hernandez-Vargas' group studied methylome profiling of breast cancer stem cells [45]. For CpG island methylation pattern analysis of breast cancer stem cells, the Illumina bead array technology (Illumina methylation cancer panel I bead arrays) is preferred because this technology is based on high-throughput and quantitative microarray methodology. After analysis of 807 genes and 1,505 CpG islands, 10 genes are demonstrated to have a significant methylation pattern in breast cancer stem cells (BCSC) compared to MCF7 cancer cell line. These methylated genes in BCSC: runt-related transcription factor 3 (RUNX3), glutathione peroxidase 1 (GPX1), Fas (TNF receptor superfamily, member 6), membrane metalloendopeptidase (MME), v-myc myelocytomatosis viral oncogene homolog 2 (MYCL2), Lipocalin 2 [oncogene 24p3] (LCN2), HRAS-like suppressor (HRASLS), signal transducer and activator of transcription 5A (STAT5A), colony-stimulating factor 3 receptor [granulocyte] (CSF3R), placental growth factor and vascular endothelial growth factor-related protein (PGF). Hypomethylation of JAK-STAT-related genes—CCND2, STAT5A, and JAK3—is also found and further assessed by quantitative RT-PCR, which demonstrates JAK-STAT pathway activity. It has been previously shown that normal stem cell self-renewal capacity is related with JAK-STAT pathway [46, 47]. Hypomethylated JAK-STAT pathway genes are further analyzed using the same methylation panel. In addition to CCND2, STAT5A, and JAK3, other significantly hypomethylated JAK-STAT pathway genes are LIF, IL12B, CSF3, EPO, CSF2, IL10, PIK3R1, and IL13. These JAK-STAT pathway-related genes are hypermethylated in BCSC but fold change is lower than hypomethylated genes: IL6, JAK2, CCND1, IFNGR1, CCDN3, IL11, AKT1, and IL12A.

Epithelial to Mesenchymal Transition (EMT)

EMT is a highly structured process which occurs during embryonic development, wound healing, and cancer progression. However, the mechanisms of regulation of EMT in any of the three types of EMT are conserved to a great extent. There is evidence to support the role of EMT in breast cancer as seen by a number of in vitro studies in normal and malignant mammary epithelial cells and in in vivo studies in mouse models of breast cancers [48, 49]. A number of transcription factors like Snail/Slug family, Twist, δEF1/ZEB1, SIP1/ZEB2, and E12/E47 [50–52] play a major role in the EMT program.

Epithelial to Mesenchymal Transition (EMT) and Breast Cancer Stem Cells

It has been observed recently that the EMT is connected to CSCs, suggesting that the generation of cancer cells with stem cell-like properties needed

for dissemination as well as self-renewal in order to establish secondary tumors is due to the process of EMT [53]. Ectopic expression of Snail or Twist or exposure to TGF-β leads to induction of EMT in immortalized human mammary epithelial cells (HMLE) which increases the ability of the cells to form tumorspheres. These cells have a mesenchymal phenotype and have an increased ability of tumor initiation [54]. About 45–50 % of breast cancer patients develop refractory or resistant disease [53]. In human breast tumors, neoadjuvant chemotherapy results in the increase of CD44+/CD24−/low cells and enhanced ability of mammosphere formation [55]. These cells with a CD44+/CD24−/low profile have been observed to be more resistant to chemotherapies, radiotherapies, and other hormone therapies, which is unlike the other population of differentiated breast cancer cells of the tumor mass [56]. The mechanism of drug resistance induced by EMT is not well understood. Increased levels of Twist seem to play a role in mediating drug resistance by EMT. Upregulation of Twist reportedly increased transcription of AKT-2 to promote cell survival and resistance to paclitaxel [57]. The expression of Snail or Slug promotes cellular resistance in MCF7 cells after exposure to doxorubicin and promotes invasiveness [58]. Once the molecular mechanisms underlying the contribution of CSCs to drug resistance are elucidated, it would enable exploring newer, more effective therapeutic targets, which would reduce the burden of tumor relapse.

Circulating Tumor Cells (CTCs) in Breast Cancer

Circulating tumor cells (CTCs) have been identified in a number of breast cancer patients and have been correlated with disease progression as reported in literature [59–65]. CTC identification is an upcoming diagnostic tool for advanced-stage cancer patients. The lower number of CTCs limits the detection and poses a technical challenge. However, recent developments in the detection methods include image-based approaches like classic immunocytochemistry (ICC), the US Food and Drug Administration (FDA)-cleared CellSearch® system (Veridex), the Ariol system, and the laser-scanning cytometry; molecular assays based on nucleic acid analysis of CTCs, such as the highly sensitive RT-qPCR methods, multiplex reverse transcription PCR (RT-PCR) assays, or a combination of molecular and imaging methods; and protein-based assays like the EpiSpot assay, which detects tumor-specific proteins released by CTCs [66].

CTCs, CSCs, and EMT

It has been observed that the expression of stem-like and EMT markers in CTCs is associated with resistance to conventional anticancer therapies and treatment failure, highlighting the need to improve technologies for detecting and eliminating minimal residual disease [67]. The association between EMT and CTCs remains unknown; however, data suggest the role of the EMT process in the formation of CTCs (Fig. 12.1) and animal models with transplantable breast tumors that help in the characterization of EMT/CTC associations [67]. Balic and colleagues were the first ones to show the existence of stemlike characteristics in the disseminated tumor cells in the bone marrow of early breast cancer patients [68]. There is evidence to show that markers like CD44, CD24, and ALDH1, which are used for characterization of CSCs, are also observed in the CTCs [69, 70]. Molecular characterization of the CTCs will enable exploring newer therapeutic targets and understanding resistance to therapies.

miRNA Regulation and Breast Cancer Stem Cells

miRNAs (microRNAs) have been studied in CSCs for their role in regulating tumor formation. It has been observed that the expression of let-7 is very low or undetectable in embryonic stem cells (ES cells), while it increases during differentiation. Breast CSCs have shown a similar pattern of let-7 expression [71]. The expression of let-7 was seen to be reduced in CSCs as compared to non-CSCs [71]. Transduction of breast CSCs with let-7 reduced undifferentiated cells and inhibited cell proliferation, mammosphere formation, and

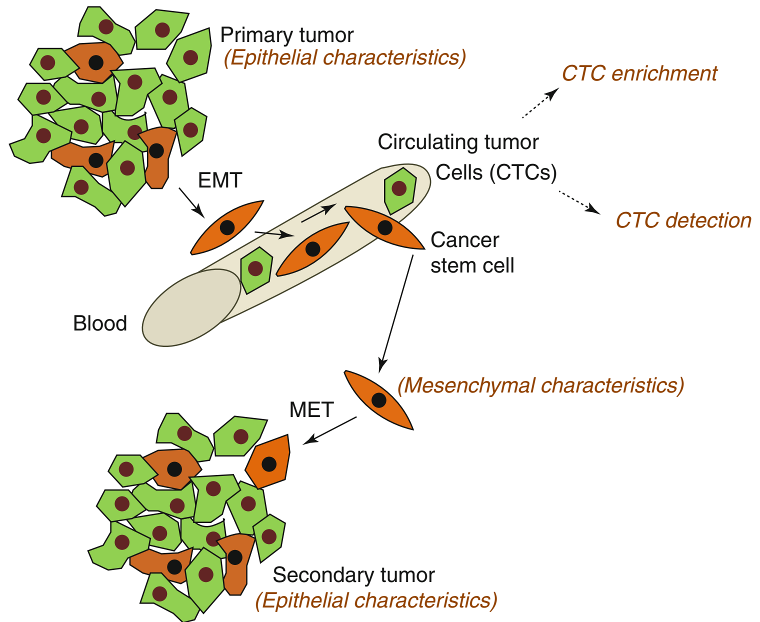

Fig. 12.1 Epithelial to mesenchymal transition (*EMT*) leads to formation of circulating tumor cells (*CTCs*) and cancer stem cells (CSCs), which pass through the bloodstream and colonize in other organs after mesenchymal to epithelial transition (*MET*)

tumor formation in vivo [71]. About 37 miRNAs have been identified which show varied expressions between human breast CSCs and nontumorigenic breast cancer cells [72]. The miR-200 family members were downregulated in human breast CSCs and normal mammary stem/progenitor cells [72], as well as suppressed EMT in breast cancer [73]. Breast cancer stem cell propagation was inhibited with the expression of miR-200 in vitro and further suppressed the tumor formation ability of human breast cancer stem cells in vivo [72]. miR-34c has been observed to be downregulated in human breast CSCs [74]. Further, ectopic expression of miR-34c expression reduced breast CSCs self-renewal, inhibited EMT, and silenced Notch4, leading to suppression of tumor cell invasiveness [74]. Zhu and colleagues studied drug-resistant human breast cancers and observed reduced miR-128 expression in human breast CSCs along with Bmi-1 and ABCC5 overexpression [75]. Increasing the expression of miR-128 led to the increase in sensitivity of breast cancer cells to doxorubicin [75]. The increasing knowledge about noncoding RNAs regulating CSCs would enable better understanding of CSCs and thereby help in elucidating novel targets for therapy.

Conclusion and Future Perspective

Omics approaches have become popular in cancer research due to the complexity of the disease. Since these technologies widely investigate the genome/proteome/transcriptome of tumors, results of these analyses enable researchers to understand disease pathology in a more comprehensive way. Although breast cancer omics analysis has been performed for long time, more specific genomic/proteomic/epigenomic examination of breast cancer has also been done since the cancer stem cell theory is accepted in the scientific world. Breast cancer stem cells are a critical subgroup of breast tumor cells in terms of therapeutic resistance and tumor recurrence. It is still not clear why those cells are specifically resistant to therapy. To be able to understand this important point, genome-wide and proteome-wide analyses of breast cancer stem cells have been performed. Some of these analyses revealed significant “signatures” of breast cancer stem cells. These signatures mostly form a unique cluster of genes or proteins. These signatures of breast cancer stem cells need to be further validated in the future. It has been elucidated recently that circulating tumor cells have an association with EMT, and further knowledge

about these CTCs will enable early detection and better prognosis of the disease. Also, miRNAs are observed to have a regulatory role in the CSCs, which make them important targets in therapy for breast cancer. As more information about breast cancer stem cells is known, designing drugs for killing the therapy-resistant CSCs would become much simpler, thereby increasing the efficacy of treatment.

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Omics of Male Breast Cancer

13

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Abstract

Male breast cancer (MBC) is rarely diagnosed. However, it has relatively poor prognosis when compared to female breast cancer. Although the importance of genetic predisposition in MBC etiology has been considerably understood, a wide range of gene and protein alterations playing various roles in MBC carcinogenesis point at its polygenic nature rather than single gene inheritance. Therefore, more comprehensive approaches including omics study and related technologies are required to thoroughly manage this malignancy, though such studies have been scarcely performed in MBC.

Keywords

Male breast cancer • Genetics • BRCA1 • Hereditary cancer • Molecular marker • BRCA2 • EGFR • Her2

Introduction

Male breast cancer is a rarely diagnosed malignancy, but has a relatively poor prognosis. In addition to various environmental, structural, and lifestyle factors—such as obesity, alcohol and estrogen intake, radiation, and some occupational exposures—lots of genetic events, including predisposition in relatives, Klinefelter syndrome, and some gene disturbances particularly in BRCA gene family, play important roles in etiopathogenesis. Although many individual genetic mechanisms have been revealed on a large scale, more comprehensive approaches such as omics study are required to be investigated. In this chapter, omics studies about male breast cancer are briefly reviewed.

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Male Breast Cancer

Epidemiology

Breast cancer is rarely diagnosed in males. Of all breast cancers, less than 1 % is observed in males [1–3]. According to data from the American Cancer Society, estimated new breast cancer cases are 2,190 for males and 226,870 for females in 2012 (male to female ratio: 1/104) [4]. The disease emerges mostly in elderly men 60–70 years old [5, 6]. In middle Africa, higher incidence rates have been reported when compared with Western countries [7, 8].

Etiology

Most of the cases of male breast cancer (MBC) are sporadically observed [9]. Familial and genetic etiological factors such as Klinefelter syndrome will be represented in the section "Genetic Background of Male Breast Cancer."

It remains controversial whether the causes of gynecomasty other than Klinefelter syndrome increase the risk of MBC. However, microscopic findings of gynecomasty have been found as high as 40 % in the histopathological specimens of the subjects with MBC [10]. Indeed the relative risk of gynecomasty in MBC development was noted as 5.86 in the US Veterans Affairs database [11]. Furthermore, MBC has been diagnosed in three males taking the drug finasteride (used in benign prostate hyperplasia), of which the most frequent side effect is gynecomasty [12].

The other causes of gynecomasty, including obesity, thyroid disorders, marijuana usage, and external estrogen intake, have only a very weak relation to MBC. In fact, MBC has been found in only two of more than 17,000 patients taking estrogen for prostate cancer treatment [13, 14]. In addition, hepatic dysfunction can also lead to hyperestrogenemia, and theoretically MBC, though most patients with advanced hepatic failure cannot live long enough to suffer from MBC [15]. For instance, schistosomiasis is an endemic parasitic infestation and can cause hepatic failure and hyperestrogenemia [7]. In some individuals with prolactinoma, MBC was diagnosed, while testosterone levels were decreased. It is not clear whether very high levels of prolactin are an actual risk factor or not, because the data are merely based on some case reports [14, 16]. Transformation from premalignant to malignant tissue by the stimulation of hyperprolactinemia may be a presumptive mechanism [14]. Expression of prolactin receptor was reported as significantly higher in MBC patients than in those with gynecomasty (60 % vs 20 %, $p = 0.003$). Moreover, as a promising potential therapeutic approach, substances antagonizing the prolactin receptor have increased the effect of doxorubicin and paclitaxel in cellular models [16].

Androgens have a protective effect on the breast tissue because they inhibit cellular proliferation. It was supposed that a mutation in androgen receptor gene would be responsible in developing MBC, but such a mutation was not determined in the tumoral tissues of 11 patients [17]. In the meta-analysis published in 1993 by Sasco, an increased incidence for MBC was noted in the testicular anomalies leading to androgen deficiency, including orchitis, undescended testicle, and testicular trauma [18]. According to the US Veterans Affairs database, the relative risk of orchitis/epididymitis is 1.84 [11]. On the contrary, it was claimed that aromatase inhibitors used to treat MBC may lead to prostate cancer through elevating serum testosterone levels [16].

Radiation is a risk factor in men as in women. Cancer develops 12–36 years after exposure to radiation [19]. The exposure to over 50–100 cGy in childhood or adolescence to the chest, especially, increases cancer risk in both genders in a similar manner [6, 20]. The prevalence is increased in employees working at high-temperature ovens, in steel plants, and in places with low-frequency magnetic fields [14]. In such professions, testicular deficiency resulting from the elevated heat increases the cancer risk [21]. It was also shown that gasoline and the steam of combustibles could play important roles in the development of MBC [22].

Alcohol intake is found as a causative factor in MBC [6]. And in patients treated for a long time

with the drugs leading to hyperestrogenemia, including estrogens, digitalis, cimetidine, methyldopa, and spironolactone, the risk of breast cancer was found to be higher [23].

Obesity has become a social concern due to its increasing frequency in economically developed countries. Hence, elevated body mass index (>30 vs <25; RR = 1.79) was found to be related to MBC [24]. Obesity developed while younger than 30 years old is a risk factor in males as well as in females. The mechanism of developing cancer in obesity may be due to an increase of androgen to estrogen conversion in the abundant adipose tissue [6]. Perhaps in concert with obesity, physical activity was inversely related to this disease [24, 25].

Spinsterhood, Jewish individuals, the previous benign breast disease, delayed puberty, and hypercholesterolemia have also been proposed as causative factors [23]. The other risk factors are diabetes (RR = 1.30) and cholelithiasis (RR = 3.45) [11]. The risk factors responsible in development of MBC are shown in the list below.

Risk Factors in MBC

1. Gynecomasty and hormonal imbalances
   (a) Finasteride
   (b) Obesity
   (c) Thyroid disorders
   (d) Marijuana usage
   (e) External estrogen intake
   (f) Hepatic dysfunction
   (g) Schistosomiasis
   (h) Prolactinoma
   (i) Testicular pathologies
2. Radiation
3. Occupational environment
   (a) High temperature
   (b) Low-frequency magnetic fields
   (c) Gasoline
   (d) Steam of combustibles
4. Alcohol intake
5. Drugs leading to hyperestrogenemia
   (a) Estrogens
   (b) Digitalis
   (c) Cimetidine
   (d) Methyldopa
   (e) Spironolactone
6. Various disorders
   (a) Diabetes
   (b) Hypercholesterolemia
   (c) Cholelithiasis
7. Other risk factors
   (a) Spinsterhood
   (b) Jewish individuals
   (c) Previous benign breast disease
   (d) Delayed puberty
8. Klinefelter syndrome
9. Family history

Clinicopathological Features

Clinically, an MBC patient is presented with a unilateral, painless, and stiff subareolar mass, generally, and a discharge, on occasion [6, 16]. The lesion is frequently eccentric and somewhat irregular, and the discharge is bloody in 75 % of cases [6]. Nipple discharge may be an early symptom of noninvasive disease [16]. Nodal disease is widely observed, particularly in elderly patients, and may be a sign of advanced stage [6]. Axillary node metastases are more frequently observed in males [16]. Diagnostic tools are physical examination, mammogram, breast ultrasonography, nipple discharge examination, fine-needle aspiration, biopsy, estrogen and progesterone receptor tests, human epidermal growth factor receptor 2 (HER2) testing (by immunohistochemistry or FISH or by PCR), ploidy level and cell proliferation rate test (by flow cytometry, image cytometry, and Ki-67 tests), tests for gene expression patterns, and imaging tests [6, 16]. Estrogen and progesterone receptors (ER and PR) are positive in about three of four MBC subjects [6].

Of MBC cases, 96 % are carcinoma and 4 % are sarcoma [6]. Approximately 90 % of the cases are pathologically presented in infiltrating ductal carcinoma subtype [3, 14, 17, 26]. Inflammatory carcinoma and Paget disease of the nipple are found as well [26]. Lymphogenous and hematogenous metastases resemble those in female counterparts [5].

Prognosis

MBC survival is generally comparable to that of female cancers. In fact, in the report of the American Cancer Society, the numbers of estimated deaths due to breast cancer are 410 in males compared to 39,510 in females (male to female ratio: 1/96), and this rate is considerably close to that of estimated new breast cancer cases (male to female ratio: 1/104). The perception of relatively poorer prognosis for MBC might be due to later diagnosis predisposition [3, 27, 28].

Tumor size and lymphatic invasion are considered as prognostic parameters [5, 27, 29, 30]. The lymph node metastasis seems to be the most important one [14]. With nodal involvement, 5- and 10-year survival rates decrease from 90 and 84 % respectively to 65 and 44 % respectively, even as low as 14 % in terms of 10 years' survival in the cases with positive lymph nodes equal to or more than four [14, 31].

The other factors, which are being studied, are ploidy and S phase; ER, PR, and androgen receptor status and human HER2/neu gene amplification; BRST2; cyclin D1; bcl-2; p16; p21; Ki67; cytokeratin (CK) 5/6; CK14; p53; and epidermal growth factor receptor (EGFR) [32–34]. Kornegoor et al. reported that increased mitosis and higher grade correlated with high Ki67, HER2, p53, p21, and low PR and bcl-2 expression. According to their multivariate analyses, negativity in PR and accumulation of p53 were independent markers and correlated with poor prognosis [34]. Johansson et al. offered N-acetyltransferase-1 (NAT1) as a possible prognostic biomarker for MBC [35].

By means of molecular subtyping including ER, PR, HER2 and EGFR, CK5/CK6, CK14, and Ki67, it suggested that MBC could be divided into five prognostic subgroups [36]. According to the study, aggressive forms of MBC had a combination of (ER + and/or PR+, and HER2+ or Ki67 high/luminal B), and in addition to this combination, a combination of (ER + and/or PR+, and HER2− and Ki67 low/luminal A) represented a large part of MBC [36]. Prognosis was found to be better in overexpression of apolipoprotein D and male breast tumor-associated antigen (MBTAA) [37].

Current Treatment Options

The treatment for MBC is not stationary and can vary according to clinical stage, pathological grade, and genetic features, though the main remedy is surgery [14]. The other options used as adjuvant and neoadjuvant approaches are radiotherapy, chemotherapy, hormonal therapy, and their combinations [14]. Orchiectomy and medical castration with LHRH analogs and antiandrogen treatments are other choices [6]. Targets of drugs used for MBC include estrogen receptors in tamoxifen from selective estrogen receptor modulators (SERM), aromatase enzyme in letrozole from nonsteroidal aromatase inhibitors, and DNA replication in anthracyclines and microtubules in paclitaxel [38]. Lapatinib, a tyrosine kinase inhibitor, and trastuzumab, a monoclonal antibody, can be used in HER2 gene (HER2/neu or ErbB2) positive MBC cases [38].

In BRCA-deficient patients, drugs targeting poly(ADP-ribosyl)ation polymerase (PARP) were suggested to be used. Because PARP complements BRCA-related repair processes, with the therapy of olaparib—a PARP inhibitor—promising responses were obtained with tolerable side effects in a heavily refractory population [16].

To optimize treatment and to avoid the adverse effects of the drugs in cancer generally and MBC herein, biomarkers and molecular pathways in the background of the disease should be discovered; thus, pharmacogenomics and targeted approaches should be popularized. Another topic on which studies should be done is phytochemistry to reduce unfavorable outcome of cancer therapy [38].

Genetic Background of Male Breast Cancer

Genetic predisposition is suggested in up to 10 % of cases (5–10 % of all cases) [6, 39]. According to the National Institute of Health (NIH)-AARP Diet and Health Study, having a first-degree relative with breast cancer increased the risk of disease development (RR = 1.92) [24]. In a study investigating familial features of the males with breast cancer, in families with MBC, female breast cancer or ovarian cancer is reported at a rate

of 30 % [40]. The risk of breast cancer increases by two to three times in the sisters and daughters of patients with MBC [41]. In one study, MBC has been reported in both brothers, with one of those also having prostate cancer [13]. Breast cancer probability increases in subjects having female relatives with breast cancer [5].

The genes which are suggested to be related to MBC include BRCA1, BRCA2, HER-2/ERBB2, P21/Waf1, P53, MYC, AR, CYP19A1, ESR1, PGR, PPM1D, ZNF217, CCND1, KRAS, CHEK2, MMP2, MMP9, CYP17A1, PCNA, PTEN, HFE, MSH2, MLH1, PMS1, and PMS2 [38]. These genes generally play important roles in MBC pathogenesis in groups rather than individually [38]. The mutations were reported in BRCA1, BRCA2, CHEK2, P53, PTEN, CYP17A1, HFE, MSH2, MLH1, PMS1, PMS2, AR, KRAS, and CYP17A1; upregulation in BRCA2, HER-2/neu or ERBB2, ESR1, PGR, MYC, PPM1D, ZNF217, CCND1, P53, CYP19A1, MMP2, MMP9, P21/Waf1, and PCNA; and downregulation in AR and BRCA1 [38].

BRCA1 and BRCA2 mutations can play an important role in etiology [8]. The overall rate of BRCA1/2 mutation in MBC families was reported as 34 % [16]. BRCA1 has been recently isolated. It is a tumor suppressor gene and localized in 17q. With a germline mutation of this gene, the breast cancer risk increases. The accumulation of BRCA1 occurred by hereditary mutations is responsible for hereditary breast cancer at a rate of 100 %. In this type, the disease arises in earlier ages. Almost 1,200 mutations have been described to date.

In families with BRCA2 mutation on 13q, MBC has been found as increased [42, 43]. They are with an autosomal dominant transmission and related to some extent to familial properties. For BRCA2 gene, nearly 900 mutations have been described. BRCA2 mutation in the MBC cases without family history is an unlikely event [40]. The breast cancer subjects with BRCA2 mutation have generally similar prognostic features compared to those without mutation, though nuclear grade tends to be higher and the frequency of p53 mutation is increased in those with mutation [40]. Of MBC patients, about 10 % have BRCA2 mutations versus BRCA1 mutations, which are less frequent [44]. It has been demonstrated that MBC is more related to the BRCA2 gene in the studies. It has been reported that BRCA2 is more related to hereditary breast cancer compared to BRCA1. In fact, in familial MBC cases, BRCA1 and BRCA2 are found at the rate of 19 and 77 %, respectively [45].

In contrast, although BRCA3 has been studied, conclusive data are not available. Remaining genetic factors may arise from a polygenic model, which should be further studied [46]. Except for BRCA1 and BRCA2, other factors with increased expression levels are proto-oncogene C-erb-2, tumor suppressor gene p53, cyclin D1, epidermal growth factor receptor, bcl 2, CHEK2 and PTEN mutations, and CYP17 polymorphism [6, 14, 44, 47]. As predictive values, kinase inhibitor proteins p27Kip1 and p21waf1 have been found in MBC [48]. An important relation was observed among CD31, CD34, and CD105 markers and microvascular density in MBC [49].

The expression of both survivin and COX-2 was reported in a large amount of the patients (69 and 36 %, respectively). As known, survivin, an apoptosis inhibitor, may be responsible for resistance to HER2-directed therapies, and COX-2 metabolites may enhance the tumoral angiogenesis and suppress antitumoral immunity [16]. Additionally, it was reported that vitamin D receptor (VDR) polymorphisms did not increase breast cancer in men [50].

Klinefelter (XXY) syndrome [7] is characterized by rudiment gonads, gynecomasty, aspermatogenesis, and elevated FSH levels. The highest risk of MBC is seen in Klinefelter syndrome. Indeed, relative risk was found as 29.64; in other words, the patient with this syndrome has increased risk up to 50 times compared to the male with normal genotype [11, 17, 51]. Hypertrophy in the breasts of these males resulted not only from gynecomasty but also from the acinar and lobular development [20]. There is a marked hyperestrogenemia in the patient with Klinefelter syndrome [14].

Biomarker studies have argued that breast cancer arises as different diseases in females and males. In fact, Shaaban et al. and Kornegoor

et al., by using molecular determinants, such as ER, PR, HER2, Ki67, and CK5/CK6, showed that breast cancer phenotypes were different between genders [52]. Genetic factors and related molecules which have been studied in MBC are shown in Table 13.1.

Due to genetic diversity in MBC, and the paucity of specific markers and standardized treatment modalities, novel diagnostic and therapeutic approaches should continue to be developed. Omics and related technologies should become crucial at this point.

Table 13.1 Genetic factors and related substances studied in MBC

BRCA 1	CD105
BRCA 2	Survivin
HER2 gene (*HER2/neu or ErbB2*)	COX-2
MYC	Ki67
ER	Tropomyosins
PR	NAT1
BRST2	RAD51B (14q24.1)
AR	TOX3 (16q12.1)
C-erb-2	rs13387042 (2q35)
p53	rs10941679 (5p12)
p16	rs9383938 (6q25.1)
p21	rs2981579 (FGFR2)
P21/Waf1	rs3803662 (TOX3)
p27Kip1	rs6504950 (COX11)
ESR1	CCND1 (11q13)
PGR	TRAF4 (17q11)
PPM1D	CDC6 (17q21)
ZNF217	MTDH (8q22)
CCND1	Tropomyosin-1
KRAS,	Cathepsin D
MMP2 and MMP9	Galectin-1
HFE	Enolase1
MSH2	MSH6
MLH1	CDH13
PMS1	PAX5
PMS2	PAX6
Cyclin D1	WT1
EGFR	GATA5
Bcl 2	GSTP1
PCNA	HOXD10
Apolipoprotein D	VEGF
MBTAA	HSP27
CHEK2 (*Li-Fraumeni syndrome*)	PDI
PTEN (*Cowden syndrome*)	PPIA
PARP	Prx1
CK 5/6, 8, 18, 19 and 14	MicroRNAs
CYP17A1 and CYP19A1	Hypermethylation
CD31 and CD34	

Adapted from Refs. [6, 8, 14–16, 32–34, 36–38, 40, 42–45, 47–49, 58, 61, 62, 64, 67–75]

Omics and Related Subterms

With this neologism, a set of terms with -omics suffix are implied in fact. The best known samples include genomics, proteomics, and metabolomics. Omics aims to reveal a holistic approach to genome and proteomes [53]. According to Merriam-Webster, the medical definition of genomics is “a branch of biotechnology concerned with applying the techniques of genetics and molecular biology to the genetic mapping and DNA sequencing of sets of genes or the complete genomes of selected organisms using high-speed methods, with organizing the results in databases, and with applications of the data (as in medicine or biology).” The medical definition of proteomics is “a branch of biotechnology concerned with applying the techniques of molecular biology, biochemistry, and genetics to analyzing the structure, function, and interactions of the proteins produced by the genes of a particular cell, tissue, or organism, with organizing the information in databases and with applications of the data (as in medicine or biology)” [54].

Genetic sequences and even fully genetic blueprints can regrettably give, to a certain extent, information about the functional and dynamic processes related to physiological and pathological situations. Therefore, the need for genetic studies intensely remains, even though the Human Genome Project (HGP), completed in 2003, tried to reveal identifying all genes up to 22,000 in human DNA, determining the sequences of the three billion base pairs in human DNA, and storing and improving all this data [55]. Thus, no information about genetic productivity, functionality, and final protein metabolism can be obtained by looking at a

Table 13.2 Omics progress

	Genomic age		Postgenomic age
Terms	Gen	→	Genomics
	mRNA	→	Transcriptomics
	Protein	→	Proteomics
Analyses	Structural	→	Functional
	Static	→	Dynamic
	Individual	→	Global

genetic sequence. For especially polygenic diseases and conditions such as aging, diabetes mellitus, cardiovascular disorders, and cancer, more functional and global approaches should be developed. Thereupon following HGP, we have entered into the postgenomic age, in which functional studies rather than structural ones and dynamic studies rather than static ones will gain importance (Table 13.2).

Indeed, proteomics make a global description of proteins possible, yielding the new useful markers like oncomine platform in more accurate diagnosis and exact prognostic designation of certain important diseases such as malignancies [30, 56, 57]. Proteomics is also useful to reveal postgenomic events, which play important roles in tumoral phenotype [57]. Tumoral behavior will be understood much better by advanced mass spectrometry and proteomics studies.

Omics and Male Breast Cancer

Unlike female breast cancer, the prevalence of protein alterations in MBC is scarcely known [58, 59]. The proteomics studies can lead to exploring original modalities of this disease from diagnosis and treatment to prognosis [58].

It is supposed that breast cancer is a very different disease between genders. This difference can result from a particular genetic background and thereby lead to different clinical approaches modified as to this fact [59]. Indeed, genetic identification is very important in revealing gene function and so in improving medical treatment [60].

Barh and Das investigated sophisticated interactions among 25 genes involved in MBC pathogenesis by using a broad bioinformatics approach [38]. These genes were BRCA1, BRCA2, HER-2/ERBB2, P21/Waf1, P53, MYC, AR, CYP19A1, ESR1, PGR, PPM1D, ZNF217, CCND1, KRAS, CHEK2, MMP2, MMP9, CYP17A1, PCNA, PTEN, HFE, MSH2, MLH1, PMS1, and PMS2 [38]. In this study, the authors found that these genes were involved in ER and EGFR signaling, in addition to DNA repair pathways in MBC pathogenesis [38]. MYC genes found as downstream target of the pathways were regulated by TNF, EGF, TGF, and estrogen through ERBB2, and estrogen was the major regulator of these MBC pathways [38]. Additionally, any abnormal AR expression had to affect all three critical pathways according to their key node analysis [38]. With their results, the authors claimed that a pharmacogenomics approach will be more effective than conventional treatment modalities due to the broad genetic variability among MBC patients [38]. Chemopreventive dietary and some phytochemicals targeted on the individual genetic event, such as inducing apoptosis or arresting tumoral proliferation which has not had adverse effect on normal cells, can be safer treatment options [38]. Within this framework, the authors stated that some phytochemicals including resveratrol, indole-3-carbinol, glyceollins, genistein, lycopene, tangeretin, EGCG, curcumin, sulforaphane, retinoic acid, beta-carotene, grape seed extract, and dibenzoylmethane are effective on some key molecules such as ESR1, BRCA1, BRCA2, PTEN, beta-catenin, aromatase (estrogen), VEGF, TGF, MMP-2, MMP-9, NF-kappaB, AKT, EGFR, PKC, and HER2 to inhibit tumoral growth. Furthermore, in this interesting report, it was suggested that curcumin, resveratrol, ATRA, genistein, and EGCG could be used in all MBC types irrespective of their molecular profile because these five phytochemicals covered and targeted all genetic pathways, which makes them promising potential cancer drugs used singly or in combination with acceptable adverse effects [38].

In their report, Orr et al. performed a genome-wide association study (GWAS) of MBC and control cases and found that a single-nucleotide polymorphism (SNP) in *RAD51B* at 14q24.1 was significantly associated with male breast cancer

risk (odds ratio (OR) = 1.57) and clarified an association at 16q12.1 to an SNP within *TOX3 as well* (OR = 1.50) [61].

In another GWAS by Orr et al., SNPs showing a statistically significant association with MBC were rs13387042 (2q35) (OR = 1.30), rs10941679 (5p12) (OR = 1.26), rs9383938 (6q25.1) (OR = 1.39), rs2981579 (FGFR2) (OR = 1.18), and rs3803662 (TOX3) (OR = 1.48). They found that OR values between male and female breast cancer were different in terms of the following SNPs: rs13387042 (2q35), rs3803662 (TOX3), and rs6504950 (COX11), which points out the heterogenic nature of the disease and difference between genders [25]. These and similar studies strongly show the importance of GWAS in identification of renewed risk loci in MBC, which represents new perspectives on the molecular background of this malignancy [25].

Using multiplex ligation-dependent probe amplification (MLPA), copy number gain of the genes CCND1 (11q13), TRAF4 (17q11), CDC6 (17q21), and MTDH (8q22) was found as important in MBC carcinogenesis and as higher than 40 % in male breast cancer. Copy number gain of one or more genes was related to highly malignant phenotype [62]. In this study of Kornegoor et al., independent prognostic parameter, namely, the most important single gene in prognosis, was CCND1 amplification in MBC [62].

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry can be used in the determination of differential protein expression, leading to new biomarkers in malignancies [16]. Underexpression of tropomyosin-1, a tumor suppressor, and alterations of cathepsin D and galectin-1, mediators of cellular invasion, and metastasis were reported [16]. Also, using comparative genomic hybridization (CGH), gains were most frequently observed at 1q, 8q, and 16p, and losses were most frequently observed at 8p, 16q, and 13q [63].

Hypermethylation is an important epigenetic change accompanied by genetic events. Promoter hypermethylation, which is a notable gene-silencing mechanism, may be an earlier event in cancer development [64]. The reversible nature of this mechanism makes it a suitable therapeutic target and useful marker in monitoring the response to treatment and prognosis [64]. Indeed, methylation can play physiological roles in genomic imprinting and many malignant processes [64]. Kornegoor et al. studied methylation of some tumor suppressor genes in MBC by using the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) [64]. In their study, in more than 50 % of tumoral samples, there was promoter hypermethylation in MSH6, WT1, PAX5, CDH13, GATA5, and PAX6, while in normal tissues, there was uncommon or absent. Methylation was correlated with poor survival. Thusly, methylation was significantly correlated with high grade ($p = 0.003$) and an independent predictor of poor survival ($p = 0.048$). Hypermethylations of ESR1 and GSTP1 were found to be associated with high mitotic count ($p = 0.037$ and $p = 0.002$, respectively) and high grade (both $p = 0.001$). Methylation was less frequently observed in MBC than in female breast cancer in some genes, especially ESR1 ($p = 0.005$), BRCA1 ($p = 0.010$), and BRCA2 ($p < 0.001$). The most commonly methylated genes (MSH6, CDH13, PAX5, PAX6, and WT1) were shared by male and female cancer types. The authors concluded that promoter hypermethylation indicates poorer prognosis in MBC and has different characteristics between genders [64].

In the study by Chahed et al. in 2008, a first application of proteomics performed for evaluating protein alterations in male breast tumors, two-dimensional gel electrophoretic analyses (2-DE) coupled with mass spectrometry was used to identify protein alterations in infiltrating ductal carcinoma (IDC) of the male breast [58]. The authors found some alterations in the expression of cytokeratins 8, 18, and 19. Cytokeratins (8, 18, and 19) and tropomyosins (Tms) are structural proteins, and they may have an important role in diagnosis and prognosis. Cytokeratins 8 and 18 are related to increased migration and invasion in several breast tumor cell lines [65], while cytokeratin 19 levels are found to be higher in micrometastases of breast cancer [66]. Tropomyosin1 found to be underexpressed in cancer tissues in Chahed's study is known to play a role in suppression of the malignant phenotype, suggesting an important role in male breast carcinogenesis. Chahed et al. found the simultaneous

downregulation of tropomyosin 1 and upregulation of tropomyosin 4 in MBC [58].

There is a co-upregulation of molecular chaperones (heat shock protein [HSP27] and protein disulfide isomerase [PDI]), and stress-related proteins (peroxiredoxin 1 [Prx1] and peptidylprolyl isomerase A [PPIA]) in male breast tumors. These findings related to chaperone activity may show a stress response during malignant transformation [58, 67]. Increasing Prx1 and PPIA may provide protection against oxidative damage for tumor cells, due to their antiapoptotic and antioxidative feature [68, 69]. In a similar way, HSP27 may also have protective properties against oxidation and involve multidrug resistance in malignant cells [58]. HSP27 is related to high probability of metastasis as well [70]. Enolase1 was also found to be increased in the study. This enzyme is important in the glycolytic pathway and represents increased energy needs during carcinogenesis [71]. Cathepsin D is a factor related to invasion, and galectin-1 is related to cell-extracellular matrix adhesion, tumor progression, and metastasis [72]. hnRNPs, a group of proteins functioning in mRNA processing and telomere maintenance, were found as upregulated, and the authors proposed hnRNPs as a potential molecular target in MBC [58].

MicroRNAs (miRNAs), which are a recently described nonprotein coding RNAs modulating pleiotropic functions, expand our horizons about explaining tumoral development and progression [16, 73, 74]. With miRNA researches, novel molecular mechanisms have been suggested and new diagnostic markers and potential therapeutic targets exposed [73]. The studies focused on miRNA analyses in MBC are very scarce.

In a study investigating the role of miRNA on male (for benign and malignant tissues separately) and female breast cancers, of miRNAs analyzed, 17 had increased expression and 26 decreased in MBC as compared to benign gynecomastia tissues. In addition, of 17 miRNA genes analyzed in male and female breast cancer tissues, 4 were upregulated and 13 downregulated in males as compared to females [75]. In this first study on miRNA analyses in MBC, quantitative real-time polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) analyses were used to reveal the outcomes of miRNA expression. In this study, expressions of HOXD10 and VEGF were found to be higher in MBC specimens. As known, miR-10b, one of the downregulated miRNAs in the study, suppresses the expression of *HOXD10* which is important in cell migration and extracellular matrix remodeling. In addition, miR-126, another downregulated substance in this study, suppresses the expression of vascular endothelial growth factor (VEGF) which is important in tumor-related angiogenesis [75]. The authors concluded that miRNA gene expression profiles were significantly different between MBC and benign tissues, suggesting the important role of miRNAs in MBC development, though gynecomastia was taken as benign male breast glands in their study. These findings seem to lead to novel VEGF-related therapeutic approaches [75].

Lehmann et al. described the identification of differentially expressed miRNAs in human MBC by using fluorescence-labeled bead methodology [73]. They found that MBC samples could be differentiated from control ones according to their miRNA expression pattern and that miR-21, miR519d, miR-183, miR-197, and miR-493-5p were identified as most prominently upregulated and miR-145 and miR-497 as most prominently downregulated in MBC [73]. In addition, the authors pointed to the important differences between male and female breast cancer with their findings. In brief, a large number of protein alterations were shown in their proteomics study. In a single work, multidimensional assays could be performed, and many proteins attendant in cellular mechanisms, including heat shock proteins, cytoskeleton proteins, antioxidative enzymes, proteins related to invasion and metastasis, and intracellular signaling proteins, could be analyzed by means of omics and proteomics technology [58]. These outcomes suggest the importance of omics methods in all aspects of the cancers and MBC herein once again.

Conclusion and Future Perspective

Male breast cancer is a very rare malignancy but with relatively poor prognosis. Because of the familial factors in etiology, genetic predisposition

is most likely to occur in this disease. Indeed, the fact that many gene and protein alterations have been described demonstrates its polygenic nature. Overcoming such a malady would be possible by more integrated approaches like omics study and technologies, though omics and relevant studies have been scarcely performed in MBC. Nonetheless, these scarce studies in this area promise prudential hope.

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14 Omics of Chemoresistant and Metastatic Breast Cancer

Margarita Aguilera and Juan Antonio Marchal

Abstract

Advances in research and knowledge, especially in the two last decades, have elucidated key pathways involved in the pathobiology, heterogeneous phenotype, molecular classification, risk, diagnosis, prognosis, and treatments for breast cancer. In spite of these advances, breast cancer still remains one of the major causes of death in developed countries. While advances in personalized medicine have allowed for management of the disease within a large and diverse number of clinical cases, and targeted treatment approaches that are less aggressive and more effective have been an important development, chemoresistance and metastasis are still the principal unsolved biological pitfalls of breast cancer. These areas deserve special attention from biomedical researchers. Thus, this chapter summarizes and highlights important analyses with regard to omics technologies, genomics, epigenetics, pharmacogenetics, transcriptomics, and metabolomics that integrate data and elucidate causes and putative solutions within the complex biological system that frames the recurrence status of the breast cancer patient. The most important milestones have been the discoveries of specific gene expression signatures, specific pathways, and miRNAs. In this context, some recent hypotheses about breast cancer stem cells or initiation tumor cell theories have gained a foothold. Nevertheless, greater efforts and higher-throughput investigations will be necessary to overcome many fundamental obstacles in the remission of the disease.

Keywords

Breast cancer • Omics • Chemoresistance • Metastasis • Biomarkers

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Introduction

Heterogeneity defines the complex pathobiology of breast cancer. Consequently, it has been possible to investigate, analyze, and explain the multiple characteristics of its biology, diagnosis, prognosis, treatment, remission, recurrence, metastasis, relapse, and individualized medicine management [1, 2].

Although one million women worldwide are diagnosed with breast cancer every year, big challenges have been met in the last decade due to complete genome sequencing. Moreover, there exists an International Cancer Genome Consortium (ICGH) that is devoted to cancer genetics data [3], specifically mutation obtained by genomic, epigenomic, and transcriptomic studies. However, they are still a long way from managing the complexity of present and future biomarker data and being able to use these data at a clinical level with relevance and impact on diagnosis, prognosis, and treatments so that this disease becomes a chronic controlled disorder. While the development of personalized medicine and pharmacotherapy for breast cancer patients has substantially improved survival rates—allowing for a cure in up to 70 % of new breast cancer cases worldwide—it is necessary to highlight the chemical resistance and metastasis that are the two major and uncontrollable problems present in some cases. These problems are worthy of scrutiny as they cannot be addressed by new advanced therapies and can result in a rather bleak prognosis for the patients concerned. Postgenome era should also be targeted at both population and individual levels to develop novel risk assessment and prevention [2].

Omics technologies have contributed to the immediate challenge of learning more about using the molecular characteristics of an individual to improve detection and treatment and ultimately to prevent the development of breast cancer [4].

A major achievement has been the molecular classification and molecular pathogenesis that should not be analyzed independently of global clinical data. The classification has been based on variations in global gene expression patterns, using complementary DNA microarrays. Thus, individual cancers could be categorized, based on their gene signature, into at least six distinct subtypes: luminal A, luminal B, normal-like, HER2-like, basal-like, and unclassified (Table 14.1). Normal-like tumors resemble normal breast tissue, HER2-like are characterized by HER2 overexpression, luminal A and B are estrogen receptor positive, and basal-like are triple negative (estrogen receptor negative, progesterone receptor negative, and HER2 negative) and a group of an unclassified type, not corresponding to any of the previous groups described [5].

One of the most aggressive phenotypes that present chemoresistance and metastasis is the triple-negative breast carcinomas (TNBC), characterized by the absence of ER, PR, and HER2 expression and with a poor prognosis. It is epidemiology relevant because of its prevalence in younger patients (<50 years) [6].

Omics technologies have contributed toward finding and establishing important molecular aspects of the pathogenesis involved in another key area: the BRCA1 connection, apoptosis resistance, proliferation, epithelial-mesenchymal transition, angiogenesis, and microRNA expression [5, 7].

It is important to highlight that host germline genetics may determine the tumor subtype, and this has been demonstrated because of the presence of BRCA1-associated breast cancers cluster within the basal-like subtype [2]. Moreover, the assessment of those women with inactivating germline mutations in BRCA1 or BRCA2 shows that they have up to an 85 % chance of developing breast cancer during their lifespan [8].

The translation from the bench to bedside of the clinical molecular findings gives power to gene expression signatures (GES) that can become specific and validated biomarkers used by the FDA and other international expert panels. These markers will shed light on prognostic responses to breast cancer treatments, for example, mutated HER1/HER2, BRCA1/BRCA2 genes, etc. Moreover, new, targeted therapies will have had their fundamental basis in the specific molecular profiles and pathways discovered [9].

However, the complex nonlinear relationship between the driver mutations and the phenotype of individual patients introduces difficulties in developing a next generation of biomarkers and therapeutic biological agents [10].

In spite of the great advances in overcoming these difficulties, in reality there is a lack of robust and validated biomarkers for predicting the responses to specific therapies. Moreover, the

Table 14.1 Fingerprint gene of types of breast cancer

	Luminal A	Luminal B	Basal-like or TNCB	HER2/ErbB2	Normal-like	Claudin-low
Genetic signature	Luminal A (estrogen receptor positive and/or progesterone receptor positive, HER2 negative)	Luminal B (estrogen receptor positive and/or progesterone receptor positive, HER2 positive)	Basal-like (estrogen receptor negative, progesterone receptor negative, HER2 negative, cytokeratin 5/6/17 positive, and/or epidermal growth factor receptor positive)	HER2-like (estrogen receptor negative, progesterone receptor negative, HER2 positive)	Gene expression of adipose and other nonepithelial genes, high basal-like and low luminal gene	Unclassified (negative for all five markers)
	HER2 –, *ER+*, *Ck5/6*, *and EGFR +/–*	*HER2 +*, *ER+*, *Ck5/6*, *and EGFR +/–*	*HER2 –*, *ER –*, *Ck5/6*, *and/or EGFR +*	*HER2+*, *ER+/–*, *Ck5/6*, *and EGFR +/–*	*NA*	*ER –*, *HER2 –*, *Ck5/6 –*, *and/or EGFR -*
Clinical outcome	Most favorable prognosis	Low response	Worst prognosis and high incidence of metastasis	Shortest relapse-free and overall survival	Intermediated prognosis	NA
Treatment response	Low response to chemotherapy previous to surgery	Low response to chemotherapy previous to surgery	High complete response to chemotherapy previous to surgery	High complete response to chemotherapy previous to surgery	Low response to chemotherapy previous to surgery	NA
			Low response to tamoxifen and aromatase	Targeted therapies		

NA not applicable

discovery of key targets for the development of effective drugs has not yet been achieved. For this reason, systematic studies of more than 25,000 cancer genomes at omics (genomic, epigenomic, transcriptomic, etc.) levels will reveal the catalogue of oncogenic mutations and enable the development of new cancer therapies. The aim is to link the global omics data with clinical data and establish algorithms for the classification of risk, prognosis, and therapy, as performed already by Glinsky [1], a stemness cancer therapy outcome predictor (CTOP). The approach in this new era of knowledge requires a multidisciplinary management of breast cancer patient healthcare.

Genetics in Breast Cancer

A short panoramic view of known genetics in breast cancer is necessary to understand the complexity of the theme developed in this chapter. Thus, familial breast cancer comprises approximately 20–30 % of all breast cancers. Currently, mendelian approaches or empirical data models can be used in clinical practice for risk prediction of breast cancer in order to facilitate genetic counseling.

Family linkage studies have identified the high-penetrance genes, BRCA1, BRCA2, PTEN, and TP53, that are responsible for inherited BC syndromes (Table 14.2). Moreover, a combination of family-based and population-based approaches has indicated that the genes involved in DNA repair, such as CHEK2, ATM, BRIP, and PALB2, are associated with moderate risk [11]. However, not all familial breast cancer can be linked to a single gene. Susceptibility alleles in other genes, such as *PTEN*, *ATM*, *STK11/LKB1*, and *MSH2/MLH1*, are also rare causes of inherited breast cancer. The majority of women with sporadic or nonhereditary breast cancer have common low-penetrance genetic risk variant genes such as *FGFR2*, *TNRC9*, *MAP3K1*, *LSP1*, *2q35*, *5p12*, and *8q24* of the eight variants discovered thus far and, together, account for 60 % of breast cancer in the general population of women of European ancestry [2].

Table 14.2 Familial genes in hereditary breast cancer

Gene	Percentage
BRCA1	20
BRCA2	20
TP53	<1
CHEK2	5
Emerging genes: CASP8, MAP3K1, LSP1, TNRS9	5
PTEN, ATM, STK11/LKB1, BRIP1, PALB2, NBS1	<1
Unknown candidate genes	50

Molecular biology and gene expression signatures of breast cancer are critical for developing novel approaches to the prevention and healthcare management of this disease. Despite intensive work already developed, new molecular pathways should have to be identified to further contribute to the understanding of breast cancer genetics.

Omics Applications Toward Chemoresistance in Breast Cancer: "Chemoresistome"

Omics discoveries are transforming the traditional approaches to breast cancer management. However, to date, there are no validated sensitivity and/or resistance predictive factors available in clinical settings, and the mechanisms involved in cancer cell chemoresistance are still largely unknown. All the biomarkers included in this category will be referred to as "chemoresistome" for identifying present and future key molecular marker determinants of resistance to diverse therapies.

Up to now, multiple molecular pathways may contribute to the sensitivity/resistance of cancer cells to chemotherapy. Specifically, the mechanisms that may contribute to chemotherapeutic resistance in breast cancers could include decreased intracellular drug concentrations, mediated by drug transporter's overexpression and metabolic enzymes; antiapoptotic factors and DNA repair; cell cycle deregulation or arrest by impaired cellular responses; the promotion of progression of cancer cell populations; perturbations in DNA methylation and histone modifications; and alterations in the availability of drug targets [12, 13].

Moreover, tumor heterogeneity may also contribute to resistance, where small subpopulations of cells may acquire or stochastically already possess some of the features enabling them to emerge under selective drug pressure.

The present chapter has identified the main omics approaches pursued by several authors that can explain key factors involved in the chemoresistance of breast cancer (Table 14.3). In this context, the most important aim will be to validate those biomarkers for use at a clinical level.

Genomics in Breast Cancer Chemoresistance

While the genomic predictors are of current interest, the evolution of patients' characteristics and treatments requires a permanent optimization. Several studies have attempted to report these genomic predictors. Frati et al. [14] established a genetic profile involved in the chemoresistance and extrapolated the drug sensitivity to breast cancer. ER+/HER−/low gene expression proliferation tumors are categorized as chemoresistant, whereas tumors with an ER−, HER2+, or ER+/HER2-/high proliferation gene expression profile can be categorized as chemosensitive tumors. Moreover, there is scientific evidence of chemoresistance in patients with ER+/HER2−/low proliferation tumors when treated with endocrine therapy, and evidence exists among oncologists to allow clinical use of gene expression tests to identify patients who do not require chemotherapy among node-negative ER+ patients [15]. A genomic predictor combining ER status, predicted chemoresistance, predicted chemosensitivity, and predicted endocrine sensitivity, identified patients with a high probability of survival following taxane and anthracycline chemotherapy.

Epigenetics in Breast Cancer Chemoresistance

Epigenetics and RNA-related markers are promising for the explanation of dysregulation cell pathways involved in cancer. Indeed, miRNA profiling has been convincingly demonstrated to classify tumor and non-tumor samples, sensitivity/resistance to drugs therapies, etc. Thus, evidence of miRNA-mediated reversal of multidrug resistance in human cancer [16] warrants further studies of miRNA-based approaches for treating drug-resistant tumors. Some specific results indicated that miRNA-125b played an important role in chemotherapeutic resistance in breast cancer cells, specifically in primary breast cancer cells. Experiments were carried out on breast cellular models showing that the expression of miR-125b decreased 5-FU-induced cytotoxicity and increased 5-FU resistance under various concentrations of 5-FU treatment in MCF-7 cells [17].

Pharmacogenomics in Breast Cancer Chemoresistance

It is well known that treatment efficacy is optimal for one certain genotype group, while a similar drug is most efficacious for another genotype group, so that therapy can be personalized to achieve maximum success in patient care. If a polymorphism is identified to be associated with drug dosing, physicians can change clinical care using genotype in the dosing algorithm. Likewise, if a polymorphism is associated with a serious adverse event, an alternative treatment could be selected for such patients [18].

The aim of pharmacogenetics and the main targeted drug therapy of breast cancer are to determine whether there is a correlation between genetic polymorphism, such as in targeted molecular structures, and response to treatment or the development of drug-associated toxicity [19, 20].

Table 14.4 compiles the genes that have shown significant variability according to each drug that can be prescribed in breast cancer. These data have been obtained through www.pharmagkb.com database, which stratify the validated SNP according to clinical impact and outcome, FDA mandatory label, and other parameters. The majority of the genes are related to transporters, CYP450 alleles, and specific targeted therapies.

Table 14.3 "Chemoresistome and metastasisome in breast cancer": omics methodologies for chemoresistance and metastasis

Omics methodology applications	Key short definitions	Techniques	Biomarkers type	Databases	References	References
					Chemoresistome	Metastasisome
Genomics Genetic variability	Interindividual variability gene profiles	Affymetrix/Luminex PCR-RFLP	SNP, CNV	ICGC HAPMAP SNP	Chen et al. [4] Frati et al. [14] Kim and Paik [15]	Thomassen et al. [44] Dawson et al. [45]
Genomics Epigenenetics	Methylation pattern independent of primary DNA sequence	Immunoblot for histone hyperacetylation ChIP-Seq (specific epigenetic modifications	SCFA, HATs, HDACi	*Databases* and Tools EPIGENIE_*Epigenetics*	Kutanzi et al. [12] Fang et al. [26]	Rodenhiser et al. [49] Samantarrai et al. [50] Bentires-Alj et al. [81]
Genomics Pharmacogenomics	Interindividual variability gene profiles in drug therapy	Affymetrix/Luminex TaqMan real PCR, KASPAR PCR-RFLP	Drug transporters Drug targets Pathways	Cypalleles Pharmagkb	Cascorbi and Haenisch [19] Cascorbi [20]	Hill et al. [48]
Transcriptomics	Gene expression profile	RNA microarrays; RT-PCR; Qpcr	Transcripts of RNA cDNA	H-DBAS – H-invitational *database*	Gröger et al. [29] MacCartan et al. [30] Flamant et al. [31]	Saunus et al. [42] Lorusso and Ruegg [56]
Proteomics	Determinations of protein differentially expressed	2-DE analysis, multidimensional, chromatography, LC-MS/MS, MALDI-TOF, SELDI-TOF	Proteins, peptides, oligopeptides	UniProt *Proteomics databases* | EBI	Chuthapisith et al. [33] Hortobagyi et al. [32]	Terp et al. [58] Niu et al. [59] Qin and Ling [82]
Metabolomics	Quantitative measurement of multiparametric metabolic responses	NRM analysis, MS, GC, HPLC CE/MS, FTIR analysis	Metabotype: lipids, sugars, vitamins, antioxidants	The Human *Metabolome Database* (HMDB)	Martinez-Outschoorn et al. [35]	Martinez-Outschoorn et al. [35] Tenori et al. [64] Oakman et al. [62]
Nutrigenomics	Correlated data between genomics and diets collections	Programmed diets/ sequencing	Metabolic enzymes; key diet parameters	*European Nutrigenomics Organization*	Saukko et al. [36] Riscuta and Dumitrescu [37] Corella and Ordovás [38]	Ozdemir et al. [65] Nieman et al. [67] Taniya et al. [83]
Lipidomics	Lipid global levels in clinical measurements	GC, HPLC	Lipid profile	*Lipid database*	Xue and Wong [39] Antoon et al. [40] Aluise et al. [41]	Nieva et al. [69]

Table 14.4 Drugs therapy for breast cancer and target genetic variability

Drugs	Chemical structure	Gene (PHARMAGKB)
Anthracyclines	Variable general structure	ABCB1 ABCB4 SLC28A1 SLC22A2 ABCC1 ABCB4 SLC28A3 SLC10A2
Doxorubicin FDA drug label at DailyMed: 102358 dc-2109-4829-b333-b99417334e39	–	ABCB1 ABCC2 ABCC4 CBR3
Antiestrogens *Tamoxifen* FDA drug label at DailyMed: 7ee3d3d2-85d1-4018-8e70-5ed8a64ae1f0	–	CYP2D6 CYP3A ABCC2 SULT1A1
Antiestrogens *Fulvestrant* FDA drug label at DailyMed: 83d7a440-e904-4e36-afb5-cb02b1c919f7	–	ESR1 ESR2
Aromatase inhibitors *Letrozole* *Anastrozole* FDA drug label at DailyMed: 11628cdc-4e3c-4063-ee9f-c51e2386a820	–	CYP19A1 GLDN CYP2A6
Docetaxel FDA Drug Label at DailyMed: 82731db6-92 fc-483b-9d73-9b2aed79b104	–	ABCB1 SLCO1B3 ATP7A ABCC6 SLC10A2 CYP1A1 GSTP1
Paclitaxel FDA drug label at DailyMed: 88d7cdd2-e650-4a16-adec-873927e03e93	–	ABCB1
Platin derivatives *Cisplatin* Therapeutic targets database: DAP000215	–	ABCB1 ABCC2 ABCC4 SLC31A1 SLC19A1 SLC22A2
Monoclonal antibodies	*Herceptin*	ERBB2
FDA drug label at DailyMed: 492dbdb2-077e-4064-bff3-372d6af0a7a2	Anti-HER2 Ig gamma-1 chain C region	FCGR2A FCGR3A
Clinical trials	*Lexatumumab*	
Monoclonal antibodies	Anti TRAIL-R1 *Mapatumumab* Anti TRAIL-R2	TRAIL-R1 TRAIL-R2

(continued)

Table 14.4 (continued)

Drugs	Chemical structure	Gene (PHARMAGKB)
Clinical trials	Inhibitors pathway	
New combined therapies	Hedgehog	SNAIL, GLI1
	Notch	Notch receptors
	WTN	NA
	IPARP	BRCA1/BRCA2
	Olaparib	

In the context of chemoresistance, among others, special attention should be paid to drug transporters, including ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters, which are also expressed at cancer cells.

The ABC transporters are a family of large proteins in membranes and are able to transport a variety of compounds including metabolites and drugs through membranes at the cost of ATP hydrolysis. Physiological functions of ABC transporters include the transport of lipids, bile salts, toxic compounds, and peptides for antigen presentation or other purposes, such as ion channel regulation. The human genome contains 48 ABC transporter genes; at least 14 of these are reportedly associated with heritable human diseases [21].

Commonly, polymorphisms in P-glycoprotein or ABCB1, ABCG2 have been widely described as affecting treatments of different diseases [22]. Moreover, the potential impact of ABCC11 genetic polymorphisms on the physiological function, breast cancer risk, and patients' response to nucleoside-based chemotherapy has been described [13].

An important discovery has been the breast cancer resistance protein (BCRP), whose expression in the liver is needed for in vitro to in vivo extrapolation of the biliary clearance of a BCRP substrate drug. A polymorphic variant C421A (rs2231142) allele was significantly lower in breast cancer patients than that in the wild-type livers [22]. Currently, there are no arguments to determine the pharmacogenetic status of HER2 to individualize trastuzumab treatment [9]. The searching of biomarkers of trastuzumab resistance are focused on several mechanisms—i.e., deficiency of phosphatase and tensin homologue and activation of phosphoinositide 3-kinase result in greater activity of the Akt-mammalian target of rapamycin signal transduction pathway [23]. Also, the overexpression of other surface receptors, such as insulinlike growth factor, provides alternative growth factor signaling and is related to lower trastuzumab sensitivity [24].

Genome-Wide Association Studies in Breast Cancer

Genome-wide associations (GWAS) based on large prospective randomized trials have the potential to greatly elucidate the genomic basis for optimizing drug efficacy and reducing toxicity. The studies on breast cancer have been mainly focused on how both disease susceptibility loci and risk prediction may lead to a better understanding of the biological mechanism for BC in order to improve prevention, early detection, and treatment. They are of clinical importance and may explain an appreciable proportion of the genetic variance in BC risk. There are few studies that specifically describe results affecting chemoresistance [25] and metastasis [26]. The power of GWAS may be increased by enlarging the number of samples in both the cases and the controls and by identifying clinical and molecular subtypes [27]. There is a long way to go toward improving the controversial results of different GWAs, and thus a novel multi-SNP GWAS analysis method called pathways of distinction analysis was developed. This method includes GWAS data and pathway-gene

and gene-SNP associations to identify pathways that could permit the distinction of cases from controls [28]. Thus, new GWAS in breast cancer generation of large-scale association studies, in combination with replication analyses and multiple scans, could have the potential to identify many more loci.

In order to further exploit the transcriptome and genome data, it is usual to carry out an integrated analysis of transcriptome, proteomic, and metabolomic data, observing how this corresponds to the varying physiological and phenotype status.

Transcriptomics in Breast Cancer Chemoresistance

One of the main aspects studied by transcriptomic studies is the epithelial to mesenchymal transition (EMT) that represents a crucial event during cancer progression and dissemination. EMT is the conversion of carcinoma cells from an epithelial to a mesenchymal phenotype that is associated with a higher cell motility as well as enhanced chemoresistance and cancer stemness [29]. This issue will be developed further in the discussion below on metastasis of breast cancer. Moreover, some specific markers have been determined to impact the chemoresistance on breast cancer patients treated with endocrine therapy. The steroid coactivator protein SRC-1 drives tumor adaptability through ADAM22, a non-protease member of the ADAM family of disintegrins [30]. Taxol and etoposide resistance have been demonstrated to be mediated by TMEM45A [31].

Proteomics in Breast Cancer Chemoresistance

Based on gene expression profiles, or proteomics of three or four validated biomarkers, it is apparent that there are multiple subtypes with different clinical characteristics, clinical courses, and sensitivities to existing therapies (ER, PR, HER2, and Ki-67 represent critical molecular markers that identify the largest molecular subsets and therapeutic decisions [32]). Current sensitivity levels may allow whole-cell proteomics approaches and subcellular fractions to now be sequenced at the protein level with success [33, 34].

Metabolomics in Breast Cancer Chemoresistance

Important molecules to be determined for classification are the metabolic enzymes and metabolism mediators that affect individually the impact of drug therapy [12, 18]. In particular, some authors have found important metabolism enzymes required for ketone body production that are highly upregulated within cancer-associated fibroblasts. L-Lactate and ketone body metabolisms are critical for tumor progression and metastasis [35].

Nutrigenomics in Breast Cancer Chemoresistance

There are nutrigenetic testing companies, which provide DNA-based nutritional advice analyzing the individual genome of patients or healthy people. The validity of biomarkers is not accurate in all cases and requires expert counseling [36]. Some authors have studied the impact of single nucleotide polymorphism, copy number, epigenetic events, and transcriptomic homeostasis as factors influencing the response to food components and breast cancer risk, among them the dietary n-3 fatty acids [37, 38]. There are also foods that can induce or inhibit the action of CYP450 enzymes involved in metabolism of drugs.

Lipidomics in Breast Cancer Chemoresistance

Lipid pathway analysis is acquiring importance in many clinical areas, as diet-controlled components, as metabolic mediators, and even as therapeutic molecules for breast cancer chemoresistance [39, 40]. Moreover, it is known that

oxidation of membrane phospholipids is associated with cancer. Oxyradical damage to phospholipids results in the production of reactive aldehydes that adduct proteins and modulate their carcinogenic function [41].

Omics Application Toward Breast Cancer Metastasis: "Metastasisome"

"Metastasisome" is the term used to denote all of the important molecular omics developments investigated by several authors with respect to breast cancer metastasis (Table 14.3). Metastasis is defined as the spread of cancer cells from a primary site resulting in the establishment of secondary tumors in distant locations [42, 43]. These steps for metastasis include escape from the primary tumor (intravasation), dissemination via the blood or lymphatic system, survival within the circulation, arrest and extravasation into a secondary site, initiation of growth into micrometastases, and maintenance of growth as vascularized, clinically detectable macrometastases.

Cancer initiation, progress, and metastasis are driven by mutations in genes that are key components in the signaling pathways. These so-called "driver" mutations deregulate signal transduction from outside the cell through cell receptors to the nucleus. This deregulation of cell proliferation, growth, cell death (apoptosis), and angiogenesis results in cancer development and metastasis. Breast cancer targeted therapy with the biological agent trastuzumab inhibits key signaling pathways responsible for cancer progression through drugs, cancer progression in the metastatic setting, and recurrence risk in the adjuvant setting [10].

Genomics in Breast Cancer Metastasis

Gene expression analyses of primary tumors have been used to predict metastatic propensity with high accuracy [44]. The detection of metastatic cells in circulating blood is an important area of research. In spite of the fact that cancer patients may have hundreds to thousands of single disseminated cancer cells detectable in the bloodstream and sites that are remote from the primary tumor [45], only a very small percentage progress to form overt macroscopic metastases.

Moreover, in the same direction, the integrated CSC hypothesis may be the most appropriate model to explain human tumor progression and particularly metastasis. EMT is associated with tumor progression in correlation with the loss of epithelial characteristics and the acquisition of a metastatic phenotype [46]. Tumors cannot form metastatic colonies unless these cells contain CSC properties. To incorporate the tumor metastasis model and CSC hypothesis, Brabletz [47] proposed a new concept of "migrating CSCs (mCSC)," which contain both stemness and mobility characteristics. The precursor metastatic cancer cells with stemlike properties are characterized by their ability to self-renew and to regenerate cell variants, which have high plasticity and intrinsic invasive properties required for dissemination and tropism toward specific organs. Several approaches have been developed for targeting precursor metastatic cells, and these have taken on greater priority in therapeutic drug discovery research by biomedical and pharmaceutical researchers.

Epigenetics in Breast Cancer Metastasis

Methylation processes in breast metastasis—microRNA and small RNA (metastamirs) [26, 48, 49]—are part of the epigenetics-miRNA regulatory circuit whose global deregulatory effect became a hallmark of different cancer types, including breast cancer and its metastasis [50].

Transcriptomics in Breast Cancer Metastasis

Numerous gene expression studies (GES) have been conducted to obtain transcriptome signatures and marker genes to understand the regulatory mechanisms underlying metastasis-related hypotheses such as EMT. The first report using a

meta-analysis of 18 independent and published GES of EMT focusing on different cell types and treatment modalities is presented by Gröger et al. [29]. They identified those up- and downregulated genes which were shared between the multiples of GES. During EMT, epithelial cells dissociate from each other, in part due to loss of E-cadherin expression; upregulate mesenchymal markers; acquire a fibroblast-like morphology; reorganize their cytoskeleton; and become more motile and invasive. Several transcription factors, including members of the SNAI family, have been shown to promote EMT and thus tumor dissemination [51].

Experimental approaches of differential gene expression through microarrays have been performed in order to analyze and validate genes, proteins, metabolites, and miRNAs that could be involved in organ-specific breast cancer metastasis [52].

The main organ-specific breast cancer metastases have been found in the bone, lung, and brain. In order to determine which genes lead to metastasis in which organs, the breast-to-bone, lung, and brain tropisms have so far been investigated in several in vivo studies [53–55]. The authors described a set of genes differentially expressed according to tissue tropism metastasis. Experimental analyses have demonstrated the role of different hypoxic gene response programs and that they have different dependence on the angiogenic response. Moreover, they have identified the physiological role of the metalloproteinases MMP1 and ADAMTS1 in breast cancer bone metastasis and uncovered a role for epidermal growth factor receptor inhibitors in targeting the reactive stroma in osteolytic metastasis. In lung metastasis, the combined effects of *COX2*, *EREG*, and *MMP1* and *2* were shown to promote primary tumor angiogenesis and extravasation of metastatic cells from the lung capillaries. There is a greater propensity of primary breast cancer with a basal-like immunophenotype to metastasize to the brain. Moreover, patients with germline BRCA1 mutations who develop breast cancer have a higher incidence of brain metastasis compared to germline BRCA2 carriers and non-BRCA1/2 patients [42].

Tumor Microenvironment and Breast Cancer Metastasis

Metastasis is an extraordinarily complex process, where the tumor microenvironment may promote tumor growth and possibly metastasis through invasive cancer cells that actively recruit stromal cells and interact with them. Some specific molecules have been shown to be important for this process. The expression of matrix metalloproteinases, MMPs, play a key role in the tissue remodeling associated with various physiological and pathological processes (e.g., morphogenesis, angiogenesis, tissue repair, chronic inflammation, rapid tumor growth, invasion, and metastasis) by degrading the ECM. Lymphovascular invasion (LVI) is related to the presence of lymph node metastasis and is a marker of poor prognosis. Mobilization of bone marrow-derived cells induced by primary tumor (PT) generates premetastatic niche (PMN), epigenetic regulation, and HGF-HDAC-CXCR-4 interactions related to invasive phenotype. Microenvironment of CSC is also actively investigated because of the impact on putative metastasis [56].

Signaling Pathways in Breast Cancer Metastasis

The metastatic signaling program could be driven by classic oncogenes or other well-known signaling cascades that can adapt to promote changes in metastasis-specific gene expression [52]. Clearly, well-known signaling pathways, such as the Src, H-Ras, E2F3, Myc, β-catenin, TCF/Wnt, and transforming growth factor (TGF)-β, are driving some components of breast cancer metastasis progression. Thus, in vivo analyses showed that *SATB1* was both necessary and sufficient to promote both lung metastasis and primary tumor progression. Microarray analysis of SATB1 signaling indicated remarkably penetrant gene expression changes, with significant regulation of multiple pertinent signatures, such as the 70-gene poor prognosis signature, and both the bone metastasis and lung metastasis.

The *Metadherin* gene (*MTDH*) also represents another novel mediator of malignant breast cancer progression with exciting, yet inconclusive, effects on breast cancer signaling.

Some biomarkers such as the cancer antigen 15–3 (CA 15–3) and circulating tumor cells have been widely studied. However, circulating cell-free DNA carrying tumor-specific alterations (circulating tumor DNA) have not been extensively investigated [45].

Secretory clusterin is a secreted glycoprotein that is upregulated in a variety of cell lines in response to stress and enhances cell survival. To be more specific, it is frequently upregulated in breast cancers by common therapies, including estrogens, and may play a significant role in tumor growth and metastatic progression [57].

Proteomics in Breast Cancer Metastasis

Proteomics is widely used in breast cancer metastasis studies with different approaches (murine model, CSC, patient tissues, etc.), all of which are concerned with researching validated biomarkers. Thus, Terp et al. (2012) [58] recently found key proteins correlating with the aggressiveness of metastasis rather than metastasis colonization per se: leucine-rich repeat containing 59 (LRRC59), while CD59 and chondroitin sulfate proteoglycan 4 (CSPG4). It was known that insulinlike growth factor 1 tyrosine kinase receptor (IGF-1R) and the chemokine G protein-coupled receptor, CXCR4, have been shown to play an important role in breast cancer metastasis. Moreover, a novel role has been found for PI3Kγ in facilitating cell migration and metastasis by regulating phosphorylation of eEF2 [59]. A glycoproteomic analysis [60] suggested the protein cadherin-5 as an emergent potential marker of metastasis, with the power to discriminate between patients with recurrent breast cancer from those with no sign of recurrence, with 90 % specificity.

In CSC studies, the protein ferritin heavy chain 1 (FTH1), which is involved in iron metabolism and iron depletion, had a significant effect in decreasing the self-renewal of CSCs. In silico analysis confirms that the FTH1 gene could represent a putative molecular target [61].

Metabolomics in Breast Cancer Metastasis

Metabolomics is an evolving field that will allow more accurate identification of patients with residual micrometastases. It can be applied to serum samples from women with metastatic breast cancer to explore outcomes and response to treatment. Thus, some authors have demonstrated that innate serum metabolomic differences exist between early and metastatic patients [62]. Specifically, some metabolites such as lactate and ketones were observed that promote the growth of bona fide ES cells, providing functional validation [35]. The metabolite markers for further investigating the role of CXCR4 in metastasis have been developed by Vermeer et al. [63]. In general, metabolomics plays a role in selecting patients with positive disease markers with greater sensitivity to specific drugs, e.g., HER2+ to paclitaxel plus lapatinib [64].

Nutrigenomics and Lipidomics in Breast Cancer Metastasis

Some dietary-related experiments have been carried out in menopausal women with metastasizing carcinoma of the breast in order to look for the effect of hyperlipoproteinemia additive administration (Primobolan) on lipid metabolism. Drug treatments exerted their effects in lung metastasis of breast cancer patients and had marked hyperlipoproteinemia with giant fatty liver, high plasma triglyceride levels, and increased levels of very-low-density lipoprotein (VLDL) and intermediate-density lipoprotein (UDL). Ozdemir et al. [65] suggested that hypercholesterolemia impairs angiogenesis and, therefore, lowers the risk of metastases in cases of invasive breast carcinoma. In general, hyperlipidemia is significantly associated with distant metastasis in breast cancer patients. Monitoring of the serum lipid profile may be helpful for predicting the occurrence of distant metastasis in breast cancer patients [66].

Obesity can contribute to cancer metastasis. The abundant availability of lipids from adipocytes

in the tumor microenvironment supports tumor progression and uncontrolled growth. Thus, many tumor types (breast, colon, renal, and ovarian) grow in the anatomical vicinity of adipose tissue. During their interaction with cancer cells, adipocytes dedifferentiate into pre-adipocytes or are reprogrammed into cancer-associated adipocytes (CAA) [67].

Conversely, good effects have been described. For instance, the mechanisms of calcitriol (vitamin D) have shown anticancer actions including cell cycle arrest, stimulation of apoptosis, and inhibition of invasion, metastasis, and angiogenesis [68]. Nieva et al. [69] have developed a promising technique for characterizing and classifying the malignant phenotype of breast cancer cells on the basis of their lipid profiling "Raman microspectroscopy." It constitutes a classificatory model for segregated metastatic cells and nonmetastatic cells without basal-like phenotype with a significant sensitivity and specificity.

Breast Cancer Dormancy and Recurrence

Disease recurrence originates from residual treatment-resistant cells, which regenerate at least the initial breast cancer phenotype. Residual cancer cells following administration of chemotherapy are termed cancer stem cells or tumor-initiating cells because of their ability to give rise to new tumor cells to the repopulating ability of treatment-resistant cells and are therefore responsible for relapse [70].

Tumor cell dormancy is also a major clinical concern. Highly aggressive recurrences can arise years or even decades after breast cancer symptoms have disappeared [71]. Very little is known about the molecular basis and mechanisms of dormancy, although it is known that single cells can lie dormant in bone marrow. Alternatively, small groups of cells lacking a proper blood supply can lie dormant in the parenchyma of visceral organs. Even with the huge support of omics technologies, the mechanisms underlying tumor dormancy in breast cancer remain poorly understood, and this represents significant challenges for both the experimental investigation and clinical management of breast cancer.

Breast Cancer Stem Cell Hypothesis

This chapter has revisited research work that supports the breast cancer stem cells hypothesis and the putative link with chemoresistance and metastasis. However, it should also be acknowledged that there is a varied body of ongoing research that can shed light on these problems using other rather different hypotheses. Such work, however, lies beyond the scope of the current chapter.

The breast cancer stem cell theory has gradually been accepted by most oncologists [72]. The terms tumor-initiating cells and CSCs are often used interchangeably. However, a tumor-initiating cell refers to the cell type within the stem-progenitor-mature cell hierarchy of adult tissue from which the cancer originates, whereas CSCs are cells that can help tumors progress, contribute to tumor heterogeneity, withstand the effects of therapy, and reinitiate tumors subsequent to treatment. Single-nucleus DNA sequencing studies have identified at least three populations of cancer cells within a breast cancer (hypodiploid, pseudodiploid, and aneuploid cells) [73]. Most CSCs are believed to be resistant to chemo- and/or radiation therapy, indicating the important roles played by CSCs in cancer relapse and metastasis [74].

The normal tissue stem cells are capable of self-renewal by symmetric or asymmetric cell division. Progenitor cells are generated to produce more committed progenitor cells or differentiated cells to fulfill the tissue-specific functions. A common element in the different versions of the CSC hypothesis is the concept of a cellular hierarchy in solid tumors and hematological malignancy cancers similar to that of normal tissues [75]. CSCs are thought to be capable of asymmetric cell division that maintains the CSC population and produces pluripotent "progenitor-like" cells. These cells, in turn, give rise to the "bulk" tumor cells through proliferation and

aberrant "differentiation." Because of their ability to regenerate all cell types in a tumor, CSCs, and possibly progenitors, are thought to have higher tumorigenic potential than "bulk" tumor cells. In any case, medical researchers avoid discussing in detail the origins of CSCs, their differentiation and dedifferentiation, genetic heterogeneity, symmetric and asymmetric modes of cellular division, and clonal evolution, until new findings emerge [71].

According to Badve and Nakshatri [76], CSC phenotype is a characteristic that represents aggressiveness of a tumor with an inherent ability to adapt, lie dormant or rapidly proliferate, and meet the demands of a (sometimes rapidly) changing environment. The CSC phenotype represents plasticity to undergo EMT and give rise to metastases. The presence of powerful intracellular transport mechanisms in these cells permits expulsion of toxins (including chemotherapeutic drugs). It is very likely that a linear correlation exists between stemness phenotype and the level of tumor heterogeneity, aggressiveness, or metastasis.

Immune surveillance by the innate and possibly adaptive immune systems also contributes to the CSC microenvironment, with effects that, at least in mice, are inhibitory. CSC are produced through EMT, which restores a stemlike phenotype and has the ability to metastasize to some cancer cells. The niche and the environment play a major role in stem cell maintenance. Disrupting the cross talk between the niche and the CSC may be one of the critical steps to circumventing resistance to therapy.

In addition to the involvement of the immune system, an additional complicating factor is the role of cellular microenvironment or CSC "niches," where CSCs survive inside the primary tumor or at distant sites. This may be particularly important, given that the stemlike phenotype may be inducible by paracrine signals such as TGF-β, Wnt, and hedgehog and signals transmitted by cell-cell contact such as Notch. CSCs exist in the context of "niches" formed by neighboring cells and extracellular matrix (ECM). The hedgehog (Hh), Notch, and Wnt pathways mediate short-range interactions with neighboring cells. Soluble mediators such as TGF-β and the related BMPs, or growth factors such as hepatocyte growth factor (Met ligand), as well as signals from ECM proteins, may all participate in regulating the maintenance, self-renewal, and differentiation of CSCs. These are characterized by slow replication, ability to generate partially differentiated progenies (pluripotency), highly effective DNA repair, ability to eliminate xenobiotics including chemotherapeutics through ABC family transporters (ABC), and expression of primitive membrane markers (CD133, Met). Transcription factors such as Bmi-1, Musashi, Sox2, Oct4, and others are commonly expressed in putative CSCs. They also overexpress the common set of stem cell genes NOTCH1, ALDH1, FGFR1, and SOX1.

Besides EMT, other mechanisms of dedifferentiation have been described and may contribute to restoration of stemness in transformed cells. In a widely accepted model, asymmetric cell division of CSCs produces pluripotent "progenitors," which in turn generate one or more bulk tumor cell types through proliferation and aberrant differentiation. CSC and "progenitors" are more tumorigenic in xenografts and less chemosensitive than bulk cancer cells. The influence of the niche on its stem cell results in selection for a more malignant phenotype. Transcription factors such as Twist or Snail will induce EMT upon exposure to transforming growth factor β (TGF-β). Stromal release of TGF-β can lead normal tissues, such as mammary epithelium, to exhibit characteristics such as invasion and metastasis as tumors develop. Thus the microenvironment may adaptively select the tissue stem cell and induce a malignant phenotype.

Cancer stem cell markers are recognized by their capacity for proliferation and self-renewal. ALDH1, CD44+/CD24−, NANOG, OCT4, and SOX2 are markers of breast cancer stem cells, which showed association with poorly differentiated breast cancers that are suspected to be enriched for cancer stem cells with NANOG, OCT4, and SOX2 (NOS markers) expression. Oak et al. [77] demonstrated elevation of HER2 levels and its differential expression in the individual cells of mammospheres. Sorting of

HER2high and HER2low populations from 3D culture system revealed overexpression of stem cell markers such as NANOG, OCT4, and SOX2 and stem cell-like properties in the HER2low cell fraction. In addition, xenografts of HER2low sorted cells from MCF-7 mammospheres showed elevated levels of stem cell markers NANOG, OCT4, and SOX2 as compared with xenografts of HER2 high, confirming stem cell-like properties of HER2 low cells.

More clinical evidence supporting the existence of CSCs and their role in treatment chemoresistance has emerged, particularly in breast cancer. Liu et al. have shown that tumorigenic cells with stemlike markers are selected by neoadjuvant chemotherapy [78]. Moreover, it has recently been shown that breast cancer cells surviving in patients after treatment with either docetaxel or letrozole have gene expression signatures characteristic of stemlike and EMT phenotypes. Interestingly we observed that in tumor grafts with high content of CD133, only subpopulations of CD133+ABCG2+ and CD133+CXCR4+ are strongly enriched after in vivo cisplatin treatment, indicating that highly resistant cells could represent only a subset within the CSC compartment. While debate continues as to the precise identity and function of CSCs, there is general agreement that CSCs display increased chemoresistance and radioresistance. Therefore, understanding the biology of the chemoresistance potential of CSCs may contribute to our understanding of tumor biology and would have far-reaching clinical implications. Although several molecules have been reported to confer chemoresistance to CSCs, much remains unknown about whether stem cell factors play a role in chemoresistance of tumor cells, including CSCs. BMI1 is reported to play an important role in self-renewal of stem cells and is associated with a number of human malignancies. Recent studies suggest that BMI1 is involved in the initiation of cancer, and targeting BMI1 by gene therapy abolishes chemoresistance in tumor cells [79]. In CSCs niche, the embryonic signaling pathways for Hh, Notch, Wnt, and others lead the planar-spatial aspects of cellular aggregation. These signaling pathways have therefore become the central focus of study for the development of new targeted therapies.

Metastasis and the Primo-vascular System

Recently, some studies have been performed on a putative new vascular system which may be an additional metastasis route, complementing the lymphatic and hematogenous routes, which facilitate the dissemination and colonization of cancer cells at secondary sites. This vascular system has been described as an independent fluid-conducting system called the primo-vascular system [80]. However, a more detailed examination of this approach is necessary in order to verify this hypothesis.

Conclusion and Future Perspectives

While the past decade has witnessed significant advances in the prevention and treatment of breast cancer by targeting the estrogen receptor and HER2 oncogene, the options for women with triple-negative disease remain suboptimal. The triple-negative molecular subtype is characterized by marked heterogeneity, chemoresistance, and metastasis that further complicates clinical trial design. Personalized approaches for the prevention and treatment of breast cancer will not be realized by any one approach, but rather through multiple approaches acting in concert. Knowledge of biomarkers should be integrated into future trials that incorporate molecularly targeted biological agents. With the availability of novel technologies, such as microRNA profiling and genome-wide association studies, we are just beginning to improve our understanding of the role of host genetics in the optimization of therapy. MicroRNA profiling has revealed that they are frequently deregulated in human tumors. The next decade will usher in novel targeted therapies for other subtypes of breast cancers because we recognize that breast cancer is not only one disease but a heterogeneous group of diseases.

Individual oncology management needs to be more cost-effective as most hospitals and physicians focus on developing electronic medical records and heterogeneity in drug metabolism, leading to variability in drug efficacy and toxicities.

The dosage of drugs will be based on a better understanding of pharmacogenetics and pharmacodynamics. Knowledge of CSC will allow for the control of chemoresistance and metastasis and a more elaborate new targeted drug, but the most important step is to initiate cancer prevention interventions with predictive genetic testing of BRCA1 and BRCA2 that will ultimately reduce overall mortality, especially for younger women. Promising clinical trials designed to evaluate anti-CSC investigational agents will need to critically evaluate CSC biomarkers and may require novel designs and endpoints for validation.

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Animal Models of Breast Cancer

15

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Abstract

Several animal models have been used to investigate the etiology and to understand the mechanism of breast carcinogenesis. Advances in cancer management relied on the use of animal models to discover and develop new cancer preventive and therapeutic strategies. Animal models have the advantage of the presence of stromal and 3D structures, which were lacking in the in vitro cell culture preclinical evaluations. Xenograft animal models combined in vitro and in vivo models to overcome the dissimilarity between genetics and other biomarkers of animals and their human counterparts. This chapter will illustrate the various animal models used in breast cancer and their relevance to human breast cancer.

Keywords

Animal models • Breast cancer • Canine • Chemoprevention • Feline • Rodents • Syngenic • Xenograft

Introduction

Worldwide, breast cancer is the most common, and the second leading cause, of cancer-related death among women [1]. Europe and Australia have the highest incidence rate of breast cancer worldwide. Western population has an age peak at 60–70 years. It was expected that the incidence number will rise from 49.814 to 64.621 in Europe between 2005 and 2018. In the USA, it was estimated that 39,510, 226,870, and >2.9 million women will die from breast cancer, be diagnosed with breast cancer (median age at the time of diagnosis is 61 years old), and live with invasive breast cancer, respectively by 2012 [2, 3].

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According to National Cancer Institute expectations (http://www.cancer.gov/cancertopics/types/breast), these numbers will increase by 2013. New cases of breast cancer are expected to be 232,340 and 2,240, while the deaths from breast cancer will be 39,620 for females and 410 for males. Consequently, 1 in 8 or even 4 [4] women is expected to have breast cancer at some point in her life, while the ratio in men is 1 in 1,000 [5]. In Africa, the incidence rate is the highest in Garbiah (Egypt) and the lowest in North Africa, except in Algeria [4], a population which mimics the European community. In Asia, the highest rate is in Israel (Westernized population) and the lowest is in India, Korea, and Thailand with an age peak at 40–50 years [4].

The nature and the cause of breast cancer have been extensively studied in human beings. Risk factors include advanced age, family history, personal history, reproductive history, lifestyle factors, and environmental pollution [6, 7]. The female adult breast consists of 15–20 fat-covered lobes that branch into many lobules, ending with milk-producing glands [7]. Each gland has a separate duct that opens in the nipple [6]. Male breasts contain small amounts of duct tissues that can develop breast cancer [6]. Breast adenocarcinomas usually are ductal (80 %) or lobular (10 %) epithelial tumors [5, 8].

Animal Models Used in Experimental Cancer Research

Many experimental procedures are impossible to perform in human subjects. Therefore, animal models that include rodents, canines, and felines are utilized to understand breast cancer pathogenesis in the hope to eliminate the disease [9]. Cancer models used so far fall into several categories: (a) animals in which cancers occur spontaneously without any alteration of the animal's genes or initiation of cancer by prior treatments; (b) animals whose genes are altered so that they develop spontaneous tumors of the same types and with similar properties to that of humans; (c) animals that develop spontaneous tumors if they are exposed to other factors, such as chemicals or radiation; and (d) animals whose natural, unaltered genetic makeup permits researchers to identify the genes that generate susceptibility to cancer development. The utility of these models varies by species, available research reagents and tools to support the studies, and similarities to the human tumors.

Chicken Models

The chicken model was the first to be used to investigate the causes of cancer. Peyton Rous successfully inoculated small pieces of sarcoma extract of a Plymouth Rock hen into another chicken of the same species that developed the same type of cancer. Transplanting the tumor from one chicken to another enabled the experimental cancer research. Subsequently, Rous was able to induce tumors by using cell-free filtrate, proposing that cancer is caused by viruses [10, 11]. Mice were then used due to their small size, rapid breeding, and their susceptibility for many human diseases, including breast cancer.

Rodent Models

Among animal models, the murine cancer model is the most extensively utilized model that has been extremely useful in providing valuable insights into cancer biology. Mouse models for human breast cancer are generally categorized into three main groups: (a) chemically induced, (b) xenograft models, and (c) genetically engineered mice (GEM, such as transgenics and knockouts) [12]. A combination of particular methodologies can be used to generate the three main types of mammary cancer models. For instance, transgenic mice are being treated with chemical carcinogens to accelerate mammary tumorigenesis.

However, despite the unquestionable importance of the murine model in cancer research, they do not adequately model some essential features of human neoplasms [13]. For example, the pathological features of most murine tumors differ from the ones seen in humans [14]. Rodents

and rodent tumors differ in their biology, detailed morphology, and sometimes in detailed histology (rats) than humans and human tumors [15]. Rodents also differ in their developmental programs as manifested in size, life span, cellular targets for oncogenic transformation (number, degree of maturation, and differentiation), the ease of rodent cells to be transformed in vitro and become immortalized, and the metastatic pattern [15, 16].

Development of Syngenic Mouse Model

Sporadic cancer is quite rare in wild rodents. Siblings were mated for many generations to achieve genetic identity [17]. However, inbred strains differed in developing spontaneous breast tumors. Thus, reciprocal crosses between high and resistant tumor strains were allowed. The transmission of mammary tumors was attributed to the mouse mammary tumor virus (MMTV) [18, 19]. Genetic susceptibility and hormonal influences of multiple pregnancies were also reported as cofactors in mammary carcinogenesis [20].

Hormonal Effect on Rodents' Mammary Glands

The morphology, number, and location of mammary glands differ between humans and rodents. The development of mammary glands is affected by sex, age, hormonal stimulation, and reproductive status in mouse and rat [21]. The mammary gland usually appears as an atrophic organ in the male mouse that rarely develops cancer. However, in a reproductively active female mouse, breast tissue undergoes dynamic changes throughout its life span. Terminal end buds (TEBs) are formed in nonpregnant females under the influence of pituitary ovarian integrated hormonal system. Terminal differentiation into milk-secreting alveoli occurs during pregnancy and lactation. After weaning, the secretary epithelium of the mammary gland involutes into an adult nulliparous-like state by apoptosis and redifferentiation.

To test the hormonal effect on breast tumors, it was necessary to study first the influential effects on the normal mammary gland [21]. Several differences in hormone response were reported between humans and rats. Spontaneous breast tumors were linked to viral infection in mice and detected only in long-term studies. The studies illustrated that the breast carcinogenesis is a multistep process initiating in hyperplastic lesions that then develop into hormone-dependent adenocarcinoma. Spontaneous malignant tumors are also rare in rats. They are age, strain, and hormone dependent. The spontaneous rodent models were limited by a long latent period and low incidence rates. However, other rodent models such as chemically induced models have been developed to investigate the effect of chemotoxins as breast cancer inducers.

Chemically Induced Rodent Model

A link between chemicals and carcinogenesis (nasal cancer and tobacco snuffing, skin cancer and chimney sweeping, aniline industry and bladder cancer, asbestos and lung cancer, and mesothelioma) was discovered more than 200 years ago [5, 6]. Occupationally induced cancer identified many chemical carcinogens and led to the creation of the Occupational Safety and Health Administration (OSHA) to formulate regulations of health safety and protection of workers. Chemical carcinogens have been classified according to their chemical structure or reactivity into carcinogenic polycyclic aromatic hydrocarbons (PAHs), aromatic amines, N-nitroso compounds, alkylating agents, natural products, and inorganic substances [5, 6]. Some drugs and hormones also have been shown to be carcinogenic (DES [diethylstilbestrol] was linked to vaginal cancer; estrogens linked to breast and ovarian cancers; and testosterone linked to prostate cancer) or promote carcinogenesis (immunosuppressive and chemotherapeutic drugs) or inhibit cancer growth (rapamycin) [5, 6, 22]. The effect of chemical carcinogens depends on dose, potency, and the affected tissue. Environmental pollutants were thought not to represent a major cancer risk due to lower exposure dose [23].

Due to the inability of epidemiological approaches to detect the finite increase in cancer incidence, animal models were used to assess the effect of low-dose exposure [24]. Apart from the

irrelevance of animal models to human breast cancer (in the mode of exposure, metabolism, and genetics), animal models were very important in detecting the hazard, the dose-response effect for genotoxic chemicals, and in identifying the premalignant stages of the disease. Testing dioxins in rats revealed that high doses are carcinogenic, but low-dose exposure is protective [25]. A link between DDT (dichlorodiphenyltrichloroethane) and breast cancer was identified in rats but still suspected in humans [26, 27]. AAF (2-acetylaminofluorene) induced cancer in rats but not in guinea pigs due to their lack of activating enzymes, yet humans have the activating enzymes. Saccharin-induced bladder cancer occurred in rats but not in hamsters, guinea pigs, mice, or humans due to low protein concentration in their urine [28]. Bladder cancer was induced in laboratory animals by feeding 2-naphthylamines but not when it inserted directly into the bladder. Thus, it was clear that chemicals can be reclassified according to their actions into precarcinogens (acts indirectly; needs metabolic activation to cause cancer) and carcinogens (acts directly). The activation steps usually occur in the liver by cytochrome P450. The involvement of cytochrome P450 was confirmed using a mutant mouse strain that expressed high levels of cytochrome P450 1A1 [6]. Cigarette smoking also activates cytochrome P450 1A1 and thus increases the risk of developing lung cancer.

Animal studies revealed that DNA is a direct target of chemical carcinogens. It was clear that carcinogenesis is a multistep process that requires a permanent unfixed DNA damage (by genotoxic or non-genotoxic means) resulting from direct or indirect (after metabolic activation) interaction of the chemical carcinogen with the DNA (DNA adducts; DNA–carcinogen complex) [5, 6] as an initiation step. Activation of chemical carcinogens results in formation of electrophilic groups (epoxide, nitrenium ions, carbonium ions, or free radicals) that attack electron-rich atoms in DNA, causing DNA mutations. Chemical carcinogens may also cause double-stranded crosslinks, bases dimmers, hydroxylation or removal of base(s), or single- and double-stranded breaks. Cancer studies in rabbits and mice revealed that a second promoting step is required to sustain tumor development via prolonged proliferation. Accordingly, if the chemical is able to cause initiation and promotion, it is called complete carcinogen, and if it causes only one step, it is called incomplete carcinogen. A third stage of tumor progression involves lesion propagation via clonal selection, cellular proliferation, altering signal transduction pathways to support autonomous growth, acquisition of invasion, and metastatic capacity.

In vitro models have shown that several chemicals (including PAHs, aromatic amines, nitrosamines, alkylating agents, reactive species, and non-genotoxins) are mutagenic and induced neoplastic transformation of human breast epithelial cells [29–32]. Mice and rats were extensively used to explore mammary carcinogens, with emphasis on rat models due to comparability with the human in hormonal dependency and pathological progression [21, 33–36]. Several chemicals induced mammary tumors [especially DMBA (7,12-dimethylbenz(a)anthracene) and MNU (N-methyl–N-nitrosourea)] in different strains at different doses according to the route of administration [21].

DMBA and MNU induced mammary cancers in Sprague-Dawley female rats. Other carcinogens and other rat species such as Fisher or Wistar Furth rats have been studied as possible animal models to induce mammary cancers [35]. DMBA- and MNU-induced mammary carcinogenesis models are similar in many respects. Both carcinogens induce mammary adenocarcinoma with a single dose. The tumor induction is mammary gland specific. There is well-established dose-dependence, and tumors are developed without any systemic toxicity. Both models are extremely reproducible. The multiplicity can also be adjusted with carcinogen dose. Basic differences, however, exist—for example, DMBA requires metabolic activation; thus, the model is more suited to studying initiation and promotion or for evaluating effects of agents that may affect parameters of carcinogen metabolism and activation. MNU, on the other hand, is a direct-acting carcinogen and is not well suited to studying stage-specific activity of a chemopreventive

agent. MNU-induced mammary tumors are largely adenocarcinomas, whereas tumors induced by DMBA consist of 60 % adenocarcinomas and 40 % benign fibroadenomas [36]. Thus, DMBA-induced tumors require histopathology for confirming the carcinoma incidence. Finally, unlike DMBA, MNU-induced tumors are locally invasive and metastasize to distant sites.

Both mouse and rat models showed hormonal dependency of chemically induced mammary tumors. It has been shown that pregnancy considerably shortens the latency of mammary tumorigenesis. Rats also showed age dependence at the time of exposure.

In general, it appears that epithelial cells in mammary terminal end buds are the targets of carcinogenic initiation and that a series of morphologically identifiable steps are involved in the development of mammary carcinoma. The premalignant steps include ductal hyperplasia of the usual type and carcinoma in situ of the cribriform or comedo type; atypical ductal hyperplasia has not been reported [37].

Many external, internal, and biological factors can modify the susceptibility of the mammary gland to neoplasia [21]. Tumorigenic response is affected by gland topography, differentiation, genetic and dietary influences, interaction with the stroma, and hormonal, neural, and growth factors. The in vitro cell culture model was established to test which lobule type (1, 2, or 3) is more susceptible to carcinogenesis [33, 38].

Radiation-Induced Rodent Model

Radiation is a form of energy that travels from its source as waves or energized particles [39]. Atomic radiation (alpha, beta, gamma, and neutrons), X-rays, and UV (ultraviolet) are carcinogenic. The carcinogenic effects of ionizing radiation was evident by four main bodies of studies: (a) of atomic bomb survivors (who suffered from leukemia and other types of cancers including breast cancer); (b) of individuals who were medically irradiated for diagnostic or therapeutic purposes; (c) of occupational exposure of healthcare workers—in manufacturing and mining and among the various sectors of the nuclear weapons/nuclear power industries—(d) and environmental epidemiological studies of communities exposed to indoor radon and to radiation across the nuclear fuel cycle [40].

Exposure to sunlight (containing UV radiation) induces skin cancer due to formation of pyrimidine dimers in DNA and mutation of p53 gene leading to permanent DNA mutations. Therefore, radiation acts primarily as an initiator for tumor growth. Animal studies showed that exposure to X-rays induced leukemia in a proportional rate to the received dose. Animal studies also reported DNA damage due to RF (radiofrequency) waves. In addition, human studies reported high rates of leukemia, brain cancer, and other pediatric cancers among children living near high-voltage power lines due to ELF (extremely low frequency) waves [6].

α-particles (2 protons and 2 neutrons) are positively charged particles that result from the decay of the heaviest radioactive elements, such as uranium, radium, and polonium. The health effect from exposure to α-particles depends greatly on the exposure route. α-particles lack the energy to penetrate even the outer layer of skin, but can be very harmful if they are inhaled, swallowed, or get into the body through a broken barrier (skin wound). They can cause severe damage to cells and DNA in sensitive living tissue via energy dissipations and ionizations [41]. People who have been exposed to radioactive radon (α-emitter present in cigarette smoke and some poorly ventilated buildings) had increased rates of lung cancer. Radium paint has also been linked to bone cancers [6].

β-particles (electrons) are emitted by certain unstable atoms such as hydrogen-3 (tritium), carbon-14, and strontium-90 during radioactive decay. They are more penetrating than α-particles but are less damaging to living tissue and DNA because the ionizations they produce are more widely spaced. Some β-particles are capable of penetrating the skin and causing damage such as skin burns. However, as with α-emitters, β-emitters are most hazardous when they are inhaled or swallowed [41, 42]. A few years after the Chernobyl explosion, juvenile thyroid cancer rates increased due to the ingestion of β-emitting forms of iodine [43].

γ-rays (photons) are weightless packets of energy that are often emitted along with α- or β-particles during radioactive decay. They represent radiation hazard for the entire body. They can easily penetrate barriers, such as skin and clothing that can stop α- and β-particles. Gamma rays can pass completely through the human body easily; as they pass through, they can cause ionizations that damage tissue and DNA [41, 42]. Ionizing radiations exert its effect via water hydrolysis and release of hydroxyl free radicals, which interact with DNA bases leading to various mutations or directly interact with DNA by direct stripping of electrons and breaking bonds. Subsequently, different forms of DNA damage—such as deletions, single- and double-stranded DNA breakage, sequence rearrangements, and chromosomal translocations—occur, leading to genetic instability that favors tumor progression [6].

Human breast tissue showed high susceptibility for radiation-induced cancer [44]. Accidental or therapeutic radiation exposure was reported to be associated with breast carcinogenesis due to high sensitivity of breast tissue to radiation [45]. Although human exposure was age dependent (incidence rate increases if the exposure occurs in age <19 years), a phenomenon which was not observed in irradiated rodents. Animal models (especially rats) were essential to study the effect of radiation, the effect of fractionated dose, and the dose-response curve [46]. Whole body or localized irradiation by single or fractionated sublethal dose of g-rays or X-rays or neutrons was able to induce mammary tumors [21, 47]. Hormones have a synergetic effect upon administration prior to radiation exposure [48].

In Vitro Cell Culture Model

Cell culture models were used to explore the deregulatory mechanisms (due to the genetic, epigenetic, and environmental interactions) of proliferation, apoptosis, and migration in progressive breast cancer. Cells are easily propagated, more relevant to the human model (steroid dependent, genetic and genomic features, and representatives of breast cell subtypes), can be genetically manipulated, can be grown as xenograft (both in vitro and in vivo), and can give reproducible and quantifiable results under specific conditions. Apparently, it was evident that not a single cell line can represent the human breast cancer, but a panel of cells represented the heterogeneity of the disease [49]. Although most of the cells used were isolated from advanced grades, new cell lines were isolated and provided insights about the molecular and cellular variations between tumor-initiating and non-tumor-initiating cellular subpopulations and their role in drug resistance [49]. In addition, the developed 2D and 3D and heterotypic co-cultures revealed variations in post-translational regulatory mechanisms and allowed the study of tumor–stromal interaction, respectively [50–54].

Xenograft Models

Xenograft tumors are produced by injecting human cell lines ($0.5–5 \times 10^6$ cells, hormone or non-hormone dependent) into the skin (subcutaneous) or into the mammary fat pad (orthotopic) of an immunocompromised mouse. Isaacson and Cattanach were the first to report that some human breast cancer cell lines form tumors in immunodeficient mice [55]. However, procedures were complicated and not widely used until the introduction of the mutant nude mouse. Recently, the nude (Foxn1) mice and severe combined immunodeficiency (SCID) mice are the most commonly used research models in xenograft experiments. These types of animal models have naturally occurring single-gene mutations that affect their immune system. Nudes have a chromosome 11 autosomal recessive mutation that causes failure of hair growth and other defects, including thymic epithelial dysgenesis, which renders them T-cell deficient [56]. The SCID mouse has a spontaneous mutation inactivating DNA protein kinase resulting in the lack of functional T cells and B cells [57, 58].

Xenograft models are used to study the different steps of tumor formation and progression (genetic signature, interaction with tumor microenvironment, and metastasis) [49]. Preinvasive disease samples (ductal carcinoma in situ) have shown better success in xenograft models [59, 60].

Xenograft breast cancer models also play an important role in preclinical trials and drug efficacy as they are relatively inexpensive, easy to generate, and have short latency. They correlated with prognostic outcomes and showed relevance to that of human breast cancer [49]. Estrogen receptor (ER)-positive cancer cell xenograft models are currently indispensable for preclinical testing of inhibitors of steroid receptor signaling and drug resistance studies. It was reported that steroid and peptide hormones have a considerable effect on the initiation of mammary tumorigenesis in mice [34]. In addition, investigation on steroid signaling and mammary cancer are being performed in rat models that, unlike mice, exhibit a significantly higher frequency of ER-positive lesions, which hold great promise for the improvement of rat models to study estrogen signaling and tumorigenesis in vivo [61].

Xenograft models were used to study metastasis in spontaneous and experimental assays [62]. There are many ways to establish breast cancer metastasis, depending on the site of injection and the specific tropism of the chosen breast cancer cell line. Orthotopic or ectopic implantation of cancer cells in the mammary fat pad, with subsequent formation of primary tumors and metastatic lesions, partially resembles the multiple stages involved in human breast cancer [63]. The achieved metastatic rate reported so far ranged from 7 to 20 % according to the variability related to the site of implantation (orthotopic being better), the age and strain of mice, and the type of hormonal supplementations. Injecting cells into the tail vein results mainly in lung metastasis, whereas portal vein injection provokes colonization of the liver, and intracardiac infusion gives rise to a broader target organ spectrum, including bone.

Xenograft models suffer many limitations. For instance, the used cell lines have been adapted to grow in culture with different environmental requirements than those of the primary breast tumor. In addition, the cell selection process during the conversion to continuous culture line leads to changes in later generations of cell lines (genetic drift). Also, cells in culture are subject to viral and/or mycoplasma infection [64]. Established experimental models in which dissociated cells from surgical breast cancer tumors, after mixing with extra-cellular matrices, have been used as xenograft in nude mice [65]. In this model, cells undergo morphogenesis that reflects their original phenotype, and they provide a much more relevant model for studying primary human breast lesions and cancers in vivo [66]. However, even these models that are derived directly from clinical samples have their limitations.

Another important difference is the stromal difference between mice and humans. Mouse mammary stroma largely consists of adipose tissue, while human stroma contains a relatively high amount of fibrous cells surrounding the epithelial compartment. Hahn and Weinberg previously reported that chimeric xenograft tumor models contain fewer stromal of murine origin. The nature of chimeric rodent/human tumors differ significantly from that of human and result in unpredictable growth, differentiation, or metastatic properties [67].

To overcome and generate the correct microenvironment for human epithelial cells, xenograft models can utilize fibroblasts derived from the human mammary gland [68]. These features will significantly improve existing xenograft models that use untransformed epithelial cells. The co-transplantation of the correct stroma is, however, only needed when normal and preneoplastic cells still depend on local growth factors. The newly designed breast cancer model, which utilizes in vitro-designed breast cancer model of transformed cells, exhibited some increase in the efficiency of tumor formation when these cells were co-implanted with normal human fibroblasts, suggesting that local growth factors have an effect on the proliferation of these neoplastic cells [69]. However, tumorigenesis of such models is not completely dependent on the human stroma.

One of the important factors to ensure correct generation of xenograft models is the transplantation of immortalized human cells (stroma and epithelia into immunocompromised animals). Furthermore, the tissue-recombination approach might reconstitute a correct epithelial–stromal interaction in the primary mammary cancer in

the xenograft model. Therapeutic regimens have been proposed to target the cancer-associated stroma in addition to cancer cells. The use of such a strategy clearly becomes inappropriate for the treatment of metastasizing breast cancer in this xenograft model. Neoplastic epithelial cells, but not normal fibroblasts, invade other organs; one should expect that, unlike in the primary tumor, cancer cells interact with the murine stroma in lung or bone metastases. For that sake, improved xenograft systems might not be different from conventional models. Kim et al. reported that the use of transformed stromal cells might metastasize along with malignant epithelial cells [15], but this strategy does not mimic what happens in human breast cancer.

It was reported that the 3D cell–cell interaction model was xenografted into immunodeficient mice. This comprised normal breast fibroblasts derived from reduction mammoplasties plus normal human umbilical vein endothelial cells in combination with normal and preneoplastic human breast epithelial cells derived from clinical samples [70]. However, the model showed difficulty in assembling such cell combinations on a long-term and reproducible basis. With the recent development of immortalized human adult mammary stromal cells [71], it has now become possible to satisfy these criteria and to perhaps develop a fully (humanized) breast cancer model in immunodeficient mice.

Appropriate microenvironment is one of the important factors, which supports epithelial cells with local growth factors and plays a role in tumor growth. In turn, epithelial cells signal back to the stroma, which then becomes competent to support epithelial proliferation and differentiation [72]. Recently, the host microenvironment has been shown to provide appropriate conditions for the tumor cells to survive and proliferate [73, 74]. The abundance of cytokines and growth factors produced by tumor and host stromal cells of microenvironment is thought to facilitate tumor cell behavior in an autocrine and/or paracrine fashion [75, 76].

Lacking immune response is another drawback of xenograft models, especially for therapeutic clinical trials that rely directly or indirectly on an intact immune system. Different strategies to suppress tumor growth by attempting to promote rejection of the tumor through cell-mediated immunity in the host have been under investigation. However, there are several potential solutions to the immune-response problem in the context of modelling immunotherapy. Generating of such a mouse model can be achieved by matching the human immune system in addition to the human stroma–epithelia graft in the mammary gland. For instance, it has been shown that non-disrupted tumor biopsy tissues implanted into SCID mice resulted in the co-engraftment of tumors plus tumor-infiltrating lymphocytes, with tumor-infiltrating lymphocytes within the tumor graft remaining functional and responding to lymphocyte cytokines [77]. In addition, human peripheral blood lymphocytes, injected subcutaneously with a human lung tumor into SCID mice, also engraft and display antitumor cytotoxic activity [78]. It could envisage the use of mice that combine the immunodeficiency phenotype of the nude/SCID with engraftment of human bone marrow stem cells.

Another strategy focuses on local growth factors that act mostly in a paracrine fashion. It is assumed that the tissue-recombination approach will model all necessary growth factors to support normal proliferation and differentiation of epithelial cells, but this approach does not reflect species-related incompatibilities of systemic factors produced by the host with the corresponding receptors of the graft.

Expression of human hormones at near-physiological levels in the immunocompromised host is an important requirement for the implementation condition in the animal model design. In addition, it is necessary to examine what effects such a "hormone replacement" with ligand-receptor incompatibility will have on the general physiology and reproductive capability of the animal model.

Advanced breast cancer commonly spreads to the bones, lungs, liver, or brain, and bone (70 %) is the most common site of breast cancer metastasis [79]. Lack of appropriate animal models that fully reflect the biology of human breast cancer metastasis to bone is a major barrier to progress in

identifying the molecular mechanism(s) of breast cancer bone metastasis and developing therapeutics. Generating such an animal model will help to identify the therapeutic target for identifying the molecular mechanism(s) of breast cancer bone metastasis and to evaluate the effectiveness of new candidate drugs during breast cancer progression. Because an in vitro experimental model may not recapitulate the tumor–stromal interaction in the bone microenvironment, an in vivo animal model would be useful to elucidate the mechanisms of tumor–stromal interaction in the bone microenvironment.

Previously, Lynch et al. developed a murine bone invasion model of breast cancer that allowed researchers to explore cellular and molecular interactions between malignant cells and skeletal tissue in a syngenic setting [80]. For such an animal model, three murine mammary tumor cell lines, 4 T1, Cl66, and Cl66M2, which are derived from a spontaneous mammary carcinoma, were implanted in a BALB/c mouse and exhibited different patterns of spontaneous metastasis following injection into the mammary fat pad [81, 82]. The histological results of the developed animal model mimicked the histological features of metastatic bone lesions in human breast cancers. The model therefore presented an exciting opportunity to examine the molecular mechanisms underlying tumor–bone interaction and breast cancer-induced osteolytic changes in bone. Anguraj et al. identified and used a tumor–stromal microenvironment-specific gene expression signature for such an animal model to extend the understanding of the metastatic bone microenvironment in human disease and to predict potential therapeutic targets, which in turn was able to mimic both the human-breast cancer bone microenvironment and osteoclastogenesis [83].

Generating animal models for preclinical testing must not only reproduce the pathology and behavior of human tumors, but must also be highly reproducible with predictable endpoints. To enable mouse xenograft models to be used in drug screening of therapeutic strategies, they should reflect the cellular composition of exiting breast tumors. Although considerable difficulties will be encountered in the generation and use of such complex models, their potential value in the longer term is such that every effort should be made to develop them.

Genetically Modified and Transgenic Models

Genetically manipulated animals are generated by transgenic, gene-targeting (knockout) technology, and combined models [15, 84]. They assisted in understanding gene function and regulation at the molecular level within the whole organism. They also enhanced exploration of breast tumor pathogenesis via induction of single and multiple mutations in gene-encoding regulators of growth factors, signal transduction, cell cycle, differentiation, matrix metalloproteinases, and apoptotic pathways. In addition, they are useful candidates for therapeutic evaluations. Although the genetically engineered mice (GEM) present fundamental differences at the level of the organism and the cell, it is designed to reproduce very specific aspects of tumor formation and progression based on knowledge of human tumor genetics. However, the relevance of the genetic milieu (type, magnitude, and background) must be considered. Several promoters were used to drive the expression of oncogenes in the mammary epithelium to initiate (double-transgenic mice model) or modulate breast carcinogenesis or develop metastasis (independently or dependently on hormonal supplementation or pregnancy) in lung and lymph nodes in mice [15, 84–88]. It has been shown that GEM is affected by the promoter used and the background strains, which in turn affect the tumor morphology and latency [88].

All tissues and cells carry the same defect in most of oncogene-bearing transgenic and tumor-suppressor gene knockout models, which does not mimic the real situation but the human familial cancer. Cell-type specific promoters and promoter-specific recombinase-based mechanisms were introduced to overcome this problem. Apart from the fact that those approaches on their own are limited by their hormonal dependency, several other models (loss of ER, HER-2 amplifications, BRCA1 mutations, and EMT) have been developed which mimic that in human breast cancer [5, 15, 88].

GEM revealed an association between delayed postlactational involution (governed by >50 genes) and increased carcinogenesis, as well as an age-related protective effect of lobular involution [89]. It also illustrated the synergetic effect of oncogenes in accelerating mammary transformation [90]. MMTV, ITAM, and MMTV LTR mice models have enriched our knowledge about the role of infection and oncogenesis, oncogene pathways mediating signaling in breast epithelial cells and invasive breast cancers [91]. In addition, GEM was used to test vaccines against mammary tumors. Growth of EMT-6 mammary tumor cells was inhibited in HSP65-GnRH6 immunized GME [92, 93]. COX-2 inhibitors also delayed the incidence of mammary tumors in both GEM and human breast cancer [94].

Despite the usefulness of the transgenic and genetically modified animal models, they were limited by their irrelevance to human breast cancer. Replacement of rodent models by another species more relevant to human (pigs or dogs) was thought to produce more relevant models for preclinical studies.

Feline and Canine Models

Dogs and cats spontaneously develop malignant tumors that offer a more appropriate and relatively natural model of the corresponding neoplasm in humans [9, 95]. Unlike laboratory rodents, felines and canines are more outbred and share a common environment with humans; thus, they may be exposed to the same carcinogen [96]. Also, the spontaneously occurring tumors in canines and felines develop twice as frequently as in humans, share similar histopathological features with the human neoplasm, and progress at a more rapid rate than the human neoplasm [95, 97]. Above all, the comparable responses to the cytotoxic agents provides a unique opportunity to use the companion animals as models to test the response to new therapeutic approaches that include new chemotherapy agents and gene therapy [95, 98].

Several studies have been carried out since 1960 on felines and canines to determine whether the spontaneous tumor formation in dogs and cats can represent a natural model of the corresponding neoplasm in human beings [9]. According to the World Health Organization (WHO), the animal neoplasm that can be used as a natural model for the human neoplasm includes leukemia, mammary glands, osteosarcoma, melanoma, and ovaries [9, 95]. Among them, breast cancer is also a major health problem in cats and dogs [9].

Felines

In cats, mammary carcinoma ranks as the third occurring cancer in frequency following lymphoma and skin cancer [99]. At least 80 % of the feline mammary carcinomas are highly malignant, rapidly metastasizing, and often fatal [95, 98]. The histological features of feline mammary carcinoma are more similar to the human mammary carcinoma than the ones in murine and canines [95, 100]. The surgical removal of the tumor in cats and humans in most cases is insufficient to eliminate the disease [100]. Also, on the basis of age onset, incidence, and pattern of metastasis, feline breast cancer provides an appropriate model for the human breast cancer [95, 98].

Feline mammary carcinoma has been proposed as a natural model of hormone-independent human breast cancer. In human breast carcinoma, hormonal status plays an important role in the behavior and treatment of breast cancer [101]. Estrogen receptors (ER) are present in 60 % of the tumors, and progesterone in more than 30 %. Patients with ER-positive tumors have a better prognosis and respond to hormonal therapy (e.g., tamoxifen) [9, 102]. However, 30 % of human breast tumors are ER negative with a worse prognosis than ER-positive tumors [95, 102]. Patients with ER-negative tumors do not respond to hormonal therapy; thus, research on new therapies is warranted. On the other hand, 80 % of feline mammary tumors are ER negative and are highly aggressive [97, 102]. The lack of estrogen dependence on most feline tumors, and the remarkable similarity of feline intraepithelial lesions (IELs) to those of human, with the tendency to lose hormone expression in IELs, suggests that the feline breast carcinoma could be

a suitable model to study ER- and PR-negative breast tumors [95, 102].

Feline mammary carcinoma has been also been proposed as a natural model for HER2-overexpressing breast tumors [95, 103]. HER2-overexpressing tumors are characterized by overexpression of HER2 receptor and the genes associated with the HER2 pathway [95]. HER-2 overexpression is found in approximately 15–25 % of human breast cancer [95, 104]. Tumors that overexpress HER2 are highly metastatic and often are associated with poor prognosis [105]. The study by De Maria et al. has shown that the HER2 kinase domain in cats has a 92 % homology with the human counterpart [95]. They have also found that HER2 protein is highly elevated in feline carcinoma samples compared to human carcinoma [95, 103]. Altogether, these data suggest that the spontaneous feline mammary carcinoma could be a suitable model for HER2-overexpressing human breast carcinoma [95].

Furthermore, overexpression of the tyrosine kinase receptor RON and its feline homologue STK has been found in both human and feline breast carcinoma, respectively [98]. Moreover, RAS mutations were not found in feline breast carcinoma, consistent with the low prevalence of RAS mutation in human breast carcinoma [95, 98]. Moreover, other reports have indicated that some similarity in the overexpression of cyclin A and the nuclear accumulation of P53 could exist between feline breast carcinoma and those of the human [95].

Altogether, these data suggests that the spontaneous feline mammary carcinoma can be utilized as a natural model for hormone-independent, HER2-overexpressing and highly aggressive human breast carcinoma. Furthermore, the availability of appropriate experimental animal models is important for the development of novel therapies or improvement of the current ones [104].

Canines

Spontaneous canine mammary carcinoma is the most common cancer among female dogs, accounting for 52 % of the diagnosed tumors [13, 99]. The incidence of the disease is two to three times more than the ones reported in humans and is often fatal due to metastatic disease [14]. Similar to human breast cancer, canine mammary carcinoma is a disease predominantly found in females, and cases in males are reported, but are very rare [13, 14]. Several studies have shown that canine and human breast cancer share similarities in terms of histological types and biological behaviors [99]. Furthermore, many epidemiological factors characteristic of mammary tumors are similar in both species. These include age-related incidence, protective effect of early pregnancy, and reduction of breast cancer in aging individuals as a result of ovariectomy in early life [13, 99].

Several studies have shown that most of the cancer-related genetic alterations and gene expression modulations that play a role in human mammary tumor development are similar to those in canine carcinoma [13, 100]. For example, the genome-wide comparative analysis between the human and canine mammary tumors has demonstrated a great overlap in the deregulation of signaling pathways that are associated with cancer development, such as the mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K)/AKT pathway, phosphatase and tensin homolog (PTEN), and Wnt-β-catenin [100]. Another genetic alteration is the genetic amplification of HER-2, which is associated with the mammary tumor development and progression in both species. HER-2 overexpression is commonly found in human mammary carcinoma and has also been detected in canine mammary carcinoma [13, 96].

Other studies have shown that deregulation in breast cancer 1 and 2 (BRCA1 and BRCA2) genes is associated with mammary tumor development and progression in both humans and dogs [13, 101, 102]. In normal cells, BRCA1 and BRCA2 genes help ensure the stability of the DNA material and prevent uncontrolled cell growth. In human breast cancer, BRCA2 overexpression is associated with poor prognosis, while downregulation of BRCA1 usually occurs during the progression of sporadic breast cancer [13, 102]. Similarly, in canine mammary carcinoma,

downregulation of BRCA1 in mammary tumors has been associated with malignant phenotype [101], while BRCA2 expression has been shown to decrease in adenomas and increase in lymph node metastases of mammary adenocarcinoma [102].

Another example of the shared etiologic factors between humans and dogs is the hormonal dependence on tumor development. In both species, steroid hormone receptors, including estrogen receptor (ER) and progesterone receptor (PR), play an important role in the development of breast cancer. Earlier studies have shown that a significant percentage of canine mammary carcinoma is also as estrogen dependent as its human counterpart. This suggests that the dog is a useful model for hormonal studies as well as for the development of endocrine therapy for human breast cancer [99]. Furthermore, genetic alteration affecting the P53 gene has been found to be associated with tumor development of both human and canine mammary carcinoma [103, 105]. P53 is a tumor-suppressor gene that plays an important role in cell growth, cell cycle, DNA repair, and autophagy [13]. In canine mammary carcinoma, genetic alterations in the P53 gene are predictors of increased malignant potential and often are associated with poor prognosis [105, 106].

Additionally, numerous prognostic factors that are associated with potential clinical application in mammary carcinoma are similar in human and canine breast cancer [13]. Among them are E-cadherin and P-cadherin. Cadherin complexes play a role in intercellular adhesion. Their altered expression is associated with tumor progression [107]. In both species, E-cadherin and P-cadherin expression in mammary tumors have been shown to play a role in tumor genesis and dissemination [13, 107].

In addition, canine mammary intraepithelial lesions (IELs) have been found to share a strong histological resemblance to those in the human breast [96]. Mammary IELs, such as hyperplasia, atypical hyperplasia, and ductal carcinoma in situ (DCIS), are frequently diagnosed among women today due to routine mammography. Detection of these lesions among women is considered a risk factor that predicts the development of invasive cancer later on [96, 108]. Canine IELs have similar frequency to human IELs, and their resemblance in terms of histology and pattern of ER-α and HER-2 expression to human IELs suggests the canine as an ideal model to study the progression as well as prevention of human ER-negative breast cancer [96]. Above all, studies in human and canine females have revealed that the number of microvessels in malignant mammary tumors correlates with metastasis [109]. Taken together, these studies demonstrate that the canine mammary carcinoma shares many similarities with the human breast carcinoma. This supports its use as a valuable and predictive model for human breast cancer.

Evaluation of Therapeutic Strategies and Preclinical Studies

Breast cancer is characterized by genetic heterogeneity, which makes its diagnosis and treatment challengeable [110]. Despite having similar histological appearance, individual breast tumors can exhibit tremendous variations in clinical presentation, disease aggressiveness, and treatment response in different patient and ethnic populations [111]. Therefore, breast cancer is currently regarded as a heterogeneous disease that has been classified into various molecular subtypes according to the gene expression profile of ER, PR, and Her-2/neu including basal cell-like or triple-negative (ER−, PR−, and HER2−), Her-2/neu (ER−, PR−, and HER2+), luminal A (ER + and/or PR+, HER2−), luminal B (ER + and/or PR+, HER2+), and normal breast-like [74, 112–114].

Other studies were also conducted to classify breast cancer according to the oncogenic signaling pathways, offering an opportunity to individualize therapeutic strategies in a preclinical study using the mouse model [115]. Creation of several transgenic mouse models (such as Myc, Ras, Neu, Wnt, MET-induced tumors, polyomavirus middle T model, EMT, and MMTV-MET) showed a linkage between the histological types and the gene expression profile. Transgenic

mouse models may allow preselection strategies based on genetic profiling for better therapeutic interventions similar to HER-2 preselection for treatment with trastuzumab. Based on the genetic signature, mouse breast tumor models were classified into normal, mesenchymal, basal, luminal, and mixed. The EMT tumors were clustered with p53−/− and DMBA tumors and showed close relation to the triple-negative human model, which makes this model a promising one for therapeutic validations. Contradictory results were observed regarding the luminal mouse models due to ER-mediated differences between human and mouse models. An MPA (medroxyprogesterone acetate) mouse ER- and PR-expressing metastatic model was developed and maintained via syngenic transplants in MPA-treated mice [116]. Also, hormone-independent tumors occasionally grow in untreated mice and some of them respond to some hormonal therapies, such as antiprogestins (RU486), estrogens (E_2), or tamoxifen. Subsequently, several cell lines (MC4, MC4-L4E, and MC4-L4F) were developed from C4-HD (with mutated p53) and C7-2-HI (highly metastatic in axillary lymph nodes and lungs) used to study the stromal–parenchymal interaction and in vitro hormone responsiveness. MPA also showed mammary carcinogenic effect in rats, cats, and dogs. It has moved to group 1 due to sufficient evidence for human carcinogenic effect.

A recent study on 466 tumors collected from 463 patients confirmed the validity of breast cancer models [117]. Based on mRNA expression microarray, DNA methylation, SNP arrays, miRNA sequencing, whole-exome sequencing, and reverse-phase protein array (RPPA) (for 348 of 466), it has been shown that the four breast cancer phenotypes have variable genomic and proteomic features with variable consequences on the corresponding pathways and clinical manifestations. The study revealed variability in both mutation frequencies and types, association of sporadic breast cancer with germline contribution in 10 % of cases, existence of two types of $HER2^+$ subtypes, similarities of basal-like subtype to serous ovarian carcinoma, and many other genomic targeting events. Based on these findings, it is possible to propose and try variable therapeutic interventions.

GEM models assisted in elucidating novel targets for prognosis and therapeutic interventions, evaluating chemotherapeutic agents. They were also combined with other models to evaluate the effect of chemopreventive agents, dietary manipulations, and chemical carcinogenesis [88].

Both DMBA and MNU animal models have been used for chemopreventive studies [118]. MNU 50-d-old rat model was used to test tamoxifen and N-(−4hydroxy)phenylretinamide chemopreventive effect. The model is relevant to humans in proliferation rate and differentiation, it is hormone dependent, does not need metabolic activation, and associated with Ras activation but sensitive to weight loss. DMBA model is also commonly used in chemopreventive studies representing a model which needs metabolic activation.

Semipurified diet with low level of pure lycopene inhibited mammary tumors [119]. Tomato carotenoid inhibited mammary tumor multiplication when injected intraperitoneal (twice a week) 2 weeks before DMBA administration to termination [120]. However, these results were not reproduced in MNU models due to differences in route of administration, preparations, doses, strains, and type of carcinogen [121]. However, animal studies and epidemiological data support that tomato consumption is organ specific with no protective effect on the breast [122].

Animal models are used in preclinical studies to test anticancer drugs [123]. In preclinical phase 0, drugs are tested for safety, efficacy, pharmacokinetic, and pharmacodynamics and to determine the maximum tolerated dose. They have been also used to test molecularly targeted drugs. However, the animal model and species should be selected carefully in order not to over- or underestimate the response in humans. Both in vitro and in vivo models were used to identify genetic signatures of breast cancer metastasis [124]. Evidence points to the inheritance nature of the metastatic characters of tumors and the role of stem cells in determining the metastatic site. Mice models were used to test for drug sensitivity. These studies improved our understanding of the metastatic

process, identified new markers, and shall help in tailoring therapeutic interventions.

Conclusion and Future Perspective

Breast cancer is a heterogeneous disease linked to many risk factors, including endocrine-disrupting chemicals (EDCs) [125], oral contraceptives [126], early life environmental exposure [127], high intake of well-done and processed meat due to the presence of heterocyclic amines (HCAs) [128], and other factors [5, 6, 21]. Animal models have improved our understanding about the development of the mammary gland and the genetic alterations, which occur during mammary carcinogenesis [129]. They also illustrated the role of stem cells in tumorigenesis and targeting therapeutic strategies [130, 131]. However, several variations between animal models and human breast cancer exist. For example, the pathology of EMT tumors differs in mice than that of humans [132]. Canine models also showed similarities and differences with human mammary tumors at the molecular level [133, 134]. However, several studies have shown that the feline and canine mammary carcinoma meet the necessary requirements for serving as natural models of human breast cancer [135–137]. Besides the spontaneous aspect, the close similarities in cancer biology of human mammary carcinoma and mammary carcinoma in canines and felines also support their use in clinical trials as preclinical models. Therefore, the two animal models could be used in carcinogenesis studies and therapeutic trials, which could contribute to a promising advance in the development of new cancer drugs and treatments.

It is difficult to imagine that one model will fit for all studies. Perhaps several models will be used for integrative conclusion. It has been suggested that new models be developed taking into consideration the choice of species and the impact of the immune status, microenvironment, stroma, and ECM (extracellular matrix) [138]. For therapeutic evaluations, it is recommended to apply imaging techniques to monitor the drug response [138], RECIST (Response Evaluation Criteria in Solid Tumors) criteria, histological, transcriptomic, and proteomic studies [139].

Altogether, animal models have been indispensable for understanding the mechanisms of mammary tumorigenesis. However, they must continue to be developed to be able to recapitulate human mammary tumors. The advances in molecular biology techniques and the high throughput data have advanced our understanding of the heterogenetic nature of breast cancer and would advance the use of animal models in therapeutic and preclinical investigations.

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16 In Silico Disease Models of Breast Cancer

Anjana Munshi and Vandana Sharma

Abstract

Breast cancer is a highly heterogeneous disease as a consequence of multiple cells and genetic aberrations. It is the second leading cause of death among women in Western countries. It has been reported that approximately 1 in 8 women is affected by breast cancer and one-third of women die from breast cancer every year. The most common type of breast cancer is infiltrating ductal carcinoma, which represents around 80 % of all malignancies. Recent advances in the area of breast cancer have increased the survival rate of women with breast cancer. The post-genomic area has provided information regarding gene mutations and their effect on pathogenesis as well as on the outcome of breast cancer. A number of interacting biomarkers belonging to different pathways have been reported to influence the progression of breast cancer. However, we need more authenticated and sophisticated technology for early diagnosis and effective treatment in the area of breast cancer. In the past few years, computational modeling or in silico modeling and simulation of disease processes has gained momentum.

Computational models of breast cancer have been developed to aid both biological mechansims and oncologists. The development of in silico models is facilitated by experimental and analytical tools which generate required information and data. Statistical models of cancer at the pathway levels, genomics, and transcriptomics have been proven to be effective in developing prognostics/diagnostics. Statistically inferred network models have been proven to be useful for avoiding data overfitting. Signaling and metabolic models with the knowledge of the biochemical processes involved and metabolism, derived from research studies, can also be

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reconstructed. At longer length scales, agent-based and continuum models of the breast cancer microenvironment and other tissue-level interactions would enable modeling of cancer cells and predictions of tumor progression.

Even though breast cancer has been studied using genomics, transcriptomics, and systems approaches, significant challenges yet remain in order to translate the enormous potential of in silico cancer biology for the betterment of patients suffering with breast cancer, thus shifting the paradigm from conventional population-based to patient-specific cancer medicine.

Keywords

Breast cancer • Computational models • Genomics • Transcriptomics • Statistical models • Continuum models • In silico models

Introduction

Breast cancer is a hyperproliferative disease of mammary epithelial cells and is the second leading cause of death among women in Western countries. Approximately 1 in 8 women is affected by breast cancer, and one-third of women die from breast cancer every year. The most common type of breast cancer is infiltrating ductal carcinoma which represents around 80 % of all malignancies. Breast cancer is a highly complex and heterogeneous disease on account of the consequences resulting from multiple cellular, genetic aberrations and epigenetic interactions. The post-genomic area has provided information regarding gene mutations and their effect on pathogenesis as well as on the outcome of breast cancer.

A number of interacting biomarkers belonging to different pathways have also been established to detect and diagnose the progression of the breast cancer. The potential response to chemotherapy, radiotherapy, and surgical procedures offers additional confounding factors in the physiological behavior of cancer cells [1–4]. In recent years, a number of disease models of breast cancer have been developed. Gupta and Kuperwasser [5] have reviewed in detail the novel models that have particular relevance for drug development in breast cancer. The authors have also discussed the advantages and disadvantages of each model with respect to the various aspects of breast cancer pathogenesis [5].

The convolution of genetic mutations and epigenetic modifications explains the complex nonlinear relationship between molecular state and cellular cancer phenotype and therefore emphasizes the need for integration of this heterogeneous data into in silico models [6–11]. The rapid advancement in experimental and analytical tools has allowed for the simultaneous measurement of thousands of biomolecules, paving the way for in silico model construction of increasingly large and diverse biological systems. Based on the biological scales, different modeling approaches are used which give an insight into the broad array of molecular and physiological events characteristic of the disease [11]. Practical analysis of high-throughput data is used to identify the molecular signatures of cancer phenotype. These signatures are indicative of aberrant function pathways. It can be used to predict the type, stage, or grade of biopsied tumor samples. More advanced methods aim at specifically inferring the structure and/or quantitative relationship among biomolecules within interaction and regulatory network of importance in cancer. Alternatively, kinetic models of biochemical reaction networks are used to simulate the mechanistic details, behavior of metabolism, and signal transduction in cancer therapy.

One of the most important biological systems that mediates cancer development is the local tumor microenvironment. This includes the extracellular matrix, cooperating tumor, and approximate host cells as well as extracellular signaling factors and the metabolic context of local tissues [12–20]. In addition to this, cancers also exhibit other major interactions with somatic tissues, concomitant malignant invasion, and tumor-induced angiogenesis. Additional confounding factors are chemotherapeutic, radioactivity, and surgical procedures. The heterogeneous nature of the tumor microenvironment poses substantial modeling challenges. However, ongoing research has sought to characterize these cancer systems, including continuum and discrete models. The earliest models describing the molecular basis of cancer implicated genetic mutations as causative for malignancy. With the sequencing of the human genome, and now individual cancers, malignant genetic transformations should be studied and modeled in the context of the entire genome. In this chapter, we describe the recent in silico models in cancer in general, and breast cancer in particular, and also examine the direction of ongoing research in cancer systems biology and discuss the opportunities for fundamental biological insights and their potential applications to clinical practice.

Table 16.1 Databases and software for building system models of breast cancer

Databases	Utility
Ensembl UCSC Genome Browser	Genome sequencing data
Entrez Gene KEGG, HPRD, DIP, MIPS, MINT, BioGRID, IntAct Gene Ontology Annotation Database Universal Protein Knowledgebase Genome Reviews	Genome annotation data
Kyoto Encyclopedia of Genes and Genomes Gene Ontology The SEED MetaCyc BioCyc Transport DB	Biochemical pathways and functional associations
Database of Quantitative Cellular Signaling Protein interaction networks Database of Interacting Proteins Molecular INTeraction Database Mammalian Protein–Protein Interaction Database Protein interaction networks Database of Interacting Proteins	Protein interaction networks
Gene Expression Omnibus 50	High-throughput genome-scale data
Stanford Microarray Database	Transcriptomics
Proteomics Identifications Database	Proteomics

Software and Database Resources for Breast Cancer

With the rapid advancement of technology for high-throughput biological measurements, it has become extremely important to construct tools for the distribution, integration, and assessment of this raw information. Multiple online databases have been created to store and distribute the genome-scale data, including proteomics, transcriptomics, and regulatory sequences [21–25]. These resources provide easily accessible high-throughput data, which can be used to construct and validate the cancer models (Table 16.1). Some of the examples of the databases are:

- Cancer Program Datasets Portal: www.broadinstitue.org/gsea/msigdb/
- Molecular Signature Databases (MsigDB): www.broadinstitute.org/gsea/msigdb/
- Achilles Project Datasets 1.0 (1.02 cell lines): www.broadinstitute.org/icbp
- B Cell Interactome: http://amdec-bioinfo.cugenome.org/html/BCellInteractome
- TransfactomeDB: http://bussemaker.bio.columbia.edu:8080/YeastTransfactomeDB/
- Memorial Sloan Kettering Cancer Center: http://www.pathwaycommons.org
- Oregon Health and Science University (OHSU), SAGE Bionetworks, SYNAPSE: heiserl@ohsu.edu

- DREAM Breast Cancer Prognosis Challenge: https://synapse.sagebase.org
- Kmethylome: http://cbbiweb.uthscsa.edu/
- Hormone Receptor Target Binding Loci Database (HRTBLDB): http://motif.bmi.ohio-state.edu/hrtbldb/
- miR2Disease: http://www.mir2disease.org/

Linear Programming

Linear programming-based machine learning techniques are used to increase the accuracy as well as objectivity of diagnosis and prognosis of breast cancer. Linear programming helps the physician and patient by providing the information to plan the treatment that may eliminate the need for the prognostic surgical biopsy procedure. One of the Xcyt image analysis programs has been used to perform the analysis of cytological features based on a digital scan in cancer patients. It diagnoses the image as malignant or benign, along with the estimated probability of accuracy, and predicts the recurrence of cancer. This system was first used by a surgical oncologist in 1993 to classify 131 cases with 100 % accuracy clinically. Another example of linear programming is the use of a recurrence surface approximation (RSA) program for predicting the recurrence of cancer after its surgical removal [26]. Linear programming also gives a probability of malignancy that allows a patient to compare the specific diagnosis with hundreds of cases reported previously.

Statistically Derived Models of Breast Cancer and Molecular Networks

Statistical models of breast cancer can be divided into two types. The first type of model employs unbiased statistical inference using appropriate algorithms, and the second one incorporates a priori constraints of specific biological interactions from data [11]. These models help researchers to develop, quantify, and test various treatment hypotheses quickly and efficiently. Statistical models at the chromosomal, genetic, transcriptomics, and pathway levels provide critical insights into molecular mechanisms and consequences of malignant tissue transformations, despite incomplete information of underlying biological interactions [11]. These methods are helpful in the elucidation of key bimolecular events and pathways involved in oncogenesis. Numerous studies have sought to infer the structure of small- and large-scale bimolecular networks in human cells.

Efforts have been made to craft network-based statistical models of cancers, including breast cancer, in which the architecture of regulatory networks for a portion of the human genome is characterized [27–30], e.g., the Bayesian model. The Bayesian model discriminates between physical and functional interactions between several thousands of genes [31]. The related probabilistic Boolean network formalism model has been used to construct other cancer types, e.g., gliomas [32].

Different transcriptional classifiers have been developed for the identification and discrimination of cancer types, subtypes, and grades: hierarchical clustering, k-means clustering, support vector machines, artificial neural networks, and classifiers based on the relative expression of gene pairs [33–36]. Transcriptomic signatures have also been applied to model relapse and overall survival in different types of cancers and are used to predict the tumor response to chemotherapeutic agents. For example, Gene Set Enrichment Analysis (GSEA) and related tools have been developed and applied to identify pathway perturbations in human cancers on the basis of transcriptomic data including breast cancer [37–39]. These models often start with genome-scale microarray data and through computational, or combined with, experimental analyses derive prognostic classifiers consisting of a lesser number of highly relevant transcripts. The statistical signatures have potential utility for informing small-molecule, radiological, and surgical treatment choices which cannot be measured by standard histopathological and clinical analyses [11]. Statistically inferred network models can be used to study the topology of complex cellular systems and to explain important genetic interactions and

control points [11]. These are used to map the status of regulatory agents into qualitative states. The availability of larger high-throughput datasets encoding different facets of regulatory interactions, combined with innovative methods for their integration, would enable the construction of precise numerical network models [11].

Breast Cancer Risk Assessment Tool (BCRAT) and International Breast Cancer Intervention Study Model (IBIS)

Different breast cancer risk models are used by clinicians for patients considered at average and above-average risk based largely on their family history as well as genetic factors [40]. The Breast Cancer Risk Assessment Tool (BCRAT), based on the Gail model, is used to determine whether a woman meets the minimum risk threshold of a 5-year risk of at least 1.67 % in order to consider tamoxifen for chemoprevention [41, 42]. This is the most frequently used breast cancer risk assessment tool in the United States [43]. Current age, age at menarche, age at first live birth, number of previous biopsies, history of atypical hyperplasia, race/ethnicity, and number of affected first-degree female relatives are included in this model. However, this model does not include information on the BRCA1/2 mutation status or extended family history. In comparison, the Intervention Study Model (IBIS) includes BRCA1/2 mutation status and extended family history, along with other nongenetic risk factors, including age at menarche, parity, age at first live birth, age at menopause, history of hormone replacement therapy used, etc. The BCRAT model has been used in several large cohorts and has been found to be well calibrated for women at average risk [44–47]. However, the short-term and lifetime breast cancer risks assigned to a woman by the BCRAT and IBIS models vary considerably. The BCRAT model tends to assign lower risk than the IBIS model to women who have a strong family history of breast cancer [48]. Therefore, the BCRAT model is not recommended for risk assessment for these women, nor for women under the age of 35 or those with a personal history of lobular or ductile carcinoma in situ. Quante et al. compared the two models and concluded that the IBIS model performed better in a cohort of women whose risks span the continuum of breast cancer risk [49]. Extending models that already capture the extended family history and genetic information like the IBIS model may help risk models play a major role in disease prevention.

Network-Based Models

Computational prediction and prioritization have proven to be complementary to genetic mapping, in terms of integrating existing information on disease biology and relatively unbiased whole-genome measurements [50]. Interdependent interactions of genes and proteins form complex cellular networks—signaling networks, gene regulatory networks, and metabolic networks. The computational models of breast cancer involving these pathway networks provide insights into molecular etiology and consequences of malignant transformations.

For modeling and evaluating, the structure of proteins actively involved in breast cancer, amino acid sequences are retrieved from UniProtKB/Swiss-Prot. This provides descriptions of a set of proteins, their function, domain structure, posttranslational modifications, and variants. Template selection and target structure modeling includes structural homologous entries, obtained for proteins from local alignment search using Basic Local Alignment Search (BlastP). Comparison of homology models with known template reveals similarities between biochemical and biological functions to be inferred. Homology modeling is based on the notion that new proteins evolve gradually from the existing ones by amino acid substitution, deletion, addition, and three-dimensional structures and functions. This method tries to identify structures similar to target proteins via sequence comparison [51].

A brief introduction about networks is important to understand the modeling processes.

A network is defined as an efficient abstraction of biological systems [52]. Nodes and vertices in a molecular network are used to represent biomolecules, such as genes, proteins, and metabolites. Edges or links between nodes have been used to indicate physical or functional interactions, including transcriptional binding, protein–protein interaction, genetic interaction (such as synthetic lethal), biochemical reactions, and many others [50]. An edge on a network (if it happens in the cell) shows that two molecules are functionally related with each other, and the distance on a network is correlated with functional similarity [53]. Network/graph theory provides multiple definitions and tools to measure the distance/proximity between two nodes on a network, which makes network analysis particularly suitable for the quantitative modeling of gene–gene and gene–disease relationships [50]. Network analysis has been found to provide powerful tools to fully exploit the potential in human disease study; for example, in genome-wide screening studies on cancer mutation, it was found that though ~80 mutations can be present in a typical cancer, they tend to fall into a few functional pathways [54]. Network-based approaches have been used to predict the disease genes, with a much better performance than traditional approaches of disease gene prediction.

The discrete mutational events that are found in the cancer genome and epigenome substantially modulate the transcriptional profile within the cancer cells. Models based on these perturbed gene expressions can be applied for the diagnosis and prediction of disease subtypes and stratification of different tumor grades [11]. The Gene Ontology Consortium has been devised as a controlled vocabulary for describing molecular functions and biological processes of genes based on information given in the literature and from available databases. Classification of the mutated gene is available on Osprey [55]. Lin and colleagues identified 50 mutated genes and 77 mutations belonging to calcium ion binding group involved in breast cancer disease [56]. The authors used as- Different models (of multidimensional analysis of mutates genes) of sequence similarity, functional annotation, and protein interactions were used and it was found that five groups were associated with extracellular matrix organization and biogenesis, extracellular matrix cellular cell–cell adhesion, microtubule binding, and actin binding [56]. Different transcriptional classifiers have been developed, including hierarchical clustering, support vector machines, and artificial neural networks. In addition to these, classifiers based on the relative expression of genes pairs have also been developed [33–36].

Disease genes and other information are mapped to the network, and a scoring scheme scores each candidate gene according to its relative position on the network, as well as additional information. The score is supposed to reflect the probability of the candidate gene causing the disease. Finally, all candidate genes are ranked according to the score, and the top genes are predicted as disease-causing genes. The predictability of this proposed approach is often assessed by cross-validation with known gene–disease relationships. Therefore, the scoring scheme is the key to a disease gene prediction method [50].

Cellular Networks

Cellular networks are the core basis of the biological complexity of cancer cells. Cellular networks include:

- *Protein interaction networks:* encode the information of proteins and their physical interactions.
- *Signaling networks:* illustrate inter- and intracellular communications and the information process between signaling proteins.
- *Gene regulatory networks:* describe the regulatory relationships between transcription factors and/or regulatory RNAs and genes and *metabolic networks* of biochemical reactions between metabolic substrates and products [57].
- *Regulatory networks:* consist of hub genes as global transcription factors; they may govern a large amount of genes in response to signals (external/internal).

Jeong and Lee have developed candidate regulatory network in human breast cancer cells and compiled a set of 425 transcriptional factors and

548 signal transduction from the gene ontology site [58]. The curated cluster 1,424 has been found to have 49 genes related to cell cycle and 26 genes related to cell division. The cluster was having activities responsible for cell growth [59]. The authors validated the gene ontology-enriched cluster using the TRANSFEC and HPRD databases. TRANSFEC has transcription factor target relationship and HPRD has information regarding protein–protein interactions.

Networks are presented as directed or undirected graphs. Protein interaction networks are modeled as undirected graphs where nodes represent proteins and links represent physical interactions between proteins. Directed graphs are used to present gene regulatory and metabolic networks. In the case of gene regulatory networks, nodes represent transcription factors or genes, while links represent regulatory relations between regulated genes or transcription factors [57]. Signaling networks are presented as graphs containing both directed and undirected links. In these networks, nodes represent proteins, directed links are used to present the activation or inactivation relationships between proteins, and undirected links are used to represent physical interactions between proteins. Signaling networks are far more complex in terms of the relationships between proteins in comparison to cellular networks, e.g., nodes may represent different functional proteins such as kinases, growth factors, ligands, receptors, adaptors, scaffolds, transcription factors, and others. All these have different biochemical functions and might be involved in different types of biochemical reactions characterizing a specific signal transduction machinery [57].

Gene Set Enrichment Analysis and related tools have already been applied to identify to pathway perturbations in breast cancers on the basis of transcriptomic data [37–39].

Integrative Network Analysis of Breast Cancer-Associated Genes

A particular type of phenotype is the result of a collaborating network of a group of genes, which might not belong to the same functional category. Therefore, the integration of microarray-generated gene lists onto the cellular networks would help in analyzing and interpreting the biological significance of the genes in a network [57]. This provides a structured network knowledge-based strategy to analyze genome-wide gene expression profiles in the context of known functional interrelationships of genes, proteins, and their phenotypes. Mutated cancer genes were studied from literature to uncover their intrinsic properties with the help of a human protein interaction network which was constructed from the entire human genome using an ontology-based method [57, 60]. In this study, a total of 346 genes encoding 509 protein isoforms were mapped onto the network. This analysis showed that cancer proteins have, on average, twice as many interaction partners as other proteins in the network, therefore, implying the evolutionary aspects of cancer genes [57].

Cancer proteins have been reported to display a high ratio of highly promiscuous domains, in terms of the number of different proteins with which they interact. This indicates that they play central roles in many biological processes and mutations in these proteins, which could lead to a higher cancer incidence [57]. The most frequently found domains in the cancer protein population have functionalities particularly focusing on DNA regulation and repair, such as zinc-finger, PHD-finger, BRCT, and paired-box domains (i.e., all transcription factors) [57].

The work carried by researchers in this direction provides a biological insight into the global protein interaction network properties of cancer proteins and uncovers one of the most striking properties of cancer proteins—that cancer-associated proteins are network hubs playing a central role in biological systems. Each hub of cancer proteins reflects a specific domain of a cellular function, which suggests that mutations of an individual or a few hub proteins together may lead to oncogenesis or cancer progression [57]. However, these studies provide little insight into the oncogenic mechanisms, simply because protein interaction networks have limited information compared to signaling networks in which

protein regulatory (activation and blocking) information is encoded.

In a biological system, cells use a sophisticated communication between proteins to perform a series of tasks such as growth and maintenance, cell survival, apoptosis, and development. Signaling pathways are important in order to maintain cellular homeostasis and determine cell behavior. Therefore, alterations in the expression of genes and their regulators would reflect on these cellular signaling pathways, thus leading to tumor development and/or the promotion of cell migration and metastasis. In fact, mutations in genes which encode for signaling proteins have been commonly seen in many types of cancers, including breast cancer [61]. Structural analysis of a literature-mined human cellular signaling network containing 500 proteins has shown that signaling pathways are intertwined to manage the numerous cell behavior outputs [62]. This work provided a framework for the understanding of signaling information processing within the cells. For example, in an examination involving receptor tyrosine kinases, it was observed that the complex and overlapping cross talk involved in signal transduction can be explained by linear combinations of docking affinities for downstream proteins [63]. Furthermore, interactions between microRNAs and the signaling network revealed the principles of microRNA regulation of the network [64]. These approaches hint that an integrative analysis of signaling networks with cancer proteins would highlight the characteristics of cancer proteins [57].

Computer-Aided Early Diagnosis of Breast Cancer

Computer-aided early diagnosis of breast cancer helps the physician to optimize the treatment [65, 66]. In order to improve the accuracy of diagnosis, as well as prognostic risk, a number of computer-aided diagnostic approaches have been proposed for breast cancer. The Bayes classifier combined with feature selection to diagnose breast cancer was applied by Butler and Web [67]. It reached 90 % accuracy by using X-ray scatter images. Abonyi and Szeifert obtained 95.7 % accuracy by applying supervised fuzzy clustering technique [68]. In a study carried out by Osareh and Shadgar [69], the authors investigated the issues of breast cancer diagnosis and prognostic risk evaluation of recrudescence and metastasis by using three well-known classifiers: support vector machine (SVM), K-nearest neighbors (KNN), and probabilistic neural networks (PNN). These classifiers were combined with signal-to-noise ratio, feature ranking method, sequential forward selection and principal component analysis, and feature extraction based on dataset I and gene microarray dataset II, respectively. They concluded that the best overall accuracy for breast cancer diagnosis is achieved equal to 98.80 and 98.33 %, respectively, using support vector machine classifier models against two widely used breast cancer benchmark datasets [69].

Microcalcifications

Clustered microcalcifications have been considered as important indicators of the presence of breast cancer. This system is based on the analysis of optimized visual examination of certain cancer indices. The detection of microcalcification is implemented via an algorithm based on (a) high-pass filtering, (b) variance normalization, and (c) adaptive filtering. Each microcalcification is given an estimated risk based on the flow chart built with expert's rule. The final diagnosis consists of an estimation of risk of the suspected microcalcification cluster. The four main virtual zones of risk include:

- Zone1: risk between 0 and 35 % (benign)
- Zone 2: risk between 35 and 55 % (benign with doubt)
- Zone 3: risk between 55 and 70 % (malignant with doubts)
- Zone 4: risk between 70 and 100 % (definitely malignant)

The image-processing algorithms have helped in revealing microcalcifications from the noisy and low-contrast mammograms.

Cryosurgery

Cryosurgery (also called cryoablation or cryotherapy) is currently used as the surgical method to treat localized tumors because of its advantages over other applications. Optimization and even integration of patient-specific modeling, meshing, thermal analysis, post-processing, and prediction of the treatment outcome into a single software have become essential. In a study by Jung, a computerized treatment planning tool was developed for cryosurgery of breast cancer, taking into account patient-specific diagnostic information [70].

Finite Difference (FDTD) Modeling of Breast Cancer

Microwave-based imaging is the most promising technology to detect breast cancer. This technique exploits the dielectric constant between normal and malignant breast tissue at microwave frequencies. Finite difference (FDTD) modeling is a numerical modeling technique used to model the propagation of electromagnetic waves in biological tissues [71]. The FDTD model critically represents the dielectric properties of normal and cancerous breast tissues and helps in the detection of the cancerous tissues. In a study carried out by Lazebnik et al., it was shown that the Debye parameters can be readily incorporated into numerical breast phantoms used in breast cancer detection and treatment applications [72].

Correlating Protein Interaction Network and Phenotype Network to Predict Disease Genes (CIPHER)

The data, including phenotypic similarity and protein networks, can be used in CIPHER (Correlating protein Interaction network and the PHEnotype network to pRedict disease genes), with drastically different formulation [73]. In this study, the researchers have chosen to directly model the correlation between disease phenotypic similarity and gene functional relatedness and have used the correlation to prioritize candidate genes [73]. The CIPHER approach has been found to accurately pinpoint the true disease genes from linkage loci or from the whole genome. CIPHER can be applied to de novo discovery without any modification, that is, to diseases without known disease genes (without mapped locus or with mapped but uncharacterized loci). In a case study of breast cancer to demonstrate CIPHER's ability in de novo discovery of breast cancer genes, 16 known breast cancer genes were treated as non-breast cancer genes. The whole human genome is prioritized by CIPHER.

While using a shortest path measure of distance (CIPHER-SP), the well-characterized breast cancer gene BRCA1 was ranked at the top, and the other 10 of the 16 genes are ranked in the top 300, roughly the top 1 % of the human genomes. Additionally, among the top 10 % of the prioritized human genomes, the de novo prioritization has identified 15 genes which have been suggested recently among novel breast cancer genes, including AKT1, ranked at 27. ATK1 is a novel oncogene, and a transforming mutation has been identified in human breast, colorectal, and ovarian cancers [74]. Therefore, this case study shows that all the advantages of CIPHER enable us to perform genome-wide candidate gene prioritization for almost all diseases, including breast cancer, leading to a comprehensive genetic landscape of human diseases [73].

Biochemical Reaction Network

Biochemical reaction networks are constructed to represent the relationships between genes, proteins, and the chemical interconversion of metabolites within a biological system of cancer cells. Biochemical networks are better in contrast to statistically inferred networks. In these models, network links are based on preestablished bimolecular interactions rather than statistical associations; significant experimental characterization is thus needed to reconstruct biochemical reaction networks in human cells. These biochemical reaction networks require, at minimum,

knowledge of the stoichiometry of the participating reactions. Additional information such as thermodynamics, enzyme capacity constraints, time-series concentration profiles, and kinetic rate constants can be added to construct more detailed dynamic models.

Stoichiometric Models

Stoichiometry is the study of the balance of energy and multiple chemical elements in biological systems. The stoichiometric model is the most basic mathematical representation of a biochemical reaction network. This model is helpful in explaining the interconversion of biomolecules purely in terms of a number of reactants and products in a biochemical reaction. The generation of stoichiometric models and analysis of their properties is a well-established process [75–77]. Genome-scale models of metabolism have been completed for a diverse range of organisms, including prokaryotes and eukaryotes [75, 78]. Among these, the most important is the reconstruction of human metabolism at the genome scale [79, 80]. Additionally, methods have been developed for reconstructing signaling networks; transcriptional, translational networks; and regulatory networks [79–82]. These models are analogous to reconstructed metabolic networks [11]. The reconstruction of stoichiometric equations can be represented mathematically to form the foundation of a genome-scale computable model [11]. Computational tools have been used to interrogate the properties of reconstructed network in silico and to facilitate the model-driven validation and refinement [83]. Generally, a stoichiometric network operates under the application of physicochemical and environmental constraints in the form of balances such as mass, energy, charge and bounds (flux capacities), and thermodynamic constraints [11]. The statement of constraints defines a solution space which comprises all of the nonexcluded network states, thereby describing possible functions or allowable phenotypes.

Constraint-based analysis of biochemical reaction network has been applied to a number of human systems. Using the reconstruction of human mitochondrial metabolic network, linear programming and random sampling have been applied to identify candidate steady states of the network under normal, diabetic, ischemic, and dietetic conditions [84]. In a study, the Monte Carlo sampling of flux spaces was used to study the enzymopathies on a human erythrocyte metabolic network [83]. The completion of a global reconstruction of the human metabolic network represents a significant milestone in systems biology. This is comprised of 1,496 genes and 3,798 reactions divided into 88 metabolic pathways and paves the path for reconstruction of metabolic models of all 200 cell types in the human body and their modified forms in various types of cancers.

Modeling Metabolism in Cancer

The utility of reconstructed metabolic models for cancer research depends on a number of challenges. First, the refinement of the global human metabolic map is essential for maximum accuracy. Second, each of 200 cell types in the human body exhibits only a fraction of the full metabolic capability contained within a genome [78]. The highly undetermined activities of metabolic enzymes clearly indicate the need for learning even more about this well-developed cellular process. In particular, cancer is known to exhibit diverse metabolic phenotypes compared with their progenitor cells, with an accelerated rate of metabolic activity observed in the most aggressive malignancies [85]. Similarly, multiple hallmarks of breast cancer—including angiogenesis, apoptosis, and avoidance of immune detection—have been linked to tumor metabolism [86, 87]. Metabolic targets have also been used for chemotherapeutic agents developed for the treatment of cancers; therefore, metabolic networks have the potential to be a rich focus for modeling of breast cancer disease.

The metabolic alterations in cancer can be used to predict the selective drug targets in cancer through metabolic networks. Folger et al. developed the first genome-scale network model

of cancer metabolism, validated by correctly identifying genes essential for proliferation of cancer cell lines. The authors predicted 52 cytostatic drug targets, of which 40 % were targeted by known, approved, or experimental anticancer drugs, and the rest were new. It also predicted the combinations of synthetic lethal drug targets, whose synergy was validated using available drug efficacy and gene expression measurements across the NCI-60 cancer cell line collection. The potential selective treatments for specific cancers that depend on cancer type-specific downregulation of gene expression and somatic mutations were compiled [88].

Kinetic Models

More dynamic models are required to quantify many important molecular processes, e.g., feedback regulation, competitive inhibition, post-translational modification, and transcriptional regulation. Biochemical reaction networks have been used as the basis for forming dynamic differential equation models with the addition of kinetic rate constants [81, 89, 90]. These models are composed of a set of ordinary differential equations (ODE). In these ODE-based pathway models, the rates of production and consumption of individual biomolecular species are described in terms of mass action kinetics (with forward and reverse rate constants). The Michaelis–Menten approximation or timescale separation is applied. Kinetic models are useful because they do not employ a steady-state assumption as constraint-based models typically do and thus can simulate detailed dynamic behavior of cancer cells. Dynamic models are also able to account for both the concentrations of compounds and flux through reactions. However, these models are more data intensive to create, as well as more prone to overfitting. Nevertheless, smaller-scale dynamic models have been used to study, in mechanistic detail, key pathways that are related to human cancer. Most of the pathways and molecular components do not function independently to promote angiogenesis but are connected via signaling cross talk, feedback mechanisms, and other forms of up- or downstream regulation. For example, the aberrant activity of the transcription factor NF-κB has been found to be linked to oncogenesis, tumor progression, and resistance to chemotherapy. A computational ODE model was used to identify the role of inhibitor of NF-κB kinase (IκB) isoforms in the temporal control of NF-κB [81]. This model revealed that IκBα was associated with strong negative feedback and fast turnoff of the NF-κB response to Ikappa B kinase (IKK) stimulation, while IκBβ and IκBε decreased the oscillations in the signaling module and also stabilized NF-κB response during longer stimulation [11]. The same model was used to study dynamics of IκB–NF-κB signaling module when cells were stimulated by lipopolysaccharide via Toll-like receptor 4 (TLR4) [89, 91]. Numerous models have been used to investigate the mechanisms governing mitogen-activated protein kinase (MAPK) pathway dynamics using kinetic models.

Microenvironment and Tissue-Level Models

Models of cancer at the tissue level, accounting for function-divergent parameters, can be broadly categorized into "continuum" models and discrete or "agent-based" models [92, 93]. The discrete models are often applied when the number of individual interacting units, such as cancer cells, is constrained to remain small. The continuum model is more practical at population scales where agent-based/discrete modeling can be computationally prohibitive [11]. Both methods of modeling integrate the information of biological context in which cancer develops and, therefore, represent a multiscale consideration of oncogenesis as it occurs within somatic tissues [94].

Continuum Models

In order to mathematically model cell–cell or cell–environment interaction in the context of cancer and tumor microenvironments,

extracellular parameters should be represented as continuously distributed variables. Systems of partial differential equations are used to simulate the magnitude of interaction between these factors, including the effects of hypoxia on the progression of cell cycle, and the impact of mechanical forces on tumor invasiveness, as well as extracellular matrix interaction [95–97]. Studies have examined cell population dynamics within colonic crypts in cases of colorectal cancer [98, 99]. Interaction between stem cells, differentiating cells, and differentiated cell populations are also considered by these models to quantitatively predict tissue-level invasion and the growth of tumor mass. Solid tumors as a multiphase system of both bound and mobile forms have been represented in other models. Such mixture modes take into consideration differential growth and apoptosis rate and also mass transfer and regulatory interaction between the phases. Continuum-based models provide powerful tools to simulate and characterize interaction between extracellular and intracellular factors in oncogenic processes. Rosenthal et al. created a simple continuum mechanical model for cell structure, cytoskeletal remodeling, and focal adhesion formation in breast cancer. Using these models, the authors predicted exclusively detailed experimental features of cell motility [100].

Cellular Automata and Agent-Based Models

Although the multivariate continuum models are able to represent the effect of various physiological and biochemical events on cancer development, these factors are highly heterogeneous and interact discontinuously with tumor cells in situ [101, 102]. The cancer cells are represented as discrete entities of defined location and scale which interact with one another and external factors in discrete time intervals according to predefined roles in cellular automata models. Agent-based models expand the cellular automata paradigm and include entities of different functionalities which interact together in a single special representation, including different cell types and genetic elements, as well as environmental factors [103]. Various phenomena have been modeled using agent-based models. These include three-dimensional tumor cell patterning, tumor–immune system interactions, surveillance, angiogenesis, and the kinetics of cell motility [104–108].

Macklin and Edgerton adopted the agent-based model to ductal carcinoma in situ (DCIS) of the breast [109]. Several applications to test the model's predictive powers were surveyed after developing and testing a patient-specific calibration protocol. The model was used to estimate difficult biophysical parameters pertaining to cell death. The authors estimated the time duration of apoptosis at around 8.6 h by applying a volume-averaged version of the model to histopathological data from normal breast epithelium. This parameter is difficult to observe experimentally. A numerical implementation of the model was used to conduct a parameter study on the time duration of cell calcification, arriving at an estimate of 15 days. The ability of the model to make testable predictions on cell biology was also examined. A Michaelis–Menten-type response of cell proliferation to oxygen availability was predicted by the model. The subsequent analysis of patient immunohistochemistry verified this prediction with excellent quantitative agreement. The authors also found that the agent model also had success in predicting patient-specific tumor sizes in a small group of index cases. In a study by Mukhopadhyay et al., agent-based modeling indicated that radiation-induced premature senescence of normal human mammary epithelial cells (HMEC) most likely accelerated variant HMEC outgrowth through the removal of spatial constraints [110]. This study showed that ionizing radiation can promote the outgrowth of epigenetically altered cells with premalignant potential.

The Ductal Epithelium Agent-Based Model (DEABM) represents two layered mammary epithelial ducts imagined as a portion of duct split open and laid flat. DEABM is a computational model that performs according to the rules of cellular and molecular mechanisms concerning breast duct epithelial dynamics and oncogenesis

[111]. This agent-based model implements DNA damage, repair, cell division, and genetic inheritance and simulates the local tissue environment with hormone excretion and receptor signaling [111]. Therefore, it serves as a model to examine the pathogenesis/oncogenesis of breast cancer.

Translational Models in Use

The combination of mathematics and the general utility of computers have been used to make important insights into breast cancer research and clinical aids. The need to take into account prognostic and clinical factors and the efforts needed to apply inferences to individuals rather than populations have led to the development of artificial neural network (ANN) methods. ANN is an information-processing paradigm inspired by the way the brain processes information. ANN consists of large number of highly interconnected processing system elements working together to recognize patterns [112]. ANN can easily consider variable interactions and is used to create a nonlinear prediction model. ANN involves successive adjustments like synaptic connections using a training set [113]. ANN offers a flexible prediction of survival time as compared to traditional methods.

In a study by Chih-Lin et al., an ANN model was used for breast cancer prognosis, predicting how long after surgery we can expect the disease to recur [114]. The authors compared breast cancer prognosis results in two datasets on the basis of ANN. The results showed that this model can accurately predict the survival probability of each time period after a patient has undergone a surgery [114].

An important ANN, developed by Ravdin, known as "adjuvant" was used to provide the prognosis of early-stage breast cancer after various modes of standard adjuvant therapy [115]. However, one of the drawbacks of this system is that it is not useful for adjuvant antiangiogenic and targeted pathway modalities because of its dependence on mature clinical data. The spontaneous mutation model of acquired drug resistance, which was based on exponential kinetic by Coldman and Goldie [116], was an important theoretical development for our understanding of "adjuvant" chemotherapy.

Another model known as the Norton–Simon model has played an important cultural role that assumes the Gompertzian growth kinetic. While it suffers from the continuous growth flaw, it has nonetheless added to the recent development of dose–dense adjuvant chemotherapy and significant survival gain for certain patient sets [117].

The Polynomial Neural Network (PNN) on Wisconsin Prognostic Breast Cancer (WPBC) dataset is also being used for prognosis of breast cancer. The Polynomial Neural Network (PNN), along with the data preprocessing technique called the principal component analysis (PCA), provides an accurate prognosis of breast cancer [118].

Conclusion and Future Perspective

The combination of mathematics and the general utility of computers have been used to generate in silico models that help to conceptualize, understand the test, and predict the physiological and pathophysiological phenomena being studied. Computational methods have played an important historical role in the long struggle to understand breast cancer, which is still an elusive goal. Recent computational efforts are making some progress in this direction. No doubt that the in silico oncology field holds much promise, but at the same time it faces technical challenges which it must resolve in order to reach its true potential. It must facilitate and accelerate the paradigm shift from conventional population-based to patient-specific medicine.

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17 Systems Biology and Integrative Omics in Breast Cancer

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Abstract

Breast cancer is a complex pathology. The molecular origins of the disease can be traced back to DNA genomic alterations, gene expression deregulation, hormone disruption, metabolic abnormalities, protein failure, and signaling pathway alterations. Lifestyle and other exogenous influences may also modulate the onset, development, and outcome of breast carcinomas and their metastatic events. High-throughput omic technologies provide us with unprecedented tools to study such alterations at an extremely detailed level and have been established thus as essential instruments both in basic and clinical research and in translational medicine and therapeutics. A number of challenges arise when we consider how to interpret and optimize the results obtained from studying the data produced in such massive experiments. Considering this along with the multidimensional nature of the disease calls for new ways of reasoning. One of these new paradigms, maybe even the more relevant of them, is given by systems biology. Systems biology is the name given to the study of biological systems (such as cells, tissues, etc.) when we consider them as integrated units whose constituents parts interact, often in a complex nonlinear fashion. In this chapter, we will consider a number of successful systems biology approaches to breast cancer, firmly founded on the use and integration of data generated in high-throughput omic experiments.

Keywords

Breast cancer • Omics • Systems biology • Molecular pathways • Tumor subtypes • Clinical applications

Introduction

Breast cancer is a neoplasm characterized by highly heterogeneous molecular origins. From the plethora of entangled biological processes

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involved in DNA damage and proliferative increase of tumor subclonal cell populations, there are, however, some key elements that one may isolate in order to construct a semi-mechanistic approach to the disease. Such key elements are usually encompassing modifications in one or more of the following high-level biological processes: global cell metabolism, hormone regulation, DNA repair, transcriptional control, and inflammation.

Since these processes involve a large number of molecular players, the common experimental approach today is the use of omic technologies. High-throughput omic experiments provide us with the tools to study large-scale biological processes at an unprecedented scale. However, this comes with a price. The analysis of such large datasets involves not only the use of advanced mathematical and computational techniques but also of new paradigms to rationalize and prioritize the results in order to ease the understanding of the underlying processes on firm grounds. One such new paradigm that has become central for the advancement of breast cancer research is the one of *systems biology and integrative genomics*, whose aim is to integrate the information obtained from different level omic experiments into one *unified* model able to characterize the *essentials* of the biology behind. In what follows, we will present a review on state-of-the-art systems biology and integrative genomics as applied to the field of breast cancer research.

The Systems Biology Paradigm

A primary goal in systems biology research is to reach a deep understanding of the mechanisms behind biological function, in particular of the intricacies that give way to organismal phenotypes based on molecular interactions. In order to accomplish such a goal, high-throughput omic technologies (HTOTs) are to be combined with large-scale mathematical modeling [1, 2] and ad hoc computational techniques [3–5]. A fundamental issue in contemporary omic studies under the systems biology approach is that of data integration. Data integration paves the way to *making sense* out of the huge datasets generated in HTOTs, a nontrivial task since even electronic managing of such large amounts of information [6] represents a challenge known as the *big data* paradigm [7].

Notwithstanding this, our current understanding of the organization of living matter into *systems* points out to the necessity of integrative frameworks generally referred to as the systems biology approach. In fact, it has been said elsewhere that in view of "…the continuous advent of novel techniques in high-throughput molecular biology and the 'omics maybe just one thing has been established: Complex biological systems need to be studied from several standpoints to unveil the actual mechanisms behind them…" [8].

In a nutshell, the systems biology approach consists of considering biological phenomena as *systems* conformed by a number (usually quite large) of components (usually quite complex themselves) interacting on a wide variety of fashions to give rise to the functional features of the system. Cell behavior, for instance, is based on the interplay of genomic information; gene expression profiles; protein abundance, structure, folding, and assembling patterns; cell signaling mechanisms; and complex biochemical reactions [9]. Hence, the complex interaction of DNA, all different RNAs, and proteins in transcriptional, metabolic, and signaling networks [10] is deemed responsible for the cell's organismal functioning [11–13]. In fact, in some cases (notably in tumor cells), even cell populations or microenvironment needs to be accounted for [14–16]. To have a glimpse of the complex relationships one may find with the aid of the systems biology approach, Fig. 17.1 shows a number of characteristic (and often interdependent) issues of complex biological systems.

HTOTs provide us with tools to measure each one of these types of data, in a multiplexed yet single-issued manner (i.e., we can have experiments done to measure the whole transcriptome or the proteome, even the [protein] interactome); for this reason, integrative frameworks [17, 18] are needed to organize and interpret experimental data [19, 20] in order to provide a fuller,

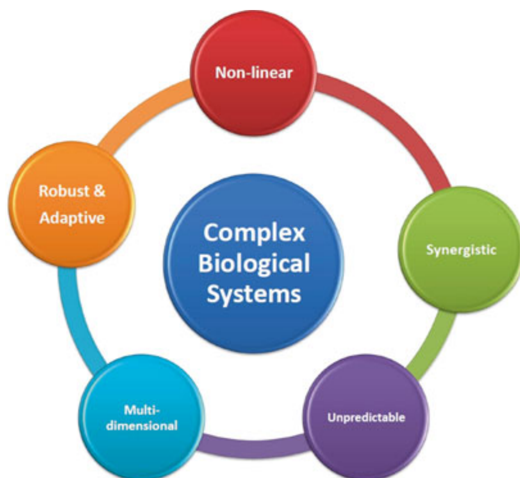

Fig. 17.1 Some characteristic issues of complex biological systems. We can notice that these are interdependent phenomena that cannot be treated separately, hence the need for integrative methodologies

deeper understanding of the way different pieces of evidence should be put in order to fit within a given biological mechanism or at least to an approximate model [21] of it [1, 8, 22, 23]. In reference [17], it is described as a multilevel data integration platform which integrates data about genes, transcripts, and proteins reported in literature as altered in breast cancer cells. Besides the data integration, the database provides an ontology-based query system and analysis tools related to intracellular pathways, protein–protein interactions, protein structure, and systems modeling in breast cancer. The rationale behind this effort is that cancer complexity needs to be studied from such an integrative standpoint as the one provided by systems biology. This is, in our opinion, the kind of studies that may provide a stronger improvement in cancer research in the future. In order to have a comprehensive view of the different omic technologies used in breast cancer research, some of them are shown in Fig. 17.2.

These challenges are specially intriguing when it comes to cancer since the complexities associated with tumor phenotypes often call for deeply multidisciplinary approaches [23–25] in order to be properly disentangling the systems' complexity to give birth to intelligible models useful in both the basic research and clinical settings. When approaching biological behavior integrating from these multidimensional studies, one is often led to reconsider well-established *foundational principles* such as the role of oncogenes [26, 27], the nature of transcriptional master regulators [28], the possibility of *rewiring* systemic metabolism to make anticancer therapies more effective [29–31], or even an entanglement of these three issues [32, 33].

For instance, when discussing the role of master regulator genes (MRGs) in breast cancer, a team led by Califano [28] presented a probabilistic modeling, computational systems biology approach that is able to infer robust prognostic markers by the identification of MRGs, causally related to the presentation of the phenotype. This way, they were able to find groups of upstream transcription factors whose expression patterns correlate with the transition from the normal cell phenotype to the malignant one. They look up for MRGs since gene regulatory networks (GRNs) work commonly as *amplification cascades*; thus, genes that are most differentially expressed tend to be further downstream from the somatic events underlying phenotype differences. These downstream genes are also often less stable, as many cofactors and potential noise sources are involved in the transcriptional cascade that leads to their differential expression. One may recall that oncogenes and tumor suppressors are not generally the most differentially expressed genes.

In order to face such tremendous challenges, systems biology has developed a multidisciplinary approach based on several milestones. In essence, biological discovery in systems biology is based on tools aimed at a global study level of the different HTOTs; such methods are means for information classification, quantification, computation, visualization, archiving, and retrieval. A particularly useful paradigm is that of complex networks. Under the network view, biological systems consist in a (usually very big) set of elements or components in the form of biological molecules: DNA segments, RNA transcripts, enzymes and other proteins, biomolecular complexes, molecular machines, etc. These components interact through a variety of mechanisms: gene regulation, DNA–protein interactions,

Fig. 17.2 Some high-throughput omic technologies (HTOTs) that are commonly used to probe the complexity of breast cancer biology. It is by integrating several of these data sources that systems biology builds a better understanding of the often complex interrelationships present at the molecular basis of breast cancer

RNA–protein interactions, metabolism, protein–protein interaction, biochemical pathways, and so on. The components are the nodes in these bionetworks, while the interactions are the links. Systems network biology is built upon two disparate but complementary ways to construct such networks, often referred to as the *bottom-up* and *top-down* approaches [5].

In the bottom-up (sometimes called kinetic) view, models are developed based on the integration of information already available in databases, and then such models are tested under a variety of experimental conditions. In the top-down (or probabilistic) approach, a data-driven process is used to infer the correlation (or interaction) structure of the networks starting with massive data from HTOT experiments; then a model is built that can be tested. Both views are complementary to each other. The first one serves to assess model fine-tuning, whereas the latter is aimed at new discovery. The top-down approach may be exemplified with probabilistic inference of gene regulatory networks [34, 35] and their theoretical fundamentally thermodynamical characterization

[36, 37], while the bottom-up approach may be devised in metabolic reconstruction [5] or mathematical models of well-known signaling pathways [1].

Classification methods for the discoveries made through either top-down or bottom-up systems biology are needed. Most of these methods are based on computational learning techniques trained with the data in public databases such as NCBI's GEO [38] which is a repository for whole-genome gene expression (and some epigenomic) experiments or the catalog of biochemical pathways in the Reactome database [39]. An archetypic example of such methods is the Gene Set Enrichment Analysis or GSEA [40–42]. GSEA is a computational method to evaluate whether a given set of genes (a gene set) is differentially expressed between two different phenotypes (i.e., it is *enriched* in one of these). GSEA may be appropriately called a systems biology method since it is based on the recognition that genes do not act by themselves at an individual level, but rather there exist synergetic, cooperative effects in their biological function mechanisms. Such cooperative effects are at the basis of the whole systems biology view [40].

Integrative Omics in Breast Cancer

The brief introduction to systems biology given in the preceding section has likely made clear the point that a technological milestone for the development of such systems-based approaches was the rise of high-throughput omic technologies. HTOTs have paved the way to a global understanding of biological phenomena that extends from the relatively straightforward (but extremely important) task of clinical and phenotypical classification to a series of deeper, more complex issues related to the mechanistic approaches of systems-level behavior of biosystems and may extend even to the (relative) control of such systems by therapeutic interventions. This section will be thus aimed to describe some of the more important applications of omic technologies in the study of breast cancer; later we will analyze the impact of such single omic approaches in a global systems biology view of breast cancer phenomenology.

One of the first applications of omic technologies (in particular of the microarray-based whole-genome gene expression analysis) was the search for molecular signatures that may be used in the phenotypic profiling of breast cancer tumors aimed at predicting clinical outcomes [43–49] or response to chemotherapeutic agents [50]. Gene expression profiling has also been widely applied to improve prognosis [49, 51] and in the subclassification of tumors [15, 52]. Gene expression profiling has also been used to determine correlation of certain molecular phenotypes with breast cancer metastasis [53].

Other HTOTs have been used to analyze breast cancer tumors. Carroll et al. [54] performed an exhaustive (genome-wide) analysis of all possible binding sites for the estrogen receptor (ER). This study's results were of foremost importance since ER is a hormone-activated DNA-binding transcription factor which is differentially regulated in breast carcinomas. Under low-to-normal hormone levels, ER is usually located in the cytosolic region. However, when estrogen binds to the receptor, it enables the migration of the receptor from the cytosol into the nucleus and eases dimerization of the receptor and binding of the ER dimer to hormone response elements in the DNA, leading to (abnormal) transcription activation. Functional tests have proved that ER is extremely important for global gene expression deregulation, and metastasis in breast cancer and as such is determinant to evaluating the patient's response to therapy and ultimately its final outcome.

In the case of proteomic approaches to breast cancer, these have also been used for the search of biomarkers, in particular due to the fact that oral-based proteomic tests may be relatively easy to perform since they are noninvasive. Some years ago, Sugimoto et al. [55] carried out a whole-proteome metabolomic study that revealed specific protein profiles not only in breast cancer but also in oral and pancreatic tumors. Their approach was based on capillary electrophoresis mass spectrometry of saliva. Other proteomic studies in breast cancer have been aimed at discovering

risk factors, such as the case of the determination of adiponectin levels and their connection with an increase of breast cancer risk [56]. Similar studies have been carried out in relation with other tumors, such as Teiten et al.'s studies of differentially expressed proteins in cancer cell lines treated with curcumin [57].

Omic approaches to cancer have also been focused on genomic instabilities, chromosomic rearrangements, copy-number variations, and other DNA mutation abnormalities. For instance, Hicks et al. have found that certain patterns of genomic rearrangement can be associated with the survival of breast cancer patients [58], while the role of mutations in the gene PIK3CA is now associated with a higher prevalence of breast cancer [59]. Other high-throughput sequencing approaches point out to UTR reorganization in breast cancer [60], a fact that may be relevant when considering the patterns of genomic reorganization found precisely in epithelial breast cancer cells—for instance, as they have been revealed by comparative genomic hybridization arrays [61].

Systems Biology Approaches to Breast Cancer

As we already mentioned, one of the paramount issues within the systems biology approach is data integration [62]. At this point, it is clear that by developing strategies that allow a research team to integrate the individual pieces of evidence provided by different HTOTs, much can be gained with regard to actually disentangling the complexities associated with life. Due to the multifactorial nature of breast cancer, being a pathology that involves (at least) metabolic and hormonal deregulation, genomic instability, inflammation, abnormal immune response, chromosomal rearrangements, mutations, gene expression anomalies, signaling cross talk, and protein folding abnormalities, one may see that it is a natural candidate to be studied under a systems biology view.

We may start by recalling the work by Sun et al. [63] that perform an integrative analysis of gene expression, methylation profiles of CpG islands, and copy-number alterations in breast cancer cells, linking such functional genomic anomalies to mutation rates as called by deep sequencing experiments in those very cells or the efforts to categorize the effects that focal amplifications of genomic regions have in homozygous deletions and other sequence alterations not only in breast cancer but also in some types of colorectal tumors [64].

There is, of course, a need for computational strategies to integrate genomic data with proper mathematical models [34, 65, 66] and that considering such issues as microRNA targeting [67] and genomic network modularity [68], as well as data mining and integration techniques [69], leads to the identification of novel biochemical pathways [70] and other molecular mechanisms of oncogenesis [71] that, although not unique [72], may lead to the improvement of our current understanding of breast cancer biology [73]. Particular emphasis should be paid to those integrative omic approaches [74] that could result in better prediction of patient outcomes [75–77]. Such integrative studies possess a very broad spectrum ranging from the influence of major chromosomal breakpoints in epigenomic regulation differences in breast cancer [78] to the role played by plant homologs of breast cancer genes [79], in particular with regard to the interplay of these in cell differentiation and proliferation [8, 80]. In this respect, a number of biochemical pathways pertaining to cell signaling, gene regulation, and other biological functions are involved in breast cancer phenomenology; in Fig. 17.3, we can see the fundamental molecular pathways determining the evolution of breast cancer along with their relation either direct (pathway cascading) or indirect (pathway cross talk).

In relation to metastasis prognosis, reference [77] discusses a common conundrum in breast cancer genomic studies: i.e., that most mutated genes are not the ones with statistical significance in their differential expression patterns. We can see that genes such as P53, KRAS, HRAS, HER-2/neu, and PIK3CA, though not always significant in expression differences, play a fundamental role through the protein

Fig. 17.3 Main molecular pathways involved in breast cancer tumorigenesis and metastasis. Direct relationships among such pathways (or pathway cascading) are shown in small, *bold arrows*, whereas indirect entanglement of biochemical routes or pathway cross talk is shown in *long broken arrows*

network by interconnecting many expression-responsive genes. Thus, in order to find out the concerting mechanisms, they overlaid the expression values of each gene with its associated protein in the protein interaction network and then look for subnetworks whose expression profiles were more fit to discriminate for metastasis. The discriminative potential of a candidate subnetwork was computed based on the mutual information (MI) metric between its activity score and the metastatic/non-metastatic disease status over all patients: i.e., high MI subnetworks were better at discriminate between conditions. These considerations shed new light in the genotype/phenotype paradigm since the phenotypic changes that are most indicative of breast cancer metastasis are not necessarily regulated at gene expression level.

In reference [66], the researchers have studied anomalous complex patterns of transcriptional regulation and its relation with other genomic alterations as central to carcinogenesis. They claim that "…the interplay of alterations in DNA copy number and epigenetic states is complex, and to understand the full picture data from multiple sources needs to be integrated. Since both copy number and epigenetic alterations result in changes in gene expression patterns, analysis of microarray gene expression data in the context of specific genomic regions is an efficient means of integrating the effects of genomic changes in cancer…."

Dexter et al. [66] even developed a new *soft-constraints* data clustering profiling method called *genomic distance entrained clustering*, which is based on a measure they propose. Such a clustering algorithm is aimed at identifying genomic regions where gene expression is coordinately altered. Aided by such a method, they were able to discover that—contrary to expected belief—high copy number correlates with high gene expression levels, and not all differentially expressed regions showed corresponding differences in DNA copy number, something that suggested them to consider alternative mechanisms of gene expression regulation like epigenetic repression are important in defining altered genomic regions. Such a class of integrative studies lies at the core of systemic approaches to cancer research.

Some systems-level studies are, however, guided by strong hypotheses deeply founded in the results of a variety of omic technology experiments in the past. Paradigmatic examples

are the role of hormone receptors such as ER or the epidermal growth factor receptor (EGFR), which belong to a family of the so-called ErbB of receptor tyrosine kinases since they are known to play a central role in breast cancer phenomenology. Along these lines, Uhlmann and collaborators [81] have analyzed the patterns of global microRNA (miRNA) regulation in protein networks characterizing changes in the cell cycle due to EGFR overexpression in breast cancer cells, since it is well known [82] that EGFR inhibitors may play quite an important role in breast cancer therapeutics. For instance, trastuzumab resistance has been linked to cell cycle dynamics modulated by ErbB proteins (in particular EGFR and Her2/Neu) [83]. Her2 transitions have been long known to have radical consequences for breast cancer patients [84].

The role that specific mechanisms of transcriptional regulation will play in breast cancer signaling was decoded by Uhlman et al. [81]. They make use of a combined strategy to analyze the multiple miRNA–protein interactions that regulate cell proliferation in response to EGFR. Such kinds of systems biology studies lead to an unprecedented view of the combinatorial effort of miRNAs to control a signaling pathway. This may be of central pharmacogenomic importance, since oncogenic pathways commonly resist inhibition signals by single regulators. Combinatorial analysis of this kind could enable us to discern the molecular basis for selecting either individual miRNAs or small sets of these with combined activity that may be used to treat breast tumors by transcriptional regulation targeting. In particular, EGFR pathway is quite relevant for breast cancer therapy, but a number of additional downstream signal transducers, such as RAS, AKT, or CDKs, are also of some importance.

Protein interaction approaches go far beyond while suggesting a definite oncogenic role to ErbB heterodimers. In particular, Holbro and collaborators have shown that ErbB2-positive breast tumor cells require ErbB3 to enhance their proliferation rates [85]. The oncogenic role of ErbB proteins in epithelial breast cells gets further support, since blocking heregulin expression (thus difficulting ErbB heterodimerization) inhibits breast cancer tumorigenicity and metastasis [86]. Further functional genomic experiments show that cell signaling is anomalous in the presence of specific kinase–kinase inhibitors [87]. An important connection can be noticed between ErbB protein modifications and signaling in breast cancer, since it has been proved that low (or null) levels of estrogen receptor signaling may give rise to differential methylation profiles (epigenetic silencing) of downstream targets [88].

This kind of signaling cascade results is extremely important to understanding breast cancer phenomenology. In fact, chemical immunoprecipitation studies have recently shown that breast cancer-associated gene regulatory networks possess a hierarchical structure; indeed, they go beyond and hypothesize a central role in the hierarchy that is played by processes centered in estrogen receptor activity [89]. Other important hubs in transcriptional networks have been already identified; among these, we can mention E2F transcription factors [90], ATF3 [91], and a broad family of MAPK-structured transcription elements [92].

Estrogen receptor alpha (ERα) is an intermediary in the process of genomic transcription regulation in breast cancer cells. The actual mechanism involves nuclear-initiated steroid signaling and non-genomic activation of various protein kinase cascades. This ER regulation actually carries importance because it is known that tamoxifen-resistant breast cancer cells have enriched expression in gene sets targeted by estrogen treatment as compared with wild-type cells [89].

In connection with the interplay of estrogen receptor signaling in these regulatory networks, a full interactome network of estrogen receptor *alpha*-bounded elements has been identified by means of whole-genome chromatin precipitation experiments [93]. Estrogen regulatory response is, as we already said, of particular importance in connection with currently available chemotherapeutics, since the response to agents like tamoxifen and fulvestrant depends on complex regulatory interactions involving both transcription factor hierarchic networks and epigenomic mechanisms, in particular differential

methylation profiles [94]. A probabilistic network biology study combining the use of HTOT data to probabilistically infer the Bayesian regulatory networks with a topology-driven analysis identified regulatory subnetworks driven by estrogen receptor α dynamics [95]. We cannot stress enough the importance of having such mathematical–computational models relating phenotypic traits (systemic estrogen response) to molecular features, since these may allow the performance of *simulations* to assess the effects of differential therapeutics, thus paving the way to individualized medicine. In this regard, it is worth mentioning that such studies have even pointed out the cyclic effects that *hormone clocks* may have at the onset of breast cancer tumorigenesis [96].

These systems-based studies, however, may go well beyond the descriptive stage to actually have a real impact in the clinic. We can mention as an example how these whole-genome functional genomic studies have identified mechanisms leading to the estrogen activation of the retinoid acid receptor (NR1B1) pathway (a well-known therapeutic target) [97]. Estrogen-mediated ErbB signaling is connected with abnormal proliferative processes in breast cancer [98], and for this sole reason it has been extensively studied at both the transcriptional and translational levels [99]. However, its impact goes much further than genomic regulation, since it is also well known the effect that it can have (by means of the so-called glucose-deprivation network) [100] in the counteraction of the cytotoxic effects of lapatinib in ErbB2-positive breast cancers. ErbB signaling thus has been subjected to extensive computational and mathematical modeling [101] to explore what are the main ligand-response mechanisms behind its complex signaling in breast cancer.

At the multiple-cell (tissue) level, we know that HER2 overregulation is able to inhibit E-cadherin transcription in breast tumors, thus destabilizing the catenin–cadherin complex, causing decreased adhesion. Indeed, high phosphorylation of catenin-d and catenin-g in HER2-positive cells under EGF stimulation contributes to lower levels of cell adhesion and increased cell motility. These facts, together with IGF signaling, may lead to discoveries of considerable pharmacological impact: IGF-1R induces cell proliferation and survival via MAPK and PI3K pathways, a phenomenon related to EGFR signaling in tamoxifen-resistant breast tumor cells by increasing their mitogenic strength indexes [98]. HER2 overexpression thus is able to increase tumor proliferation, angiogenesis, and invasiveness, and its presence correlates with bad outcomes. Pharmacological targeting of Her2 and its receptor kinase complex by using ectodomain binding monoclonal antibodies like Herceptin is often enough to reverse the malignant phenotype.

Apart from the hormone effect of ErbB-estrogen signaling networks, there are other fundamental genes/pathways to be considered when studying breast cancer. One that has recently gained importance is the RhoC GTPase [102] that has been mentioned as a transforming oncogene with the capacity to transform human breast cells into an inflammatory breast cancer phenotype. RhoC activation has even been linked to metastatic processes [103]. RhoC is a RAS homolog and as such is a small GTPase that after activation may regulate response proteins driving the expression of genes involved in cell growth, differentiation, and survival. With RAS being the most common human oncogene, it is no wonder that RhoC has been so widely studied in connection to cancer, in particular since it has been shown that RhoC overexpression is able to control the metastatic potential as well as the abundance of the so-called breast cancer stem cells [104].

Following a very detailed mathematical and computational modeling, Visvanathan and collaborators [1] proposed an integrative systems biology approach to analyze experimental data from the TNFα–NFκB signaling pathway model, integrating in a knowledge-based model database, both mathematical modeling data and literature information as well as biological data. The importance of this study relies on the fact that deregulation and overproduction of TNFα are very important components of many breast tumors; thus, by knocking off its expression by a specific monoclonal antibody, like infliximab, one may induce important drawbacks in the diseased

phenotype. The mechanism of action of drugs such as infliximab involves pathway disruption, in particular the deregulation of the TNF pathway (infliximab belongs to a class of monoclonal antibodies *anti-TNF*): its action involves binding TNF, preventing it from being recognized by its receptor, and then nullifying their cytokine activity as a second messenger. But infliximab is also able to trigger programmed cell death of TNF-activated T lymphocytes. This is relevant for its action against autoimmune diseases, but it is also important for breast cancer due to the role played by pro-inflammatory cytokines in tumorigenesis and even more in metastasis [102].

Their main interest was in shifting from intercellular signals, such as the ones caused by TNFα, to intracellular signals, such as the signal transduction pathway from the membrane receptors of TNFα to the transcription factors AP1 and NFκB and to apoptosis, that is, by following the apoptosis route of TNF extracellular signaling with a view of having more impact in cell populations (i.e., tumors) instead of single cells. They did so by considering protein–protein interaction networks in their pathway mathematical modeling that includes kinetic equations for TNFα–NFκB signaling supplemented with their constants and initial concentrations.

Since glucose metabolism is involved in both tumorigenesis and proliferation in cancer cells, another well-studied set of signaling molecules is the family of insulin growth factors. The double role of IGFBP3 [105] in malignant and nonmalignant epithelial cells and in the transformation of the latter to the former in the presence of integrin receptor complexes led to a hypothesis of *metabolic switching oncogenicity* in breast epithelial cells. Not surprisingly, IGFBP3 molecular activation mechanisms involve cross talk with other oncogenic pathways, such as those involving PKA, RhoC, and ceramide [106]. We already mentioned the systemic role of RhoC; it is noticeable that cross talk with PKA (a protein kinase involved in regulation of glycogen, sugar, and lipid metabolism), and with ceramide (involved in the sphingomyelin signaling but more importantly involved in the protein-recruiting core of processes such as differentiation, proliferation, programmed cell death, and apoptosis).

Estrogen signaling and insulin response are not the only hormone-related processes involved in breast cancer. Recently, the role of deiodinase deregulation in cell proliferation and tumor growth has been mentioned [107], and other thyroid hormones (such as tyrosine kinases) are also involved [108] via heregulin-induced activation [109]. Such genomic processes may be also related to the response to thermal stress in epithelial cells [110] and ultimately to mechanisms of DNA repair [111] such as ATR signaling [112, 113]. Interestingly enough, ATR signaling is negatively regulated by estrogen dynamics [113], so that high estrogen levels inhibit cell cycle checkpoint and DNA repair processes while at the same time is cross-regulated by BRCA1/BRCA2 [112]. Apart from this known functional feature of BRCA genes, it is worth mentioning that they are also involved in stem cell transformation [114] as well as in epigenomic-regulated genomic instability [115].

Also in connection with epigenomics and chromatin modifications, FOXA1 has gained importance in recent times as a candidate target molecule in breast cancer [116] as well as in other neoplasms [117], and it is believed that it is an important molecule, since it may be involved in cross talk of biochemical and signaling pathways in hormone response [117]. Other hormone-related endocrine and paracrine pathways deregulated in cancer have been found through the application of HTOTs studied under a systems biology paradigm. Such is the case of serotonin/prolactin cell reuptake in breast cancer tissues [118], the hormone resistance to PARP inhibitors in triple negative breast tumors [119], and the effects of cancer phenotypes over mevalonate metabolism [120].

Systems biology studies have also enable us to develop prognostic tools that may allow better clinical decisions and more appropriate therapeutic strategies, strongly dependent on patient-specific traits (in particular molecular phenotypes). We can mention the case of a 76-gene signature developed by Desmetd et al. [121] and extensively validated in node-negative

breast cancer patients in a multicentric cohort study. Other omic studies have revealed more specific traits, such as a link between inflammatory processes and survival [122], pointing out to scalability of gene expression signatures (a must for its wide clinical application), specific genes that determine breast cancer metastasis to the lungs [123], and even an integrative panel that reveals hidden relationships between p53 status to whole-genome mutation patterns and ultimately to patient survival [124]. Following similar studies, progression biomarkers have been developed, such as the protein BNIP3 [125], whose tissue levels (and even more important, serum levels) anticorrelate with tumor progression.

It is well known that most fatalities related to breast cancer are not due to the primary tumor but to metastatic processes. For this reason, whole-genome studies of tumor metastasis are extremely important from a clinical standpoint. The role of transcription factor SMAD3 in breast cancer metastasis has been discussed recently [8, 126, 127]. It has been mentioned that the mechanisms of functional interaction behind SMAD3-driven metastasis involve the activation of metastatin (S100A4) mediated by TGF-β [126] as well as antagonistic relationships between SMAD3 and SMAD2 in angiogenic processes [127]. Due to homology between SMAD2 and SMAD3, it is likely that antagonistic interactions arise due to protein moonlighting [127].

Multilevel genomic approaches to breast cancer metastasis have led us to the development of molecular signatures at both the gene expression and protein interaction levels. In the latter case, highly reproducible sets have been designed [128] by taking into account that *different* signature hubs may be altered in *different* patient sets that may affect the dynamics of *the same* pathways associated with cancer metastasis through their interaction neighbors, in other words, that cancer metastasis (some claim that cancer all in all) is not a gene-centered condition but rather a pathway (or network)-centered condition [8]. They find that pathways such as cell cycle, apoptosis, Jak-STAT, MAPK, ErbB, Wnt, and P53 signaling were among the ones more prone to be multi-gene/multi-target deregulated; that is, disease-associated pathways may depend on the co-expression changes of *different signature hubs* with the *same set of neighbors* enriched in this pathway.

For instance, changes of co-expression of interaction neighbors of either IL2 or IL6 might disrupt the Jak-STAT signaling pathway and contribute to breast tumor progression. Gene-centered approaches may overlook this disruption. Such designs are based on the hierarchical hub-centered structure of the associated protein interaction networks. This study implies that changes in the global modularity of the human interactome might be able to improve our understanding of the complex biochemical mechanisms behind breast cancer metastasis. To some degree, this is similar to what one can see in cancer pathways that may be deregulated by either abnormalities in *cancer* genes or modification of microRNAs regulating these genes. Similar network-based studies have also shed light on the rising challenge of therapy-resistant breast cancer lineages, i.e., subclonal cell populations in tumor tissues that acquire resistance to chemotherapeutics such as trastuzumab [129]. These systems-level models may enable (and are actually enabling) clinicians' and basic scientists' cooperation in the development of improved clinical strategies and therapeutic interventions. Some examples that have been to a certain degree successful will be discussed in the following section.

Clinical Impact of Breast Cancer Systems Biology

While it is true that systems biology-based research in the past has been mostly devoted to building and analyzing molecular models of disease (in particular of cancer) under a basic science perspective, in recent times a number of studies have started to move their efforts to more applied, clinical settings [130]. We are, of course, still quite far from the long-promised *individualized therapies* and *personalized medicine*; however, the systems approach to cancer biology is starting to narrow the gap.

To date, most of the clinical applications of systems biology approach belong to either improving the prognosis (and the prognostic!) or pointing out to leads on novel therapeutic strategies. The field of cancer molecular biomarkers is a tough one. It is known that improving the accuracy of prognostic biomarkers may lead to an optimal, target-specific use of coadjuvant therapies, thus improving the patient's quality of life and lowering the associated costs of unnecessary chemotherapeutics. In practice, however, at most 30 % of patients are appropriately diagnosed with available clinical biomarkers [130]. This situation, as we may see, may be drastically changed by incorporating systems biology strategies to prognosis.

A first class of improvement of molecular diagnostics and prognostics of breast cancer relies on the systems analysis of metabolic pathways and systemic alterations such as the ones prescribed by obesity and metabolic syndrome (two common comorbidities of breast cancer). The study of Krebs et al. [131], for instance, presents a clear statistical association between measurements of adiposity and increased risk of breast cancer in aged women, while reference [132] presents a systems-level cohort study that also associates the functional role of adipokines, with the development of insulin resistance and metabolic syndrome, and later with breast cancer onset and recurrence.

In a more detailed-specific molecular model, Gunter and collaborators [133] analyzed the role that insulin and the insulin-like growth factor I (IGF-1) pathway processes have in the clinical risk of breast cancer in postmenopausal women. We may recall that systems biology studies have previously pointed out to IGF signaling as having a fundamental role in breast cancer tumorigenesis. Interestingly, recent studies in papillary thyroid cancer [35] have also highlighted the role that cross talk of apoptotic pathways and IGF signaling may have in oncogenesis [134]. Systemic metabolic deregulation analyses may also allow the development of population-specific biomarkers of breast cancer prognosis; for instance, we may mention the study of Alokail et al. [135] of Saudi women in which biomarkers of metabolic syndrome and stress response correlated quite well with early onset of breast cancer in this population. This study discusses how alterations in the levels of leptin and adiponectin were followed by an increase in breast cancer risk incidence, and they also mention adiponectin as having a prognostic significance in breast cancer recurrence. Omic technologies played an important role in their findings; in this case, serum insulin, adipocytokines, and plasminogen activator inhibitor-1 (PAI-1) concentrations were measured using a customized multiplex Luminex assay. Other low-throughput technologies were used: hypersensitive C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), and angiotensin II (ANG II) were measured using ELISA.

Often prognosis is related to the degree and velocity of metastatic processes. Systems biology approaches have also identified molecular features or patterns that may result in prognostic tools for metastasis. Kim et al. [136] have recently discovered such molecular links between the presence of certain gene expression patterns and lymph node invasion in breast cancer tumors, and their findings even correlate with prognostics and survival estimates as well as with other studies [28, 70].

Important advances in breast (and in general in all) cancer prognosis deal with the discovery of molecular signatures, i.e., with a set of molecules or molecular states that may be measured by means of HTOTs or other laboratory techniques and after analysis may allow the determination of prognostic outcomes. Sophisticated mathematical and computational techniques have been developed to this aim [40, 51, 137, 138]. For instance, Kim et al. [136] discussed how a sudden increase in S1P, a growth-promoting lipid, triggered by the lack of p53, may be a regulator of proliferation, apoptosis, migration, and angiogenesis in breast tumor cells and is thus a potential therapeutic target.

We have already discussed the Gene Set Enrichment Analysis [40]. In reference [137], Carrivick and coworkers developed a statistical analysis tool based on the Bayesian correlation calculations in gene expression datasets from

whole-genome microarray experiments. Since expression microarrays are established as powerful clinical aids (next to bed in some cases), such discoveries have enormous importance. Molecular signatures of breast cancer have allowed for prospective identification in some cases [51, 138], allowing large improvements in the clinical decision-making process, and have hence established themselves as fundamental tools, in particular for extremely aggressive metastatic phenotypes that often result in mortality because of misdiagnosis and poor prognosis and therapeutics.

In the particular case of breast tumors, one of the factors causing such poor prognostics and diagnostics is the strong heterogeneity of the tumor cells, a challenge for molecular pattern-recognition strategies. However, likely the strongest achievement of systems biology approaches to breast cancer has been the discovery of different tumor subtypes. The presence of one of these subtypes or the other depends on a number of molecular features such as patterns of DNA copy-number variations [139] that have an impact on both gene expression signatures and clinical/pathological aspects of the malignancy, histologic grades [140], and again gene expression signatures [15, 76]. There is a large, yet not comprehensive, set of subclassifications of breast tumor subtypes; however, the one by Perou and collaborators is perhaps the more consistent [15]. In Fig. 17.4, we present a quick view of such classification.

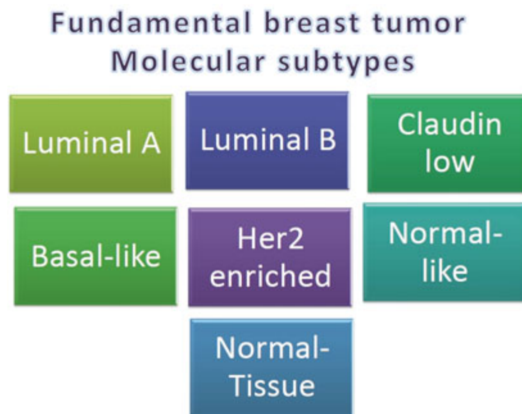

Fig. 17.4 Fundamental breast tumor molecular subtypes according to the classification of Perou et al. [15]. Tumor subtypes are color coded according with their approximate molecular likeness (i.e., closer color and brightness level indicate closer molecular profile). Color coding is, however, not quantitatively reliable, but is used just for illustrative purposes

Prognostics aside, the real concern of the clinicians is therapeutics. Also in this aspect, systems biology approaches may have resulted in significant advances. In Fig. 17.5, we can see an illustrative table of drugs designed as therapeutic agents to treat breast cancer and that instead of trying to induce generalistic responses (e.g., apoptosis) are designed to trigger (or to block) the action of specific pathways involved in breast cancer and its metastasis.

Considering the already discussed connection of breast cancer with obesity and metabolic syndrome, a systems study revealed that adipose tissue may become a *target organ* for the therapeutic treatment of hormone-dependent breast cancer [141]. A related study [142] has stressed that glucose-deprivation pathways are able to nullify chemotherapeutic toxicity induced by lapatinib in resistant ErbB2-positive breast cancer cells. If we consider that inhibition of EGF signaling in ErbB2-positive breast cancers is related to glucose deprivation and energetic stress, then we may consider how cell toxicity induced by lapatinib acts by precisely this mechanism of inhibition of glucose uptake leading to cell-level energetic stress. This mechanism is consistent with the fact that in SKBR3-treated cells, lapatinib is able to diminish (even inhibit) glucose uptake, leading them down the glycolysis pathway, while the resistant cells were not significantly affected.

The close role of estrogen status in endocrine resistance has also led researchers to the discovery of a plausible therapeutic route to overcome resistance via ER-α/PI3K targeting [143], while tyrosine kinase inhibitors may be able to sensitize bad prognostic tumors to hormone-assisted therapeutics [144]. Apart from their role in endocrine deprivation to hormone-dependent tumors, tyrosine kinases (in particular the checkpoint kinase Chk1) have become pharmacological targets in breast cancer [145] due to their central role in cell cycle.

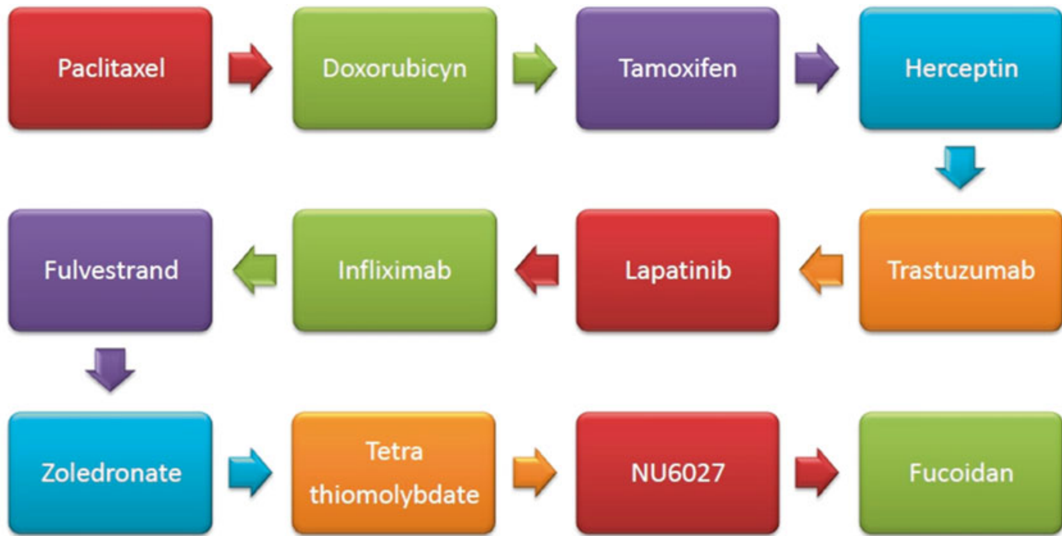

Fig. 17.5 Drugs specifically designed to treat breast cancer tumors and metastasis, in an approximate historical order (see *arrows*). We can notice that current drugs are more and more specialized and oriented to specific pathways and tumor subtypes, in line with the *personalized medicine* paradigm

Cell cycle control is particularly relevant in breast cancer treatment; along these lines, a pharmacological agent called NU6027 has been developed [146], since it has been discovered that it is a strong inhibitor of ATR response in breast (and also in ovarian) cancer cells. However, hormone-enhanced coadjuvant therapy is not for everyone, as some studies have shed light into a selective effect dependent on gene expression signatures [147].

In order to improve the implementation of coadjuvant therapies, one must take into account tumor subtypes and other molecular signatures, in particular the aforementioned role of ErbB molecules. Novel therapeutics have been designed by inducing the switch of *addictions* from HER2 to FGFR2 in Her2-positive breast tumor cells [148], since this may induce salvage after lapatinib resistance. Other related pharmacological targets are based on the interaction between the EGFR and VEGF pathways. Being that these pathways are so entangled and susceptible to cross talk, this approach may open the way to multi-target anticancer therapy [149]. Also related is the problem of the activation of the AXL oncogene that leads to increased levels of resistance to lapatinib therapy in Her2-positive tumors [150], since AXL is a tyrosine kinase receptor protein and thus susceptible to hormone control. Such cases, however, may be treated with coadjuvant tetrathiomolybdate therapy. Since its antiangiogenic effects induce protection against Her2/neu-induced breast carcinoma, it is hypothesized that the mechanism of protection involves hypoplastic remodeling of the mammary gland [151].

Other pathways that may be involved in cross talk and presenting therapeutic opportunities in breast cancer treatment include phosphorylation-dependent ubiquitination; in particular, SKP2 protein ligase is being studied with a view to

pharmacological targeting [152]. SKP2 is a fundamental constituent of the cyclin A-CDK2 S-phase kinase and is an already established proto-oncogene involved in lymphoma carcinogenesis. Skp2 is often overexpressed in p27-deficient breast carcinomas. And it is known that p27/Kip1 is a cyclin-dependent kinase inhibitor responsible for the cyclin control of the G1 phase of the cell cycle. In particular, p27/Kip1 inhibits cell division cycle by proliferative stress. Since SKP2 is an antagonistic of p27/Kip1, its action leads to uncontrolled proliferation. Cyclin-dependent proliferation is known to be a major component of metastasis in breast carcinomas [152]. Apart from cyclin kinase regulation, secondary calcium metabolism may be involved in abnormal signaling in breast cancer; for this reason, zoledronate therapy has been established to be a useful coadjuvant in some instances of breast cancer [153].

Conclusion and Future Perspective

Throughout this chapter, we have been considering quite a number of instances in which integrative systems biology-like analyses have advanced our comprehension of the extremely complex phenomenology exhibited by breast cancer tumorigenesis and metastasis, both at the molecular level and at the cell population tissue levels. This further advance is already bringing some clues and even tools and therapies as to how to treat cancer cells in a better, more comprehensive, manner.

Of considerable relevance is the role played by different omic technologies and in particular by those in the high-throughput end. Big data and complex systems cry out for new ways of reasoning and integrating such enormous corpus of information into a way accessible to the clinician and the pharmacologist, thus paving the way to the highly missed personalized medicine. It is likely that breast cancer with its many tumor subtypes, each one characterized by disparate molecular profiles, is the paradigmatic example of a *systems biology disease*. Our sincere hope is that the information reviewed here may introduce many clinical oncologists and pathologists to the systems biology approach and may narrow the gap between basic experimental and computational cancer biology and the clinical applications.

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Part II

Breast Cancer: Next-Generation Diagnosis, Prognosis, and Therapy

18 Gynecologic Considerations for Women with Breast Cancer

William R. Robinson III and Kaneez Fatima Ali

Abstract

Gynecologists are uniquely positioned to address screening issues and risk factors for breast cancer, based on their special relationship with female patients. The primary technique used for breast cancer screening is mammography, which may be augmented in special circumstances by ultrasound, magnetic resonance imaging (MRI), or computed tomography (CT) scans. Pathologic analysis of a tissue sample, which may be obtained using needle, core, incisional, or excisional biopsy techniques, remains the gold standard for diagnosis. Revised, sometimes controversial, guidelines for screening have been recommended by various organizations in recent years. Hereditary/familial concerns should be addressed by means of pedigree analysis, which may indicate genetic testing.

The utility of prophylactic treatment, including drugs, mastectomy, oophorectomy, or salpingectomy, is unclear and should be based on individual assessments. Fertility issues should be addressed early, often before treatment begins, and the options for fertility preservation are changing rapidly. Menopausal symptoms are common and may be challenging to manage. Certain treatments, including selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs), have predictable gynecologic side effects that should be addressed in advance. Posttreatment surveillance should include monitoring for recurrence as well as for other possibly related malignancies, such as colorectal, uterine, and ovarian cancers.

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Breast cancer • Gynecology • Hereditary cancers • Hormones • Fertility • Menopause

Introduction

The management of patients with breast cancer is complex. Typically, physicians from multiple medical specialties are involved, including radiologists, general and plastic/reconstructive surgeons, medical oncologists, radiation oncologists, and others. However, the interactions of breast cancer and breast cancer therapy with the function of the female genital tract are often underemphasized or overlooked altogether. Oncologists are frequently uncertain of the need for or timing of gynecologic consultation, and gynecologists may be unprepared to address the needs of breast cancer patients. This is clearly an area of unmet need in breast cancer prevention, treatment, and follow-up care.

This chapter summarizes the major gynecologic issues facing women with breast cancer. It is intended to be comprehensive and is based on distinct episodes of care in the course of women at risk for and/or diagnosed with breast cancer. Screening/diagnostic issues, including familial/hereditary concerns, are addressed first, followed by the effects of breast cancer treatment on the normal female genital tract physiology. Fertility and menopause-specific gynecologic side effects of the major therapies for breast cancer are then discussed and, finally, the issues faced by women in follow-up of treatment are examined.

The authors' intent is that this chapter serves as a useful reference for oncologists and other physicians treating breast cancer patients, as well as gynecologists, who will uniformly be faced with women affected by breast cancer in their practice. It is clear that addressing these issues will be of benefit to our patients. They deserve no less than a comprehensive assessment of the impact of this feared disease, which affects more than 10 % of our wives, mothers, sisters, and daughters.

Screening and Diagnosis

Gynecologists are in a unique position to carry out effective screening for breast cancer. Women are typically more accustomed to and comfortable with discussing, revealing, and allowing examination of their bodies by gynecologists than any other healthcare providers. Highly personal concerns (including the breasts and genital tract) are routinely included as part of the standard annual history and physical exam in the gynecologist's office. These factors combine to give gynecologists a unique advantage in breast cancer screening, and it has been reported that gynecologists are the specialty most likely to recommend aggressive screening strategies [1].

Breast cancer is a relatively common disease with 121.9 cases per 100,000 US women. This accounts for an estimated 226,870 cases in 2012. The mortality rate of breast cancer is somewhat lower at 22.5 per 100,000, with 39,510 estimated deaths in 2012. Breast cancer is therefore the most common cancer among women and the second most common cause of cancer death in women (behind lung cancer) [2].

Both the incidence and mortality rates of breast cancer vary by ethnicity. Among US women, Caucasians have the highest risk of developing breast cancer, followed by Blacks, Hispanics, and Asians. In contrast, Black women are more likely to die from breast cancer, followed by Caucasians, Hispanics, and Asians, as shown in Table 18.1.

Breast cancer screening is typically accomplished in the United States by means of mammography. Film-based mammography has rapidly been replaced by digital mammography in the past decade, which will likely become the industry standard in the near future. Interpretation of mammograms is based on the BI-RAD system (Breast Imaging Reporting and Database), a numeric (0–6) scale for predicting

Table 18.1 Incidence and mortality rates by ethnicity in US women

Ethnicity	Incidence (per 100,000)	Deaths (per 100,000)
Caucasian	129	23
Black	120	33
Hispanic	98	17
Asian	85	12

Adapted from the National Center for Health Statistics, Centers for Disease Control

the risk of malignancy from the mammographic appearance. Additional imaging techniques that may be used for screening in selected individuals include ultrasound, magnetic resonance imaging (MRI), and CT (computed tomography) scans. Ultrasound can be helpful in the evaluation of very dense breasts, particularly in young women. MRI can be more sensitive than mammography in some cases, but may miss certain cancers that mammography does not. MRI is therefore used primarily in combination with mammography, usually in women at higher risk for breast cancer based on family history or other factors. CT scans are rarely used in screening. Women with very large breasts and/or very large masses may be asked to undergo CT scan screening.

The diagnosis of breast cancer must be confirmed by a tissue sample, which may be obtained by several different methods. In general, the goal is to obtain tissue sufficient to make the diagnosis using the least invasive process. Fine-needle aspiration (FNA) is a common, clinic-based technique in which a thin (22- to 25-gauge) needle is placed percutaneously into the suspicious area. The site may be located by palpation if a mass can be felt. Image guidance, using ultrasound or mammography, is required for non-palpable lesions. In this situation, a non-hollow needle or marker may be placed to localize the area of suspicion. The clinician then uses the marker as a guide for the FNA. A syringe may be attached to the needle to aspirate a column of cells. Typically, multiple passes are taken from each area of interest. The aspirate is placed on a slide, air-dried, and then "fixed" by spraying or immersion with appropriate solutions. The slide is then stained and reviewed microscopically. The diagnosis can be made while the patient is still in the facility, allowing for counseling and treatment planning to be done at the same visit as the procedure. FNA is highly operator-dependent, requiring a skilled and experienced clinician to obtain consistent results.

Core-needle biopsy is similar to FNA but uses a larger-bore needle and local anesthetics. Core biopsy can also be done in a clinic setting to provide immediate results. Incisional or excisional biopsies are typically done in an operating room setting, with local anesthesia and intravenous sedation. Excisional biopsy requires the surgeon to remove the mass with a margin of normal-appearing tissue around it and is considered the definitive diagnostic method. An excisional biopsy may also be considered therapeutic, if the patient desires breast conservation [3].

There has been significant recent controversy in both medical and lay communities over the current breast cancer screening guidelines. The American Cancer Society Guidelines include the following:

- Yearly mammograms are recommended starting at age 40 and continuing for as long as a woman is in good health.
- Clinical breast exam (CBE) should be performed about every 3 years for women in their 20s and 30s and every year for women aged 40 and over.
- Women should know how their breasts normally look and feel and report any breast change promptly to their healthcare provider. Breast self-exam (BSE) is an option for women starting in their 20s.
- Some women—because of their family history, a genetic tendency, or certain other factors—should be screened with MRI in addition to mammograms.

In contrast, the U.S. Preventive Services Task Force has stated that insufficient evidence exists to demonstrate any benefit of annual mammography done between the ages of 40 and 49 or over 74, including clinical breast exams, self-breast exams, digital mammography, or MRI. They recommend biennial mammography screening beginning at age 50 and ending at age 75.

These recommendations have generated many negative responses from lay and medical spokespersons, with the major criticisms centered on the reduction in the use of mammography [4]. As of this writing, most professional societies, including the American Cancer Society and the American College of Obstetrics and Gynecology, have not made any changes in their recommendations for breast cancer screening. From a practical standpoint, eliminating annual mammography screening in favor of biennial screening between ages 50 and 74 is likely to have unintended negative consequences for women's overall health maintenance and disease prevention. Similar to the Pap smear, women often view the mammogram as part of an annual "package" of health maintenance measures, including a visit to the primary care physician's office. The current reality in the United States is that despite annual recommendations for these tests, many women have them done far less often. If the recommendation for that package of services is decreased from once a year to every 2 years (or an even longer gap), it seems reasonable to anticipate that many women will seek healthcare screening or maintenance even *less* often, if at all.

The gynecologist in practice should individualize screening strategies for each patient, taking recent reports into consideration as part of a frank discussion of the limitations of existing data. It is likely that screening recommendations will be further refined in the coming years as healthcare outcomes research becomes more robust, thereby requiring the gynecologist to review their practices on an ongoing basis.

Familial Risk and Genetic Counseling in Women with Breast Cancer

Genetic Evaluation

The risk to an American woman of developing breast cancer is 1 in 8 during her lifetime, giving the United States one of the highest rates of breast carcinoma in the world. The lifetime risk of an American woman developing breast carcinoma without a single risk factor is 1 in 17. Therefore, US healthcare providers routinely offer breast cancer screening to their patients on a regular basis. Risk factors for development of breast cancer include the following: family history of breast cancer, young age of menarche (younger than 16), age at birth of first child, earlier age of menopause, benign breast disease, radiation, obesity, oral contraceptive use, postmenopausal estrogen replacement therapy, and alcohol use. Unfortunately, risk factors only identify 25 % of women who eventually develop breast carcinoma [5].

Approximately 5–10 % of breast cancers have a familial or genetic link. Approximately 50 % of families with hereditary breast and ovarian cancer syndromes have germ line mutations in BRCA1 and BRCA2, which are responsible for approximately 3–5 % of cases of breast cancer. BRCA1 and BRCA2 are found on chromosome 17 and 13, respectively, and both function as tumor suppressor genes, which encode proteins that function in the DNA repair process. Greater than 1,200 different mutations have been reported for BRCA1, while more than 1,300 mutations have been found in BRCA2 [6]. Patients with hereditary breast cancer inherit one defective allele in BRCA1 or BRCA2 from either parent. If the second allele becomes dysfunctional or nonfunctional, the likelihood of a clinical cancer is very high. Women with BRCA2 mutations may have a lifetime risk of breast cancer as high as 85 % and a 15–20 % lifetime risk of ovarian cancer. Women with BRCA1 mutations have a similar 85 % lifetime risk of breast cancer and a 40–50 % lifetime average risk of ovarian cancer [7].

Approximately 1 in 300 to 1 in 800 individuals in the general population carry a mutation in the BRCA1 or BRCA2 gene. In certain small ancestral groups, such as the Ashkenazi Jews, French Canadians, and Icelanders, these mutations tend to occur more frequently. In the United States, it is estimated that approximately 1 in 40 Ashkenazi Jews carries mutations in the BRCA1 and BRCA2 genes [8].

Routine obstetrical and gynecologic practice should include evaluating a patient's risk for hereditary breast and ovarian cancer syndromes. Screening should involve questions regarding

personal and family history of breast and ovarian carcinomas. Directed screening and prevention strategies may reduce morbidity and mortality from breast cancer by identifying individuals with inherited risk. Genetic risk assessment is recommended for women with greater than a 20–25 % chance of having an inherited predisposition to breast and ovarian cancer.

The following criteria are associated with a risk of being a carrier of a genetic predisposition to breast/ovarian cancer of approximately 20 %. Genetic risk assessment is recommended for these individuals:

1. Women with a personal history of both breast and ovarian cancers
2. Women with ovarian cancer and a first-degree relative (mother, sister, daughter) or two second-degree relatives (grandmother, granddaughter, aunt, niece) with breast cancer
3. Women with premenopausal breast cancer or both ovarian cancer and breast cancers
4. Women with ovarian cancer and of Ashkenazi Jewish descent
5. Women with breast cancer at age 50 or younger or a first- or second-degree relative with ovarian cancer or male breast cancer at any age
6. Women of Ashkenazi Jewish descent in whom breast cancer was diagnosed at age 40 or younger
7. Women with a first- or second-degree relative with known BRCA1 or BRCA2 mutation

Evaluating Family History

Both breast and ovarian cancer-predisposing genes can be transmitted through either parent. Of note, families with few female relatives may underrepresent female cancer. In such cases, it may be reasonable to consider genetic counseling in the setting of breast cancer at or before age 50.

Issues Arising During Genetic Counseling

Genetic counseling for breast/ovarian cancer risk should include a discussion of possible outcomes of testing. Options in terms of surveillance, chemoprevention, and risk-reducing surgery should be discussed prior to testing. Psychological implications of test results must also be considered. The cost of genetic testing may be discussed during the genetic counseling session as this may influence the decisions of patients and family members. Another important aspect to discuss includes current legislation regarding genetic discrimination and the privacy of genetic information [9].

Genetic testing ideally begins with a family member already affected by breast or ovarian cancer. Since mutations are found along the entire length of both BRCA1 and BRCA2, full sequencing of both genes is recommended. During genetic testing, if a specific mutation is identified in an affected individual, a single-test site may be utilized for other family members. Certain ethnic groups are at increased risk of specific genetic mutations. BRCA1 and BRCA2 mutations are more often found in Ashkenazi Jewish, French Canadian, Icelandic, Netherlandic, and Swedish populations. Genetic testing for common mutations among these groups may be utilized as well.

If no mutations are found, patients should be counseled that they could still carry an unidentified mutation, an undetectable mutation in BRCA1 or BRCA2, or their family cancer history could be a result of random chance (no inherited predisposition). Management of women with a strong family history of breast cancer who have tested negative for BRCA mutation must be individualized, but may include many of the same discussions.

Risk-reduction strategies for women at high cancer risk due to BRCA mutations include surveillance, chemoprevention, and surgery. Secondary to the high risk of ovarian and fallopian tube cancer in individuals with BRCA1 and BRCA2 mutations, periodic screening for CA 125 and transvaginal ultrasonography is recommended beginning between age 30 and 35 or 5–10 years earlier than the age of first diagnosis of ovarian cancer in the family. Recommended surveillance also includes clinical breast examination and annual mammography as well as annual breast MRI beginning at age 25 or at the earliest age of onset in the family.

MRI has the greatest sensitivity for the detection of breast cancer. The combination of MRI, mammography, and breast exams has the greatest sensitivity in detecting breast cancer in high-risk BRCA mutation carriers.

Prophylactic Mastectomy

Women with BRCA1 or BRCA2 mutations may be offered bilateral total prophylactic mastectomy, starting at around age 35 or 5–10 years before the age of the youngest affected relative. Prophylactic (or preventive) mastectomy is effective in reducing the risk of breast cancer by approximately 90 % [10].

Reconstruction of the breasts may be done via a variety of methods, often at the same time as the mastectomy. Implants, typically filled with saline, can be placed under the chest muscles. The size of the reconstructed breast is determined by the amount of saline in the implant. Saline injections can be made at 1- to 2-week intervals, allowing the skin to slowly expand in accommodation. Alternatively, autologous tissue flaps can be used for reconstruction. Skin, muscle, and fat can be moved from the patient's back, buttocks, or (most commonly) abdomen to the site of the breast. The transverse rectus abdominus myocutaneous (TRAM) flap is a popular source for the donor tissues [11].

Appropriate counseling prior to prophylactic mastectomy should include discussion of body image issues, the time required for recovery and resumption of normal activities, costs, and the efficacy of the procedure. Breast cancer has been reported in women who had undergone bilateral prophylactic mastectomy, presumably due to residual or ectopic breast tissue that was not visible and therefore not removed at surgery [12].

Ovarian Cancer and Breast Cancer

Mutations in BRCA1, BRCA2, or mismatch repair genes (MLH1, MSH2, MSH6, PMS2) are associated with 5–10 % of all ovarian cancer. The cumulative risk of developing ovarian cancer by age 70 ranges from 16 to 40 % in patients with hereditary breast and ovarian cancer syndrome. BRCA1 and BRCA2 mutations are also associated with primary fallopian tube carcinoma with a lifetime risk of 1.1–3.0 % [13].

Women with BRCA1 and BRCA2 mutations may be offered salpingo-oophorectomy by age 40 or when they have finished childbearing for risk reduction of both breast and ovarian cancer. The diagnosis of ovarian cancer will be established in 2–3 % of women with BRCA1 or BRCA2 mutation before the age of 40. In women with BRCA1 mutations, the risk of ovarian cancer increases during the fourth decade of life and 10–21 % of BRCA1 mutation carriers will develop ovarian cancer by age 50. Women with BRCA2 mutations have a 24–36 % chance of developing breast cancer by age 50. The maximum impact on breast cancer reduction is accomplished by removing the ovaries earlier. Risk-reducing salpingo-oophorectomy on completion of childbearing may reduce ovarian cancer risk by 80–90 % and reduce breast cancer risk by 50–60 %. Of note, salpingo-oophorectomy may not eliminate the risks of ovarian cancer entirely, because some patients may develop primary peritoneal carcinomatosis, which is clinically and histologically indistinguishable from ovarian cancer.

Another potential surgical intervention includes tubal ligation without oophorectomy, which is associated with a 50 % reduction in ovarian cancer risk in the general population [14, 15].

Fertility Issues

Estimates indicate that 15 % of breast cancer cases will occur in women younger than 40 years of age [16]. These young patients often receive chemotherapy in addition to surgery and, as such, are at increased risk of premature ovarian failure. Chemotherapy may also increase the risk of complications during pregnancy including miscarriage, premature labor, and low birth weight. Several options to preserve fertility have emerged along with an increased awareness of these options among patients.

Breast cancer diagnoses should include a discussion of fertility concerns in premenopausal women. Patients should be reassured that

pregnancy does not increase the risk of recurrence of breast cancer. Consultations with fertility experts should be offered prior to the beginning of cancer therapy to determine if immediate intervention is warranted. It is therefore recommended that consultation with a fertility specialist be made at the time of initial diagnosis. The optimal time for fertility preservation is frequently after surgery but before beginning adjuvant chemotherapy.

Chemotherapy is a mainstay of treatment of many breast cancers. The ovaries are quite sensitive to a number of cytotoxic agents, which may induce irreversible damage and destroy great numbers of follicles [17]. Agents commonly used in the treatment of breast cancer include cyclophosphamide and adriamycin (considered moderate to severely gonadotoxic) and paclitaxel (mildly gonadotoxic) [18].

Fertility Options

Ovarian failure and decreased ovarian reserve are some of the issues women with breast cancer may face. Some possible treatments include pharmacological treatment, ovarian transposition, and donor oocytes and artificial gametes.

Pharmacological Treatment

Suppressing ovarian function using a gonadotropin-releasing hormone (GnRH) agonist, which inhibits pituitary gonadotropin secretion, has been reported to minimize gonadal damage [19]. It is recommended that treatment with GnRH agonists begins 10 days prior to the start of chemotherapy and continues throughout treatment. However, patients must be counseled that the efficacy of GnRH agonists is unpredictable.

Embryo Cryopreservation

Cryopreservation involves storing tissues or organs at very low temperatures in order to maintain viability. Embryos may be preserved and stored for future use in patients with breast cancer. Cryopreserved embryos may be used for in vitro fertilization (IVF). The resulting survival for thawed embryos ranges from 35 to 90 %, with implantation rates from 8 to 30 %. According to the Society for Assisted Reproductive Technology, the pregnancy rate with transfer of cryopreserved embryos in the United States in 2005 was 28 %, with the pregnancy rate being 34 % for fresh embryos [20]. Limitations of embryo cryopreservation include time constraints since ovarian hyperstimulation and oocyte retrieval may take 2–3 weeks, possibly delaying the onset of chemotherapy. Another limitation can be the willingness of a patient's partner to take part in IVF treatment and embryo cryopreservation. Further, supraphysiologic estradiol levels from ovarian hyperstimulation may be an adverse factor in patients with estrogen-dependent tumors. Finally, all patients should be encouraged to sign an advance directive for the use of the embryos (including donation, destruction, or research) if the patient chooses not to utilize them or does not survive.

Oocyte Cryopreservation

Oocyte cryopreservation of unfertilized oocytes may be considered as an option for women without a partner who choose not to use a sperm donor for IVF. The cytoskeleton, mitotic spindle, cortical granules, and zona pellucid of oocytes are sensitive to cryoinjury [21]. As with embryo preservation, 3 weeks may be required to stimulate and collect mature oocytes, thus delaying the onset of chemotherapy. The patient's risk of ovarian hyperstimulation is likewise increased. IVF with in vitro maturation from a spontaneous menstrual cycle has been shown to yield pregnancy rates comparable to conventional IVF treatment, but is currently only performed in highly specialized fertility centers [22].

Menopause and Hormone Replacement Issues

Breast cancer treatment is often complex and may include multiple surgical options, chemotherapy, and/or radiation therapy. Menopausal symptoms and premature menopause are frequent side effects of these treatments. The specific

mechanisms of this effect include estrogen receptor blockade (tamoxifen) or downregulation (aromatase inhibitors) [23].

Menopausal Symptoms

Common menopausal symptoms include hot flushes, night sweats, sleep disturbances, vaginal dryness, and loss of sexual interest. Menopausal symptoms may be more acute in premenopausal patients with breast cancer secondary to the acute onset of ovarian failure or suppression [24, 25].

Hot flushes or flashes appear to result from an exaggerated response of the thermoregulatory region of the hypothalamus, induced by decreased estrogen and progesterone levels, leading to an exaggerated response of the thermoregulatory center of the hypothalamus [26]. This stimulates central alpha-adrenergic receptors that modulate core temperature, causing vasodilation and sweating [27].

Vaginal atrophy results from low circulating estrogen levels or use of antiestrogen therapy using tamoxifen or aromatase inhibitors. This effect may lead to decreased sexual interest.

The type and intensity of menopausal side effects from tamoxifen and aromatase inhibitors were compared in the ATAC trial (Arimidex, Tamoxifen, Alone or in Combination trial) which showed fewer vasomotor symptoms among subjects given anastrozole in comparison to those using tamoxifen [28, 29]. Vaginal dryness and dyspareunia have, however, been shown to be more common among women taking aromatase inhibitors compared to those taking tamoxifen [30].

Treatment of Menopausal Symptoms

Lifestyle changes and pharmacological and alternative therapies may be used in the management of menopausal symptoms. The U.S. FDA considers breast cancer to be a contraindication to the use of estrogen replacement therapy. However, the safety of estrogen (and progestin) hormone therapy in breast cancer survivors is not fully known. Several trials from the 1990s ended when findings showed an increased risk of breast cancer recurrence [31]. This remains a controversial area, and hormone therapy is generally not recommended in patients with breast cancer (particularly estrogen receptor-positive types).

Hot flushes can be triggered by stimuli such as spicy food, alcohol, and anxiety. Lifestyle adaptations include dressing in layers so that clothes may be easily removed during episodes. Obesity seems to exacerbate hot flushes, while weight loss may relieve these symptoms [32, 33]. Nonhormonal pharmacological therapies for vasomotor symptoms include serotonin reuptake inhibitors (SSRIs), serotonin noradrenalin reuptake inhibitors (SNRIs), Gabapentin (gamma-aminobutyric acid), and clonidine (alpha-adrenergic agonist). Although not generally as effective as estrogen therapy, these treatments can offer some relief from hot flushes in 40–45 % of subjects [34, 35]. An important consideration is that SSRIs are potentially irreversible CYP 2D6 inhibitors, which can prevent tamoxifen from being metabolized into an active compound [36]. Gabapentin, a drug often used to manage neuropathic pain, can improve vasomotor symptoms and sleep quality at low doses, although adverse effects such as dizziness were common [37]. Of note, none of the above-mentioned nonhormonal treatments are FDA approved for treatment of vasomotor symptoms.

Other non-pharmacological treatments, such as herbal products, acupuncture, and exercise, have also been studied to determine their effects on vasomotor symptoms. Black cohosh, an herbal supplement, has shown mixed results. The efficacy of black cohosh on treatment for hot flushes remains unproven, and the safety regarding possible drug interactions with chemotherapy as well as tamoxifen has not been studied in depth [38]. Trials evaluating the efficacy of soy products and phytoestrogens in the treatment of vasomotor symptoms in breast cancer patients have shown no benefit for the treatment of these symptoms [39].

Other alternative therapies including dietary changes, exercise, acupuncture, relaxation techniques, and paced breathing have also been suggested for treatment of vasomotor symptoms.

Acupuncture has recently been found to be equally effective as venlafaxine in reducing hot flushes and produces less side effects while having a longer duration [40]. Homeopathy, acupuncture, exercise, and relaxation therapy were recently evaluated in meta-analysis form for treatment of vasomotor symptoms. Relaxation therapy showed a benefit in this review [41]. In contrast, insufficient evidence was available to determine the effectiveness of exercise [42].

Treatments for vaginal atrophy include nonhormonal lubricants and moisturizers. These lubricants can be used safely during intercourse to avoid discomfort and microtrauma of the vagina. Vaginal estrogen therapy in cream or gel form has been considered for atrophy, as the systemic absorption seems to be minimal. The estradiol vaginal ring has also been used, but there are no randomized controlled trials to assess safety of either of these methods [43].

Breast Cancer Treatments

Selective Estrogen Receptor Modulators (SERMs)

This is a class of nonsteroidal compounds that competitively bind estrogen receptors (ER) at the cellular level. The effects vary, depending on the precise structure of the individual compound. SERMs can function as ER agonists, antagonists, or mixed agonists/antagonists depending on the tissue type. The mechanism of action of SERMs is not fully understood, but appears to involve recruitment of a series of co-activator and/or corepressor proteins based on the conformation of binding to ER [44]. SERMs used in the treatment of breast cancer and their gynecologic effects will be discussed here.

Tamoxifen

First manufactured in the 1950s as a possible contraceptive, tamoxifen was not identified as a treatment for breast cancer until the 1980s, when several prospective trials showed a survival advantage, particularly in women with early breast cancer, and often in combination with chemotherapy [45, 46]. Since then, further studies have shown that tamoxifen is most effective in women with ER-positive tumors, and the drug is now widely used as a standard part of breast cancer therapy [47].

The side effect profile of tamoxifen is largely determined by its function in different tissues. Due to small variations in ER structure, tamoxifen functions as an antagonist in the breast and an agonist in the uterus. So while tamoxifen effectively slows breast tissue growth, it can accelerate uterine (especially endometrial) growth. Resulting side effects of tamoxifen therefore include uterine bleeding, polyp formation, endometrial hyperplasia, and endometrial cancer [48]. Specifically, the risk of endometrial cancer appears to increase with the extended use of the drug, which has resulted in a recommended usage of no more than 5 years [49]. A beneficial side effect of tamoxifen is its preventive effect on osteoporosis. The drug acts as an agonist in the bone and therefore mimics the bone-sparing effect of estrogen [50].

Raloxifene

Raloxifene differs from tamoxifen by functioning as an estrogen antagonist in both the breast and uterus and agonist in the bone. The drug has therefore been used to prevent osteoporosis since the late 1980s. The landmark STAR trial compared the use of tamoxifen and raloxifene for prevention of breast cancer in women at high risk. This study showed that raloxifene was as effective as tamoxifen in preventing breast cancer, but was associated with fewer cases of uterine cancer, fewer cataracts, and fewer blood clots [51]. As a result, in 2007, the U.S. Food and Drug Administration approved raloxifene for postmenopausal women at high risk for breast cancer as a preventive agent.

Gynecologic Considerations Associated with SERMs

The risk of uterine pathology is the primary gynecologic concern with the use of tamoxifen. The current recommendation is that endometrial

sampling be performed in women using tamoxifen who experience irregular uterine (particularly postmenopausal) bleeding. There is no clear evidence that increased levels of surveillance, including random endometrial sampling or ultrasonography (transvaginal or abdominal), will reduce the morbidity associated with tamoxifen, and they are not recommended.

Raloxifene is recommended for postmenopausal women at high risk for breast cancer, particularly those with or at risk for osteoporosis.

Aromatase Inhibitors

Aromatase is an enzyme that functions to synthesize estrogen in humans. Aromatase inhibitors (AIs) block estrogen production and have been used in the treatment of breast and other cancers in women. In postmenopausal women, the primary natural source of estrogen is peripheral fat, which converts circulating androgens (via aromatization) to estrogens. AIs can block this process and therefore lower total estrogen load. The large, international ATAC study showed improved survival in postmenopausal women with ER-positive breast cancer who used an AI [28]. AIs currently approved for use in breast cancer include anastrozole, exemestane, and letrozole.

Gynecologic Considerations of AIs

The effect of AIs in premenopausal women is paradoxical. AIs will block the production of estrogen in the ovary (the natural source in premenopausal women), but this effect will also stimulate the hypothalamic–pituitary axis to increase gonadotropin secretion, which in turn stimulates the ovary to produce more androgens, which potentially counteract the effect of the AI. As a result, ovarian ablation (either surgical or chemical) may be recommended with the use of AIs in premenopausal women with ER-positive tumors.

The use of anastrozole has been associated with an increased risk of bone fractures in women with breast cancer [52]. The risk of osteoporosis should be thoroughly discussed with women using anastrozole, and the use of bisphosphonates, calcium supplements, and/or weight-bearing exercises are generally recommended.

Progestins

Progestins are synthetic progestogen hormones with progesterone-like effects. They can counteract the effect of estrogen in the breast and uterus and are primarily used in a variety of contraceptive agents.

Progestins may be used as palliative treatment in women with breast cancer, particularly those with ER-positive tumors. The use of progestins in this setting has declined in recent years with the advent of SERMs and AIs [53].

Gynecologic Considerations of Progestins

Progestins can cause amenorrhea or irregular uterine bleeding in premenopausal women. High-dose progestins may also have appetite-stimulant effects.

Cancer Screening in Breast Cancer Patients

Gynecologic Cancers

Cervical Cancer

The recognition of human papillomavirus (HPV) as the probable causative agent for most cases of cervical cancer has changed screening strategies dramatically. While the basis of the screening process remains the Papanicolaou smear, most recommendations also include concurrent HPV testing and typing. The distinction between low-risk and high-risk HPV types helps identify those women who will get the greatest benefit from additional evaluations [54].

Further, this understanding of the role of HPV has made it clear that breast cancer and cervical cancer, while not mutually exclusive,

have entirely different risk factors and probable causes. There are currently no well-understood or widely accepted links between HPV infection and breast cancer, just as there is no clear data showing that the hormonal milieu (or any other factors) associated with breast cancer has an effect on the pathophysiology of cervical cancer. As a result, recommendations for cervical cancer screening can mirror those of the general population, as published in detail elsewhere [55]. What follows is a summary of the current guidelines.

The recent approval and widespread utilization of HPV vaccines as preventive measures for cervical cancer could have a dramatic effect on the incidence of cervical cancer in the future. It is predicted that comprehensive HPV vaccination of women ages 9–25 (prior to HPV infection) could prevent up to 80 % of all cervical cancers. HPV vaccination holds great promise for developing countries in particular. Many nations lack the standard infrastructure needed for Pap smear-based screening and as a result have much higher rates of cervical cancer than the developed world. Cervical cancer prevention by means of a simple vaccination could have an enormous impact on these populations [56].

Currently, there are no recommendations regarding HPV vaccination in women with breast cancer. There is no evidence to suggest that HPV vaccination would have any impact on breast cancer outcome. Thus, women with breast cancer may be vaccinated for HPV based on standard guidelines. In general, women who are not infected with HPV should be vaccinated. It should also be stated that standard screening recommendations have not changed for HPV-vaccinated women. At present, women who have been HPV vaccinated should undergo Pap smear-based screening as per standard guidelines (as described above).

Recent controversy has arisen over the cervical cancer screening recommendations of the U.S. Preventive Services Task Force [57]. Similar to the discussion on breast cancer, these new guidelines call for somewhat less frequent testing than those described above, which are based on a collaboration between the American Cancer Society (ACS), American College of Pathology (ACP), American Congress of Obstetricians and Gynecologists (ACOG), and other professional societies. Specifically, the U.S. Preventive Services Task Force guidelines were developed using cost–benefit analyses and assumptions of widespread high compliance that may not reflect real-world utilization. In fact, the women most at risk for cervical cancer are often least likely to undergo screening. Thus, it is likely that these new guidelines will result in additional cases of cervical cancer. While the overall numbers may be statistically insignificant, there is no clear way to measure the impact of a single case of cervical cancer on an individual, a family, or a community. The authors therefore will continue to recommend the ACS/ACP/ACOG guidelines for cervical cancer screening. A summary of those guidelines is shown in Table 18.2.

Table 18.2 Summarized ACS/ACP/ACOG recommendations for cervical cancer screening

Age	Recommendation	Alternatives
21	Pap smear every 3 years	–
30	Pap smear every 5 years, with HPV testing/typing	Pap smear alone every 3 years
65	Cease screening, if adequately tested with normal results previously	–
After hysterectomy	Cease screening, if no history of CIN 2, CIN 3, or cancer	–

Endometrial Cancer

There is no clear evidence that screening asymptomatic women for endometrial cancer can improve outcome. Endometrial cancer is usually diagnosed early as a result of its common association with a very specific symptom: irregular vaginal bleeding, particularly postmenopausal bleeding. Potential screening tests, including Pap smears, endometrial biopsy, or transvaginal ultrasonography, lack sufficient sensitivity and/or specificity to be effective.

The use of tamoxifen by breast cancer patients has been associated with endometrial pathology, including cancer, as described elsewhere in this chapter. However, studies of potential endometrial cancer screening techniques in this population

have not demonstrated significant benefit [58]. At present, screening of asymptomatic women with breast cancer, including those using tamoxifen, for endometrial cancer is not recommended. Symptoms of irregular vaginal bleeding should be thoroughly investigated, usually with endometrial biopsies and ultrasonography.

Ovarian Cancer

Screening for ovarian cancer in families with hereditary breast–ovarian cancer syndromes is described previously. In women without a family history of either cancer, there is no clear evidence that screening for ovarian cancer is effective. While the risk of developing ovarian cancer may be slightly increased in a woman with breast cancer, current screening strategies (including serum Ca-125 and transvaginal ultrasound) lack adequate sensitivity and specificity needed to be effective [59]. They may result in further diagnostic testing that can increase patient anxiety and carry additional procedure-related risks. While women with breast cancer should be made aware of the symptoms of ovarian cancer [60], no specific screening strategies are recommended.

Other Cancers

Colorectal Cancer

There is no evidence that the risk of colorectal cancer is changed by the diagnosis of breast cancer, although obesity is a shared risk factor. There is an unusual variant of hereditary nonpolyposis colon cancer (HNPCC), the Muir–Torre syndrome, that is associated with an increased risk of breast cancer [61]. Individuals with this variant are likely to be well known due to strong family histories of colon cancer and are typically screened very thoroughly. For most women with breast cancer, however, the recommendations for colorectal cancer screening are identical to those in the general population [62]. In summary, screening with colonoscopy should be done every 10 years between ages 50 and 75. Screening above or below those ages will have higher risk/benefit ratios and are not routinely recommended.

Lung Cancer

Again, there is no evidence that the risk of lung cancer is changed by a diagnosis of breast cancer. The most important risk factor for lung cancer, by far, is cigarette smoking. Further, screening for lung cancer has been proven to be effective only in individuals at high risk; specifically heavy smokers or former smokers with a minimum 30-pack/year history. In this group, the National Lung Screening Trial (NLST) showed that low-dose spiral computed tomography scanning resulted in 20 % reduction in mortality compared to chest X-ray alone [63]. Based on this, women with breast cancer should undergo screening for lung cancer based on the general population criteria as defined in the NLST.

Follow-Up Care

Women with breast cancer require continued gynecologic follow-up and care for all the reasons described in this chapter. Gynecologic evaluation should be a routine part of breast cancer care. Gynecologists, who commonly maintain an ongoing and long-term relationship with their patients, may be uniquely situated to allay patient concerns and correct misconceptions. Female cancer patients often maintain a level of trust in their gynecologists that is difficult for oncologists to achieve. Gynecologists should make specific inquiries about the breast cancer patients' level of understanding of the disease process, comfort with the recommended treatment plan, and confidence in her cancer specialists.

The patient will often reveal problems or concerns to the primary gynecologist that she will not tell her oncologist [64]. Concerns about appearance, femininity, and sexuality are frequent among breast cancer patients. Many oncologists do not routinely discuss these issues and many patients may perceive that their "cancer doctor" is not interested in or able to address them. Gynecologists should encourage a frank discussion of these and other personal concerns of the breast cancer patient as part of routine care. Often, simple reassurance is sufficient to alleviate fear or uncertainty. Or, the gynecologist

Table 18.3 Follow-up gynecologic care for breast cancer patients

Baseline (diagnosis)	Posttreatment	Annual	Symptom-based
Physical exam (PE)	PE	PE	PE
Cancer screening (Pap, colonoscopy, etc.)	–	per American Cancer Society Guidelines	
Counseling/reassurance	Counseling/reassurance	Counseling/reassurance (as needed—may be less frequent)	Additional studies (biopsies, imaging)

may suggest specific, commonsense interventions that oncologists are less familiar with, such as using adequate amounts of warmed lubricant as a routine part of sexual activity to alleviate chemotherapy-induced mucosal irritation. Finally, the gynecologist can communicate information that may impact therapy to the oncologist who may be unaware [65].

The authors recommend a baseline complete gynecologic evaluation for all women diagnosed with breast cancer by a clinician conversant with the concepts described in this chapter and summarized in Table 18.3. This visit will identify existing or potential problems and prepare the patient for her initial treatment, including surgery, radiation and/or chemotherapy. It is further recommended that a second visit to the gynecologist be scheduled shortly after the completion of the first-line therapy. New findings often become apparent at this visit, and/or patient concerns about the future can be addressed. After this, annual gynecologic evaluation is appropriate. Additional visits may be triggered by new symptoms, such as vaginal bleeding, hot flushes, night sweats, or mood disturbance. The diagnosis of a recurrence of the cancer should prompt strong consideration of an additional gynecologic evaluation as well. The gynecologist should therefore be an ongoing member of the patient's cancer care team. Optimal care for this most intimate of physical concerns should not be overlooked at such a critical juncture in a woman's life.

Conclusion and Future Perspective

Gynecologic care for women with or at risk for breast cancer is frequently underemphasized and should be considered a component of comprehensive cancer care. The emergence of Women's Health as a unique medical specialty should facilitate the study of these issues and their inclusion in routine management strategies. Genetic risk assessment is recommended for patients with a greater than 20 % chance of having inherited predispositions to breast or ovarian cancer. While the data regarding risk-reducing treatments is rapidly evolving, it is currently recommended that women with BRCA1 or BRCA2 mutations should be offered a salpingo-oophorectomy by age 40 or when childbearing is complete.

Advances in chemotherapy and radiation have improved survival but often compromise fertility and hasten menopause. Numerous options for fertility preservation are available and should be addressed with each patient individually. High-tech procedures for ovary and embryo conservation have great promise but may be both expensive and not covered by insurance.

Menopausal symptoms can have a major impact on quality of life in breast cancer patients, even years after primary therapy has been completed. Optimal benefit will be attained by means of continued discussions over time as the various symptoms wax and wane. Development and testing of new estrogen-like molecules hold the promise of alleviating symptoms in the future.

The future of cancer screening is likely to be primarily determined at the molecular level. The concept of personalized medicine, based on an individuals' specific genetic profile, has the potential to predict cancer risk with great accuracy. Integration of "DNA profiling" into current screening strategies will require careful analysis of the benefits in an era of increased quality/cost awareness.

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19 Imaging Technologies and Applications in Early Diagnosis and Prognosis for Breast Cancer

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Abstract

There is increasing interest in the development of imaging tests to screen for breast cancer, especially in high-risk groups where conventional technology falls short. Breast imaging has made huge advances in the last decade, and along with newer techniques to diagnose primary breast cancer, many novel methods are being used and look promising in detecting distant metastasis and recurrent disease and assessing response to treatment. While screening mammography, which is the most extensively studied technique, is recognized as the most effective method for early detection of breast cancer, and many screening procedures have been discussed, this modality has limitations that are the driving force behind efforts to refine existing mammography technologies and develop new ones offering improved detection of breast cancer. Recent studies have shown that these techniques can enhance the radiologist's ability to detect cancer and assess disease extent, which is crucial in treatment planning and staging.

Ultrasound holds promise as a method for detection of cancers in women with dense breast tissue, which is often problematic with conventional film-screen mammography. Ultrasound has also assumed an important role in breast imaging, as an adjunct to diagnostic mammography for biopsy guidance, palpable mass evaluation, and serial evaluation of benign masses. Magnetic resonance imaging is a generally accepted diagnostic procedure for a number of breast-related indications. Its greatest strength is that it is very sensitive to tumors. If a suspected area does not exhibit contrast agent uptake, the probability that it is malignant is very small. Conversely, its specificity is poorer. If the area does show enhancement,

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it may or may not be a tumor. Further imaging or biopsy may be needed to resolve the question. Digital mammography systems use digital detectors to convert X-ray photons to digital signals for display on high-resolution monitors. These systems offer capabilities not provided by conventional film-screen mammography. PET/computed tomography has a role in detecting local disease recurrence and distant metastasis in breast cancer patients.

Nowadays, despite of having a large amount of techniques to use, further studies are needed to improve diagnostic accuracy and lower the current threshold for detection, thus minimizing the false-negative rate. As imaging techniques improve, the role of imaging will continue to evolve, with the goal remaining a decrease in breast cancer morbidity and mortality.

Keywords
Imaging technique • Breast cancer • Diagnosis • Screening • Mammography

Introduction

Breast cancer remains the most prevalent cancer disease and the second cause of cancer-related mortality in women of developed countries, resulting in great social and economic impact. Therefore, prevention and screening have become important health issues all over the world. Breast imaging has made huge advances in the last decade, and along with newer techniques to diagnose primary breast cancer, many novel methods are being used and look promising in detecting distant metastasis and recurrent disease and assessing response to treatment.

In the twentieth century, eight randomized trials enrolling 500,000 women in New York, Sweden, Scotland, and Canada demonstrated up to a 30 % decrease in breast cancer mortality in the screened population [1–6]. Those smaller tumors, detected in an earlier stage by mammography, clearly improved the treatment options and the prognosis for these patients.

Breast cancer mortality has improved over the past few decades. While in the 1940s the 5-year survival rate for early-stage localized disease (without node involvement or metastasis) was around 70 %, today it has improved up to 97 % [7]. This improvement in survival is highly attributable to the increase and the effectiveness in the mammography screening programs.

Mammography is the only screening test proven to decrease breast cancer morbidity and mortality. It meets all the criteria for a screening test. First, because breast cancer is a highly prevalent disease (statistics indicate that one in nine women will develop it during her life) [8, 9], progressive at all its stages and which, when diagnosed and treated timely (when it is asymptomatic), can alter its natural course, improving the prognosis and the final outcome. Second, because mammography is a cost-effectiveness test, easy to perform, and is well tolerated by patients. Then, the principal aim of screening mammography is to detect breast cancer in an early stage to treat it, avoiding the illness and following death that accompany locally advanced or widespread breast cancer.

One study published in 2005 [10] considered separately the effects of screening mammography and adjuvant therapy on the breast cancer rate and estimated that the portion of the reduction attributed to screening mammography ranged from 28 to 65 % (median 46 %), attributing the rest to the use of adjuvant therapy. Note that this variability in the reduction of the death

rate was attributed by the authors to variations in the inclusion criteria of the participating groups.

Although mammography remains the gold standard, it does have limitations, particularly in women with dense breasts. Even when performed optimally, the sensitivity is between 69 and 90 % [11–16]. New imaging techniques are emerging to overcome these limitations and enhance cancer detection, improving patient outcome.

Many technological advances have been established after the development of mammography but, undoubtedly, the most important has been digital mammography.

The use of ultrasound has notably increased over the last decades, getting more relevance every day in the early diagnosis of breast cancer and as a valuable tool to perform breast biopsies.

Magnetic resonance imaging (MRI) is nowadays an almost indispensable technique for local staging and follow-up of breast cancer, although it needs many technical improvements providing adequate sensitivity and specificity to extend its use.

And lastly, other nuclear medicine studies are getting involved in the diagnosis and follow-up of breast cancer, with some clinical studies showing very satisfactory results.

In this chapter, we summarize the most important guidelines and also review the imaging examinations that hold promise but have not yet earned a place in routine breast cancer diagnoses.

Screening by Mammography

No screening procedure is perfect, women vary greatly in their breast cancer risk, and screening may lead to unnecessary procedures and alarm, so screening should, ideally, be tailored to the individual's cancer risk.

Radiation risk and diminished sensitivity in radiographically dense breast represent the most significant disadvantages of the mammography technique, thus limiting its usefulness in high-risk younger women. Its use must also be restricted in the detection of ductal carcinoma in situ (DCIS) without calcifications and lobular cancer, in the characterization of locally advanced cancer [17], and in multifocal cancer and also in the assessment of the breast that has previously been subjected to radiotherapy [6].

General Recommendations for Mammographic Screening

Several organizations have developed different guidelines—which are largely evidence based—for how screening mammography should be used, pointing specially to the time to start and how often it should be performed. While many issues surrounding breast cancer screening are still unresolved, general guidelines have now been implemented on the basis of data accrued over many years. Consequently, recommendations about how to screen with imaging technologies have become increasingly complex.

Experts have long agreed that screening mammography reduces the rate of death from breast cancer in women who begin screening in their 50s and 60s; however, recommendations from expert groups vary when talking about screening of women in their 40s. Meta-analyses now reveal that in this last population, screening mammography decreases breast cancer death rate around 20 % [18]. However, the absolute benefit is lower for this age group than for older women because of the younger group's lower risk of cancer [19]. There are fewer studies for women over 70 years old, but the decrease of the death rate could reach up to 55 % [20].

Taking all the results above, screening between ages 40 and 49 is not recommended by all the societies (firmly accepted by American's and rejected by Canadian's). In contrast, all the societies highly recommend screening mammography between ages 50 and 59, when it has demonstrated to be most effective.

There is no established upper age limit to the beneficial use of screening mammography. According to the 2004 revised American College of Radiology guidelines, "It is unclear at what age, if any, women cease to benefit from screening mammography. Because this age is likely to

Table 19.1 Mammography screening recommendations

Organization	Screening recommendations
AAFP	Every 1–2 years, ages 50–69; counsel women ages 40–49 about potential risks and benefits of mammography and clinical breast examination
ACOG	Every 1–2 years starting at age 40, yearly after age 50
ACS	Annually after age 40
AMA	Every 1–2 years in women ages 40–49, annually beginning at age 50
CTFPHC	Every 1–2 years, ages 50–59
NIH	Data currently available do not warrant a universal recommendation for mammography for women in their 40s; each woman should decide for herself whether to undergo mammography
USPSTF	Every 1–2 years, ages 50–69

AAFP American Academy of Family Physicians, *ACOG* American College of Obstetricians and Gynecologists, *ACS* American Cancer Society, *AMA* American Medical Association, *CTFPHC* Canadian Task Force on Preventive Health Care, *NIH* National Institutes of Health, *USPSTF* US Preventive Services Task Force

Table 19.2 Recommendations for mammographic screening in women aged 40–49 years

Organizations that recommend routine screening	Organizations that do not recommend routine screening
American Cancer Society	American Academy of Family Physicians
American College of Obstetricians and Gynecologists	American College of Physicians
American College of Radiology	Canadian Task Force on Periodic Health Exams
American College of Surgeons	National Institutes of Health Consensus Panel
National Cancer Institute	US Preventive Services Task Force

Table 19.3 Special recommendations by the American College of Radiology

Special recommendations
For BRCA1 or BRCA2 mutation carriers, untested first-degree relatives of BRCA mutation carrier, or a first-degree relative affected young, screening should be started by the age of 30
Patients with a personal history of atypical duct hyperplasia or lobular carcinoma in situ could benefit of been performed a mammography every 6 months, even could being candidates for a breast RMI
History of high-dose chest irradiation received between the ages of 10 and 30 should begin with an annual mammogram study and, recommended, an annual RMI, 8 years after the exposure

vary depending on the individual's overall health, the decision as to when to stop routine mammography screening should be made on an individual basis by each woman and her physician" [21]. Along the same line, the American Cancer Society recommends the screening "as long as a woman is in reasonably good health and would be a candidate for treatment" [22].

To establish a schedule about how often mammography should be performed for women at average risk, an interval of every 2 years appears appropriate [19]. Tables 19.1, 19.2, and 19.3 show the recommendations for screening mammography according to different societies.

Imaging Techniques for the Diagnosis of Breast Cancer

Mammography

Mammography is the only screening test proven to decrease breast cancer morbidity and mortality and it has been used during the last century. Mammography uses low-dose X-ray; high-contrast, high-resolution film; and an X-ray system designed specifically for imaging the breasts. Although it is convenient to analyze mammography independently of other imaging techniques, in the clinical practice, it is highly recommended to use it in combination with those others, especially ultrasound [23].

In the United States, the incidence of breast cancer is 3 in 1,000 women and the rate of restudy of the mammography screening is 8 %. Seven percent of the women screened will need just another mammography or ultrasound scan and only 1 % will need a biopsy to detect those 0.3 % women with breast cancer [24]. Those referred for additional diagnostic testing and further views may be studied by another mammography or special mammography views, breast ultrasound, or other adjunctive imaging such as

a magnetic resonance (MR), digital mammography, sestamibi, or T-scan.

The US Food and Drug Administration (FDA) reports that mammography can find 85–90 % percent of breast cancers in women over 50 and can discover a lump up to 2 years before it can be felt. In 1994, the equipment, quality of operations, technologies, and doctors were regulated by the Mammography Quality Standards Act (MQSA).

A mammogram is like a fingerprint; the appearance of the breast on a mammogram varies tremendously from woman to woman, and no two mammograms are alike. It is extremely helpful for the radiologist to have films (not just the report) available from previous examinations for comparison purposes. This will help the doctor to recognize small changes that occur gradually over time and detect a cancer as early as possible.

A basic knowledge is required for the analysis of the mammography. The typical equipment produces low-dose X-ray (25–30 kVp) while the breast is compressed. The compression of the breast is necessary to limit the radiation dose and to improve the quality of the images. The X-ray is radiated through the compressed breast and onto a film cassette positioned under the breast. The X-rays hit a special phosphor coating inside the cassette. This phosphor glows in proportion to the intensity of the X-ray beams hitting it, thus exposing the film with an image of the internal structures of the breast. As the X-rays pass through the breast, they are attenuated (weakened) by the different tissue densities they encounter. Fat is very dense and absorbs or attenuates a great deal of the X-rays. The connective tissue around the breast ducts and fat is less dense and attenuates or absorbs far less X-ray energy. It is these differences in absorption and the corresponding varying exposure level of the film that create the images which can clearly show normal structures such as fat, fibroglandular tissue, breast ducts, and nipples. Quality of the images can be affected by many factors, particularly high density of the breast tissue, the thickness of the breast compressed, the position, the movement, and the radiation dose.

Currently, there are two kinds of receptors approved by the FDA: film-screen mammography and digital mammography, also called full-field digital mammography, or FFDM. The last one will be explained later in this chapter. The technique for performing both is the same; what differs is whether the images take the form of photographic films or of digital files recorded directly onto a computer.

Mammography Classification

There are two types of mammography exams, screening and diagnostic:

- *Screening mammography* is the X-ray examination of the breasts when the woman is *asymptomatic*, which means in a preclinical stage. This is the goal of screening mammography and its objective is the higher sensitivity to be able to detect any anomaly. For screening mammography, each breast is imaged separately, typically from above (craniocaudal view or CC) and from an oblique view (mediolateral-oblique or MLO), as shown in Fig. 19.1 [25]. The mediolateral-oblique (MLO) view is probably the most important and most common view taken followed by the

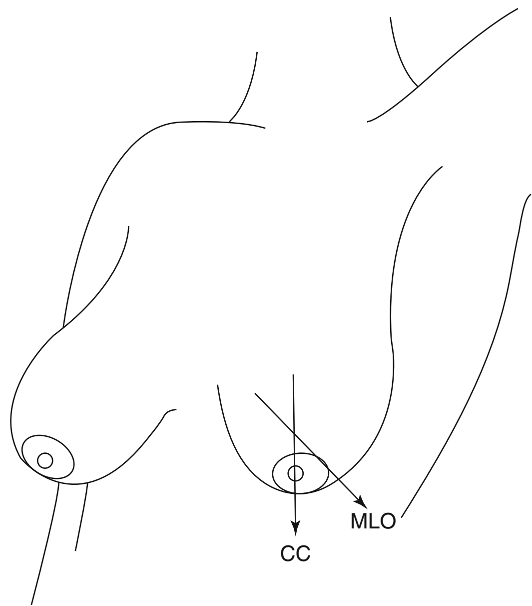

Fig. 19.1 Mammography projections. *CC* Cranial-caudal view, *MLO* Mid-Lateral Oblique view

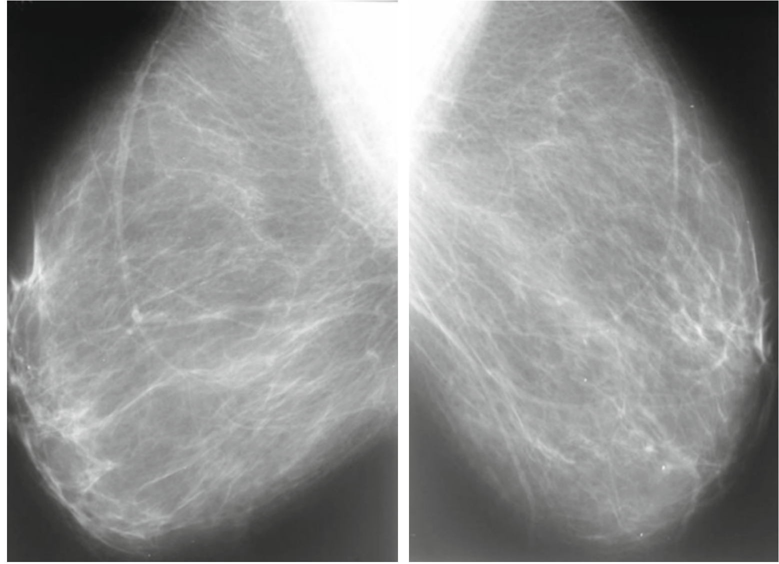

Fig. 19.2 Normal screening mammography

craniocaudal view (CC). Figure 19.2 shows a normal screening mammography.

- *Diagnostic mammography* is the X-ray examination of the breasts in a woman who either has a breast complaint (for instance, a breast lump or nipple discharge found during self-exam) or has had an abnormality found during screening mammography. Diagnostic mammography is more involved and time-consuming than screening mammography and is used to determine exact size and location of breast abnormalities and to image the surrounding tissue and lymph nodes. Typically, several additional views of the breast are imaged and interpreted during diagnostic mammography, especially in women with breast implants or a personal history of breast cancer. Thus, diagnostic mammography is more expensive than screening mammography. Figure 19.3 shows an abnormal mammography.

Mammogram Analysis

The first thing to do when a mammogram is analyzed is to detect any anomaly; the next step is its classification. The American College of Radiology (ACR) has established the Breast Imaging Reporting and Database System (BI-RADS™) [21, 26] to guide the breast cancer diagnostic routine. Each BI-RADS category is often referred as a "level" in radiologists' terms.

The BI-RADS categories are used to standardize interpretation of mammograms among radiologists. They are useful for statistical analysis of mammography practice, and the results are compiled on a nationwide basis in the United States to help refine mammography procedures everywhere. Table 19.4 shows a summary of the BI-RADS categories.

Each BI-RADS level has an appropriate management or follow-up plan associated with it (see below). Furthermore, if used correctly and consistently, each BI-RADS category has the following risk of malignancy and meaning:

- *Category 0* is a temporary category, which means that additional imaging is needed before assigning a permanent BI-RADS category. Most Category 0 findings are shown to be benign once the image study is completed.
- *Category 1* means that the screening is negative and the chance of having a breast cancer is 5 in 10,000. That also means the woman can continue with the screening established.
- *Category 2* means that the images found are benign or non-suspicious of being cancerous. It also indicates the same plan of follow-up as that of Category 1. This is the typical category

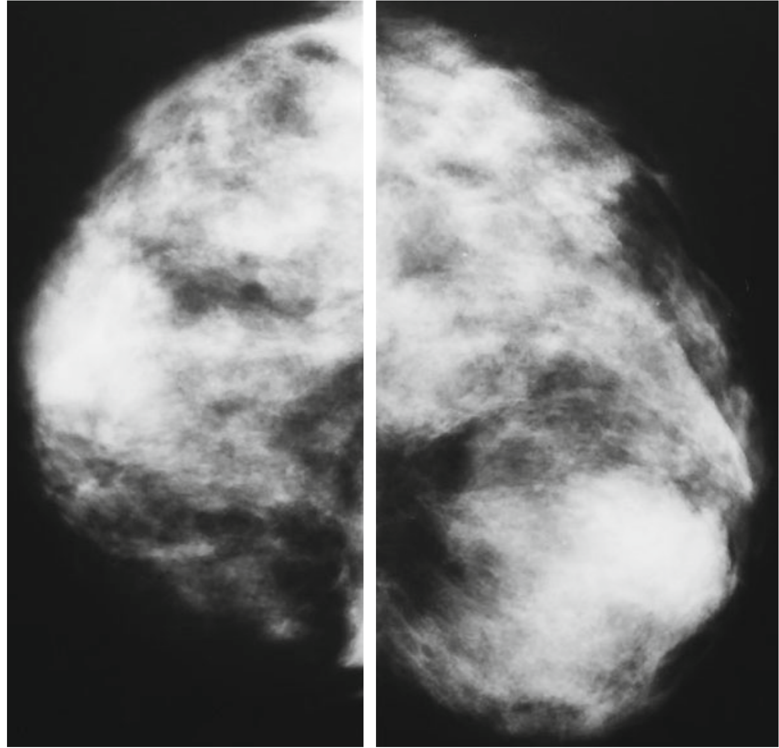

Fig. 19.3 Abnormal findings in mammography

Table 19.4 BI-RADS mammography categories according to the American College of Radiology

BI-RADS assessment categories	
Category 0	Need additional imaging evaluation
Category 1	Negative. Keep screening
Category 2	Benign finding. Keep screening
Category 3	Probably benign finding. Short interval of follow-up is suggested
Category 4	Suspicious abnormality. Biopsy should be considered
Category 5	Highly suggestive of malignancy. Appropriate action should be taken

where cases of cysts or fibroadenomas are classified.

- *Category 3* means that the image found is probably benign, but there is still less than a 2 % chance of cancer. It also means that another mammography is recommended in 6 months. However, most of the findings classified as Category 3 abnormalities do not receive a biopsy.
- *Category 4* means that the abnormality is suspicious of malignancy. Although most of the images classified as Category 4 are found to finally be benign, they require a biopsy since they have a 25–50 % rate of malignancy.
- *Category 5* means that some classic signs of malignancy are seen in the mammography, which are highly suggestive of cancer. It also means that all Category 5 abnormalities typically receive biopsy. If the biopsy results are benign, the abnormality usually receives re-biopsy since the first biopsy may not have sampled the correct area. The percentage of Category 5 abnormalities that will be cancer may vary between 75 and 99 %.

Findings Descriptions

When viewing a mammogram, it is important to know the exact orientation of the image. The breasts are best viewed as symmetric organs. Comparison of the right breast to the left breast is done for evaluation of symmetry. Perceptual psychologists have shown that the eye can more easily perceive asymmetric densities when patterns are scanned in a mirror-image fashion rather than side by side. Therefore, the conventional method is to evaluate mammograms in a

mirrorlike fashion with both the MLO and CC views mounted back to back.

What is normally identified in mammograms are masses, calcifications, areas of asymmetric density, and areas of distortion of the normal breast architecture [27]. Most of the breast cancers visible in mammographies were detected as a mass, calcifications, distortion of architecture, or a combination of these [28–32].

Masses. Masses are three-dimensional lesions which may represent a localizing sign of breast cancer. They are described by their localization, size, shape (round, oval, lobulated, irregular or architectural distortion), margins (circumscribed, obscured, microlobulated, non-defined, spiculated), X-ray attenuation, effect in surrounding tissue, and other associated findings. Depending on the morphological criteria of the mass, the likelihood of malignancy can be established.

Calcifications. Calcifications are often important and common findings on a mammogram. They can be produced from cell secretion or from necrotic cellular debris. They may be intramammary in many different locations but, alternatively, they may be found in the skin. They can appear with or without an associated lesion, and their morphology and distribution provide clues as to their etiology and its association with a benign or malignant process. Calcifications found within or around a mass provide further information about that particular mass. For example, an involuting fibroadenoma will often contain "popcorn-like" macrocalcifications. Similarly, fine curvilinear calcifications at the margin of a round or oval mass indicate a benign process. On the other hand, a mass with pleomorphic, irregularly shaped calicifications, which is also heterogeneous in size and morphology, raises much greater concern about malignancy. Calcifications are analyzed according to their size, shape, number, and distribution. The general rule is that larger, round, or oval-shaped calcifications uniform in size have a higher probability of being associated with a benign process and smaller, irregular, polymorphic, branching calcifications heterogeneous in size and morphology are more often associated with a malignant process. Certain calcification patterns are almost always pathognomic of a benign process, and in such cases, no further analysis is needed. In the majority of cases, however, a pattern of calcification deposition is inconclusive and may be attributable to either a benign or malignant process. The BI-RADS system has also classified findings of calcifications into three categories: (1) typically benign, (2) intermediate concern, and (3) higher probability of malignancy.

Areas of distortion of asymmetric density. The breasts are seen as symmetric structures and should be compared as such. Although exact mirror images cannot be expected, patterns within each breast should be similarly distributed. An asymmetric area may indicate a developing mass, a variation of the normal breast tissue, any postoperative change from a previous biopsy or surgery, or merely poor positioning and compression during imaging. The appearance of asymmetries due to positioning and compression during imaging is often the result of superimposition of normal breast structures. Nevertheless, true breast asymmetry is three-dimensional and should be present on both MLO and CC views. Once an asymmetry is determined to be three-dimensionally real, the interpreter must determine whether the asymmetry is a benign variation of asymmetric breast tissue or a focal asymmetric density that may represent a significant mass.

Areas of distortion of the normal breast. There is no one breast pattern that may be classified as "normal." Only from experience can one appreciate the variations of a normal-looking mammogram. Getting familiar with the spectrum of a "normal" appearance is to a certain extent essential to detecting abnormalities.

Common Findings

Fibroadenoma. It is the most common benign, solid growth in the breasts, especially in young women. It develops under the influence of estrogen. Its mammographic appearance is a mass with sharply well-demarcated margin, and it is virtually indistinguishable from a cyst or a well-circumscribed carcinoma. For this reason, it is impossible to identify a fibroadenoma radiographically without additional mammographic features. The additional features that allow one

to distinguish a fibroadenoma have to do with the fact that fibroadenomas often regress with menopause. During regression, the noncalcified appearance changes and calcifications develop. The typical involuting fibroadenoma contains popcorn-like macrocalcifications and is easily identified on a mammogram. Fibroadenoma has no significant risk of becoming cancer and does not put a patient at increased risk of breast cancer.

Cysts. They are harmless accumulations of fluid in the breast (and we can see them in other tissues or organs). As noted, a noncalcified fibroadenoma is indistinguishable from a cyst radiographically. That is, cysts generally have clearly defined margins radiographically when not obscured by surrounding tissue parenchyma. Cysts occur as a result of the dilatation of the lactiferous ducts within the lobules due to the imbalance between secretion and resorption. The exact causes of cysts are not known, but cysts are known to change with hormonal variations, either during normal menstrual cycles or from postmenopausal hormone replacement therapy. They commonly occur in women between 30 and 50 years of age. Cysts do not become cancer or increase the risk of cancer. Most of the time, cysts may be left alone, but sometimes a physician may drain them with a small needle (fine-needle aspiration).

Abscess. It is a benign lesion which may or may not appear round and well circumscribed. Sometimes abscesses are associated with acute mastitis and are often resolved clinically. Lacking a clinical history, only needle aspiration can diagnose them.

Intraductal papillomas. They result from a proliferation of ductal epithelial tissue. They are frequently too small to be evident on a mammogram, but if they grow large enough, they can appear as circumscribed masses and, in certain cases, may even obstruct the duct and give it the appearance of duct dilatation.

Intramammary lymph node. Normal lymph nodes are usually small, without calcifications, and have well-defined margins and shape. In the oblique mammography view, they are normally found in the axilla. On the other hand, intramammary lymph nodes are sometimes interpreted as suspicious breast masses. Magnification may be helpful to identify additional mammographic features of a hilus and central fat, in which case the likelihood of it being benign is increased.

Factors That May Affect Sensitivity and Specificity of Mammography

How mammograms can detect malignant lesions may vary in every woman. The most important factors that can modify sensitivity and specificity are the age of the patient, the density of the breast, having hormonal substitutive treatment, and the different types of breast cancer. A critical factor determining mammographic sensitivity and specificity is also the radiologist's interpretation. It is important not to forget these factors:

- *Breast density*. There is enormous variability in density among breasts, from those that are almost entirely fibroglandular in appearance to those that are almost entirely fatty in appearance. High breast density is associated with low sensitivity [33, 34]. Breast cancer attenuates X-rays and appears as a white density. A white density against a black (fatty) background is easy to detect (high signal-to-noise ratio). A white density cancer against a white background of fibroglandular tissue is difficult and, in many situations, impossible to detect. The normal dense tissue camouflages the cancer. Extensive breast density has been associated with higher frequency of false-negative mammograms. At all ages, regardless of hormone therapy (HT), high breast density is associated with 10–29 % lower sensitivity [35]. The relative insensitivity of mammography in women with dense breasts is a significant limitation of the technique.
- *Age*. Breast density generally declines with age. Therefore, excluding cases where patients were HT users, sensitivity above 65 years old is better than in younger women, not only due to this lower breast density but also due to the fibrocystic changes, and even the growing rates are lower [36, 37].
- *Hormonal therapy treatment*. HT increases breast density. That fact brings up two problems when mammograms are analyzed. The first is due to the high density itself,

which may camouflage suspicious lesions (see above); but the second concern is that the radiologist may not be able to know if that increment in breast density is due to the HT treatment or to a malignant lesion in progress [38].

- *Biological subtypes of breast cancer*. Invasive lobular carcinoma may be difficult to detect in early stages [39, 40], not only due to its characteristic growth pattern but also because it is only associated with calcifications in just 5 % of the cases.
- *Radiologist's interpretation*. There are many studies published showing substantial variability in interpretation and reading accuracy among radiologists. The clinical significance of variability in radiologists' interpretations is not clear [41]. Identifying a radiologist who is more accurate than another is difficult.
- *Technical factors*. There are many technical factors that may decrease sensitivity and specificity of the mammography. To start with, some areas of the breast are sometimes hidden in mammograms. The quality of the image is also reduced in thicker breasts due to a significant loss of contrast. Another example is breast prostheses that, when located in front of the muscle, can camouflage many lesions.

On the other hand, international comparisons of screening mammography have found that specificity is greater in countries with more highly centralized screening systems and national quality assurance programs. For example, one study reported that the recall rate is twice as high in the United States as it is in the United Kingdom, with no difference in the rate of cancers detected [42]. Such comparisons may be confounded, however, by other social, cultural, or economic factors that can influence the performance of mammography screening. No improvement in cancer detection was noted in these studies, despite the higher recall rate.

Breast Ultrasound

As a breast cancer detection procedure, ultrasound cannot replace a mammogram for breast cancer screening. Screening breast ultrasound as a possible replacement for mammography was tried unsuccessfully in the early 1970s. Supported by expert opinion, the European Group for Breast Cancer Screening concluded that there is little evidence to support the use of ultrasound in population breast cancer screening at any age [43].

Following this failed attempt, breast ultrasound fell into some degree of disrepute. In overreaction, many breast imagers in the United States loss confidence in breast ultrasound for most of a decade for any purpose other than distinguishing cyst from solid. Gradually, through the 1990s and 2000s, breast ultrasound has reemerged as the key and first diagnostic breast modality that is used after mammography. Its diagnostic and guidance roles continue to expand and evolve, and recently we have begun the process of reevaluating ultrasound as an ancillary screening tool that is used after mammography in women with high risk, dense breast tissue, or both. Moreover, it is the election technique in women who cannot be exposed to X-ray (women under 30 years or pregnant women) and is also very useful in women with breast implants because breast ultrasound is more sensitive for the evaluation of extracapsular and intracapsular rupture than mammography [44–47].

Over the past two decades, one of the advances in medicine and imaging research has been the marked expansion of the capabilities of breast ultrasound in the evaluation of breast disease. Breast ultrasound has become a fundamental component for the diagnosis and prognosis of breast cancer. However, breast ultrasound also has some drawbacks, which include its relatively higher cost compared with screening mammography, operator-skill dependence, difficulty in providing reproducible results between different facilities, and the time required to carry out the study. Perhaps the biggest shortcoming of ultrasound is its higher false-negative rate, when compared with mammography, for general screening, especially for the malignant microcalcifications that are typically better seen mammographically [48]. We may see some limitations of breast ultrasounds, as noted here:

Breast Ultrasound Limitations

- Deep location of the lesion in the breast
- Cannot detect most calcifications in breast lesions
- The ultrasound contrast between the lesion and the surrounding breast tissue
- Experience and qualification of operator
- Quality of the equipment used
- Difficulty in reevaluation of images after the exam

A key to understanding ultrasound is knowledge of the nature of the ultrasound transducer. A transducer is, fundamentally, a device that converts one form of energy to another. Modern ultrasound transducers are handheld units that convert electric signals into ultrasonic energy that is then transmitted into the tissues. Typically, a piezoelectric crystal near the face of the transducer generates high-frequency sound when voltage is applied. The sonic beams used in diagnostic breast ultrasound typically have frequencies of more than 7 million cycles per second (7 MHz). Following interaction of the sound waves with the tissues, the transducer receives and reconverts ultrasound energy back into an electric signal, which is used to create the image.

The use of high-resolution breast ultrasound equipment is important, and a dedicated breast ultrasound unit is preferable. High-frequency linear array transducers are required because linear transducers have a wider near field and can more easily guide intervention procedures. The 2000–2001 American College of Radiology (ACR) Standard for the Performance of Breast Ultrasound Examination suggests transducer frequencies of 7 MHz or higher. If broadband systems are used, the ACR standard states that a center frequency of 6 MHz or higher is needed. Current transducer frequencies are typically 10 MHz or higher. If possible, color Doppler capability should also be available, as explained below.

A transducer of the correct frequency should be used so that the frequency is appropriate to the size and depth of the area of abnormality. The ACR standard states that the frequency should be high enough to permit differentiation of fluid versus solid breast masses; however, that is not always possible.

Placing the patient in a supine position minimizes the depth of tissue penetration needed for imaging by the ultrasound beam [49]. Raising the ipsilateral hand behind the head flattens the breast and minimizes the tissue depth. For lateral lesions, the ACR standard suggests supine-oblique positioning for scanning. Turning the patient away from the side to be examined flattens the lateral tissue against the chest wall. For medial lesions, the supine position is preferred and it is highly recommended to palpate the lesion while scanning. Skin and superficial breast tissue lesions are better visualized with higher frequency transducers. Scanning of the retroareolar region is often limited by shadowing from the nipple, but angling the transducer behind and beneath the nipple helps to solve this problem.

The interpreting physician should be able to understand triangulation principles for mammographic abnormalities and to correlate breast ultrasound with mammograms [50].

Breast Ultrasound Indications

There are many different uses for a breast ultrasound, including:

- Palpable abnormalities (Fig. 19.4)
- Mammographic abnormalities
- Screening in women not able to X-ray exposition
- Pain
- Nipple discharge
- Follow-up of lesions not biopsied (mostly BI-RADS 3 lesions)
- Detecting whether a lump or abnormality in the breast is filled with fluid or solid tissue
- Measuring blood flowing in a suspicious lesion
- Second look after MRI
- Assessment of regional lymph nodes in patients with suspicious or malignant lesions
- Determination of extent of lesion in patients with suspicious or malignant nodules
- Guiding interventional procedures for a breast biopsy
- Screening in addition to mammography

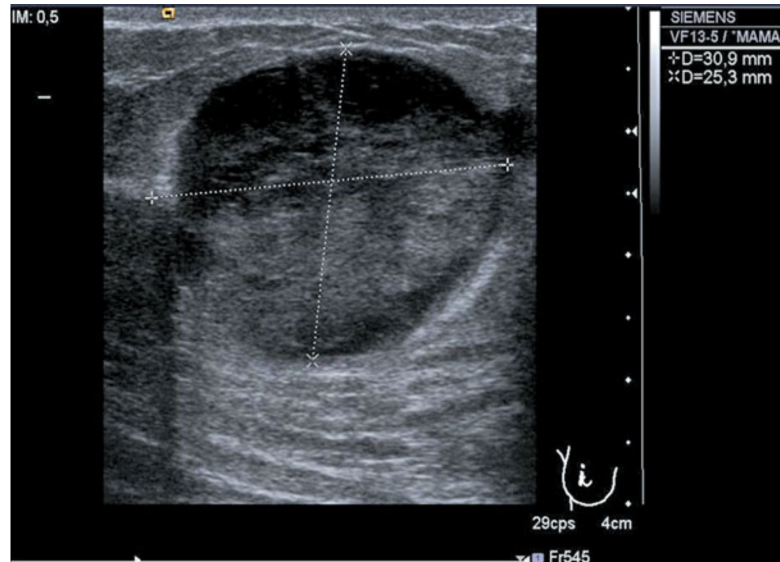

Fig. 19.4 Breast ultrasound scan evidencing a tumoral mass

Breast Ultrasound Analysis

To help standardize the examination, reporting, and recommendations, the ACR added a Breast Imaging Reporting and Database System (BI-RADS) lexicon for ultrasound to the BI-RADS lexicon that already existed for mammography. Adherence to the ultrasound BI-RADS lexicon helps us standardize descriptors and facilitates characterization of cystic and solid lesions, and assignment of a BI-RADS category helps us make more consistent recommendations for further imaging or management [51, 52]. However, the literature to date does not include sufficient data on outcomes to validate clinical use of the BI-RADS US lexicon [53].

Suspicious lesions in ultrasound analysis present similar characteristics more so than in mammogram analysis in relation to shape, size, and borders (Fig. 19.5). According to these characteristics, we can assign the lesion a BI-RADS category [54, 55].

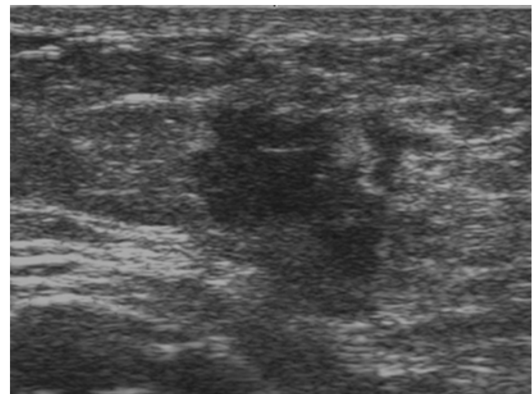

Fig. 19.5 Breast carcinoma explored by ultrasound scan

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is one of the most relevant diagnostic tools for breast cancer nowadays (Fig. 19.6). It is used widely for screening women at increased risk of breast

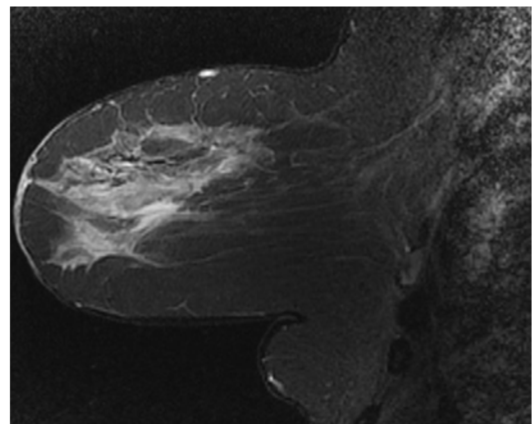

Fig. 19.6 Abnormal MRI of the breast

Table 19.5 Uses of MRI in breast cancer

MRI in breast cancer
MRI screening
BRCA carriers
Untested first-degree relatives of BRCA carriers
Individuals with more than 20 % lifetime risk of breast cancer
Extent of disease evaluation
Risk of change to a more extensive treatment due to false additional disease
MRI-guided biopsy is recommended prior to changing the treatment
Use in selected patients
Study of the contralateral breast
Risk of change to a more extensive treatment due to false additional disease
MRI-guided biopsy is recommended prior to changing the treatment
Low positive predictive value
Evaluation of axillary metastasis
High sensitivity and specificity in detecting axillary node metastases
UPSIO-enhanced MRI has the highest sensitivity and specificity
Not a replacement for SLNB
Evaluation after neoadjuvant chemotherapy
Best imaging technique in correlation between the preoperative measurements and the pathological findings
High specificity and a low sensitivity in predicting pathological complete remission
Suitable for selecting patients for neoadjuvant chemotherapy

cancer, like BRCA-positive patients; and it is also widely used in order to select the best treatment, as outlined in Table 19.5. MRI has the highest sensitivity in breast cancer imaging, but the low specificity is still its biggest disadvantage.

Compared with mammography that uses low-dose X-rays to image the breast, MRI uses powerful magnetic fields and radio waves to create the images. The MRI system is able to switch magnetic fields and radio waves to achieve views in any plane and from any orientation, while X-ray mammography requires reorientation of the breast and mammography system for each view desired.

Each total MRI exam is typically comprised of a series of three to six sequences. An "MRI sequence" is an acquisition of data that yields a specific image orientation and a specific type of image appearance or "contrast." During the examination, a radio signal is turned on and off, and subsequently, the energy, which is absorbed differently depending on the tissue, is echoed or reflected back out of the body. These echoes are measured continuously by the MRI scanner and they are reconstructed into breast images by a digital computer. A benefit of MRI compared to mammograms is that MRI can easily acquire direct views of the breast in almost any orientation, while mammography requires reorientation of the breast and mammography system for each view we want to analyze.

The most useful MRI technique for breast imaging uses gadolinium DTPA as an intravenous contrast agent. It helps to improve the quality and contrast of the images, producing stronger and clearer images and making evident the anomalies.

While there are several variations in breast MRI protocols for the evaluation of breast cancer, a sample protocol is listed below:

1. Scout (localizer)
2. T1-weighted images, without fat saturation and without contrast
3. T2-weighted images, with fat saturation and without contrast
4. Dynamic T1-weighted 3D images with fat saturation after contrast injection

It is important to note that there are differing opinions on the clinical benefits of viewing the morphology of a lesion with higher-resolution scanning versus high-temporal-resolution (low scan time) contrast enhancement analysis. Large-scale clinical studies have yet to determine the optimal imaging sequence to improve specificity and sensitivity.

Analysis of MRI

After the administration of intravenous contrast, several regions of the breast may be contrast-enhanced and these regions must be analyzed carefully because many lesions, benign and malignant, may show in this view [56, 57]. To determine the difference between benign and malignant, the interpreting physician has to consider the morphological characteristics of the

lesion (as seen in previous techniques) but also the dynamic contrast. Below, we list some of the limitations of MRI:

- Long acquisition time
- Expensive and not widely available
- Low specificity compared to mammography, which may lead to unnecessary invasive procedures
- Requires the use of intravenous contrast agent
- Cannot detect certain calcifications present in early cancers

MRI for Screening

Because of its high sensitivity in the diagnosis of breast cancer, MRI use has increased in recent years, adding sensitivity to mammography and ultrasound. Its sensitivity is important in young women with dense breast tissue and women who carry BRCA mutations because of the lower sensitivity of mammography in these cases.

Detection of breast cancer with MRI is based on tumor angiogenesis. In the tumors, there is an uncontrolled proliferation of the capillaries of the tumor and surrounding stroma, forming abnormal vessels with increased permeability. This increase in the permeability is responsible for the rapid extravasation of the contrast into the interstitial space, which results in an increased signal in the MRI, allowing the description of the shape and nature of the tumor. Due to this tumoral angiogenesis, contrast-enhanced breast MRI is the most sensitive imaging technique currently available for the detection of invasive breast malignancies.

The false negatives with MRI may occur in well-differentiated invasive ductal carcinomas as well as in some invasive lobular carcinomas [12]. Furthermore, not all ductal carcinoma in situ (DCIS) are detected by MRI, which is possibly related to a more variable angiogenesis associated with DCIS and its variable appearance in MRI. But the biggest problem is related to the false positives that can be caused by high-risk lesions such as lobular carcinoma in situ (LCIS), atypical ductal hyperplasia, atypical lobular hyperplasia, radial scars, and benign lesions such as fibroadenomas, papillomas, lymph nodes, fibrocystic changes, sclerosing adenosis, ductal hyperplasia, and fibrosis.

The sensitivity of breast MRI has been compared to that of mammography and ultrasound in multiple-prospective, observational research trials. When compared to mammography, MRI has a higher sensitivity because it is not affected by breast density. These trials have been done among patients with high risk of breast cancer, and all of them report MRI as the method with the highest sensitivity detecting breast cancer. Kriege et al. report a sensitivity for MRI of 79.5 %, while the sensitivity for clinical examination and mammography was only of 17.9 and 33.3 % [58]. Afterward, several studies have reported similar results.

The specificity of MRI is one of its disadvantages. Mammography seems to have a higher specificity of 98.5 %, while MRI has a specificity of 96.1 %. This difference in specificity between mammography and MRI is due to a larger number of false-positive examinations, because MRI detects vascular lesions regardless of whether they are benign or malignant.

MRI has other disadvantages when compared to mammography for screening. The most important disadvantages are that MRI is expensive and requires intravenous contrast. This makes screening MRI not efficient. But, related to efficiency and efficacy, it changes when MRI screening is focused only to high-risk groups because with a high prevalence, the positive predictive value for malignancy with MRI increases and there is not as much of a problem with false-positive examinations. Among these patients with high risk, the superior ability of screening MRI to detect clinically occult breast cancer is of great use, and especially in women at very high risk involving carriers of BRCA mutations. In these patients, the highest sensitivity of MRI is of special importance because it allows the cancers to be detected when they are smaller and frequently node-negative [59].

These advantages of MRI screening are summarized in the American Cancer Society (ACS) guidelines, which recommend screening MRIs for women at a lifetime risk over 20 % and recommend against them in women at a

lifetime risk below 15 %. The screening recommended consists of annual MRI screening and mammography for BRCA carriers, untested first-degree relatives of BRCA carriers, and individuals with more than 20 % lifetime risk of breast cancer, beginning annual MRI screening by age 30.

MRI in Patients with Cancer

Evaluation of Extent of Disease

Once a patient has the diagnosis of cancer, evaluating the extent of disease is of great significance. Through MRI, we have a highly sensitive approach with which to find new or unexpected lesions.

For example, multicentric cancer is detected by physical examination or mammography in approximately 10 % or fewer of breast cancer cases, while MRI can detect multicenter disease in 13–37 % of patients. Detecting a multicentric disease can change the definitive treatment as a possible contraindication to breast conservation therapy. At the same time, there is controversy over whether the use of MRI leads to too many mastectomies and excisional biopsies with false-positive results, but this may be no longer so significant since the introduction of MRI-guided biopsies.

MRI imaging must only be used preoperatively in selected patients, in order to avoid the false positives and maybe to avoid an increased reexcision rate in our patients. MRI is more effective in patients with dense breast tissue, patients with invasive lobular carcinoma, and probably in patients treated with accelerated partial breast irradiation.

Study of the Contralateral Breast

The literature has documented rates of occult contralateral cancer detection by MRI of approximately 4–9 %. MRI detects contralateral lesions in a substantial proportion of women; unfortunately, in many occasions, it does not distinguish between malignant and benign findings. Sometimes these findings are of high significance and can alter the patient's staging and treatment, but it can also make the physician recommend an unnecessary contralateral breast biopsy.

Residual Disease Evaluation

MRI can be used for assessing patients who have had a lumpectomy with positive margins. In these cases, if no previous MRI was performed, MRI may be useful for assessing the residual tumor. MRI can determine if the patient requires a reexcision or mastectomy. MRI is not indicated in the evaluation of microscopic residual disease, where the surgeon must proceed with a reexcision based on pathological margins.

Evaluation of Axillary Metastases

MRI can be useful for identification of axillary metastases. On one hand, MRI can study the size and shape of the lymph nodes; on the other hand, MRI, due to the intravenous contrast media, can study the nature of the tissue based on the pattern of uptake. A sensitivity of 90 % and a specificity of 90 % for the diagnosis of axillary metastases have been reported and a sensitivity of 98 % and a specificity of 96 % when ultrasmall paramagnetic iron oxide (UPSIO)-enhanced MRI is considered. This sensitivity and specificity make UPSIO-enhanced MRI a promising technique for diagnosing axillary metastases, but not a replacement for the sentinel lymph node biopsy (SLNB).

Evaluation After Neoadjuvant Chemotherapy

The assessment of tumor response to neoadjuvant chemotherapy has been performed with physical examination, mammography, ultrasound, and MRI. MRI seems to be the technique with the best correlation between the preoperative measurements and the pathological ones after surgery.

It has been shown that MRI has a high specificity (90.7 %) and a lower sensitivity (63.1 %) in predicting pathological complete remission after preoperative therapy in patients with breast cancer. But complete remission is not necessary for a successful breast-conserving therapy.

MRI is also used to select the patients that may be suitable for breast-conserving therapy, due to its capability to determine the size and shape of the disease; but predicting the results of the neoadjuvant chemotherapy depends more on the chemotherapeutic agent and nature of the tumor.

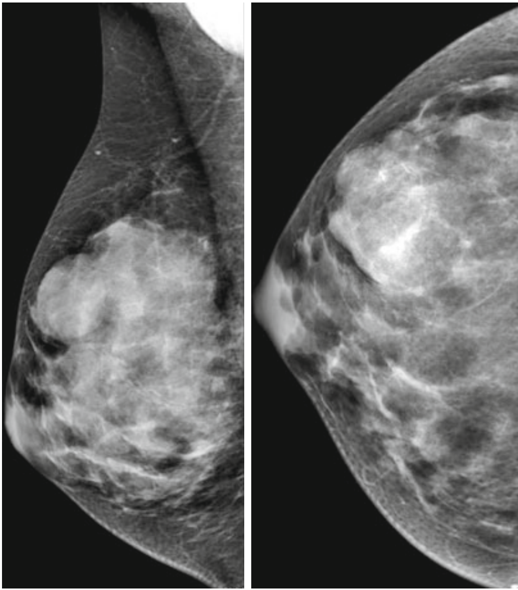

Fig. 19.7 Digital mammography in a case of phyllode tumor

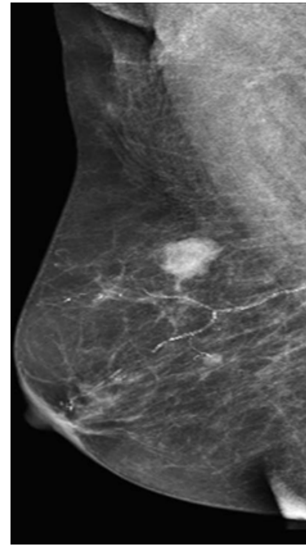

Fig. 19.8 Digital mammography showing a breast nodule and vascular calcifications

Emerging X-Ray-Based and Nuclear Medicine Technologies

There are many technologies emerging that claim to correct the deficiencies for detection and analysis in current technologies [60]. For convenience, the newer technologies can be divided into those that are based on morphology, those exploiting the physiology of malignancy, and those based on the metabolic properties of malignancies.

The first major technique change in mammography occurred with the introduction of digital mammography.

Digital Mammography

Digital mammography uses an electronic system to record an image of the breast that can be stored on a computer instead of on hard copy films. Image-processing algorithms allow manipulation of fine differences in image contrast. As a result, subtle differences, even in dense tissue, can be appreciated, as shown in Figs. 19.7 and 19.8 [61, 62].

Potential advantages include improvements in image contrast, post facto manipulation of the image, elimination of the problem of lost films, reduction in film library maintenance costs, and the ability to transmit the images over long distances (telemammography). Full-field digital mammography optimizes the lesion-background contrast and gives better sensitivity, and it is possible to see through the dense tissues by altering computer windows; this may be particularly useful in younger women with dense breasts. The need for repeat imaging is reduced, with the added advantage of reduced radiation dose to patients.

Early experience has shown that digital mammography reduces the number of patients recalled for additional views, reduces the number of false-positive breast biopsy results, and can potentially enable detection of breast cancer at an earlier stage [63–65].

Challenges and potential problems for digital mammography include a need to prove equivalence in detection and diagnosis with conventional mammography, the high cost of digital mammography equipment, and cumbersome workstation technology. Computer-aided detection systems may help the radiologist in interpretation of both conventional and digital mammograms.

Digital mammography, computer-aided detection, breast ultrasound, and breast MRI are frequently used adjuncts to mammography

in today's clinical practice. Recent studies have shown that these techniques can enhance the radiologist's ability to detect cancer and assess disease extent, which is crucial in treatment planning and staging.

Nuclear Medicine Breast Imaging Technology

Nuclear medicine techniques provide functional information on the pathophysiology of both normal tissues and disease. The introduction of radionuclides as possible agents for breast cancer detection had its start with the observation that technetium Tc 99m sestamibi (^{99}Tc-sestamibi), an energy emitter centered at 140 kiloelectron volt (KeV) and evaluated as a cardiac agent, was also seen to concentrate in women with suspected breast cancers. This increased uptake is thought to be due to increased vascularity and mitochondrial activity in and around malignancies. A 2005 review reported on 5,660 cases of ^{99m}Tc-sestamibi scintimammography [66]. The sensitivity for detection of breast cancer ranged anywhere from 80 to 90 % with a mean of 84 %. However, the sensitivity for lesions measuring less than 10 mm was low and nonexistent for those less than 5 mm. The specificity averaged 86 %.

Two of the most interesting techniques offered by nuclear medicine are positron emission tomography (PET) and lymphoscintigraphy with the intraoperative detection of handled γ probe [60]. Sentinel node detection has achieved a large consensus for reliability, and at present, it keeps having an important place in the clinical management of breast cancer. On the other hand, many authors have acknowledged the value of PET in the differential diagnosis of breast lesions and in locoregional staging, since breast cancer is strongly avid for glucose. PET has also demonstrated its efficacy in detecting axillary lymph node metastases [67, 68].

Whole-body PET may be substituted for other diagnostic assessments by examining the different regions of potential cancer dissemination. Currently, diagnosis for pre- and postoperative staging includes several studies—chest X-ray, abdomen and breast ultrasounds, mammography of the contralateral breast, and, in some patients with large lesions or symptomatic, bone scintigraphy with 99m Tc-diphosphonates—and other laboratory test may be considered. At this point, computerized tomography (CT) and MRI may be useful, and its application will depend on the individual risk of metastatic spread. Bone scintigraphy and PET may be also useful in monitoring therapy response and in detecting tumor relapses during follow-up, but there is still necessary to analyze its efficiency.

Radionuclide lesion localization (ROLL) is a simple technique used in occult breast malignant lesions that could also be removed by placing a Kopans wire into the breast close to the suspect site of mammography and ultrasound. But this is a simple technique that injects 10 MBq of 99m Tc MAA into the tissue adjacent to the suspected tumor. At surgery, a handheld probe (the same that is used for sentinel node) is used to identify the "hot site" and it is completely excised [60, 69, 70].

Imaging Axillary Lymph Nodes in Patients with Newly Diagnosed Breast Cancer

The presence of axillary lymph node metastasis in patients newly diagnosed with breast cancer carries significant prognostic and management implications. As a result, there is increasing interest to stage accurately the axilla with preoperative imaging to facilitate treatment planning. Currently, the most widespread imaging techniques for the evaluation of the axilla include ultrasounds (Figs. 19.9 and 19.10) and MRI. In many settings, the ability to detect axillary lymph nodes containing metastases with imaging and image-guided biopsy can allow surgeons to bypass sentinel lymph node dissection and proceed with full axillary lymph node dissection. However, no imaging modality currently has sufficient negative predictive value to obviate surgical staging of the axilla if no abnormal lymph nodes are detected. Promising advanced imaging technologies, such as diffusion-weighted imaging and magnetic resonance lymphangiography, hold the potential to improve the accuracy of

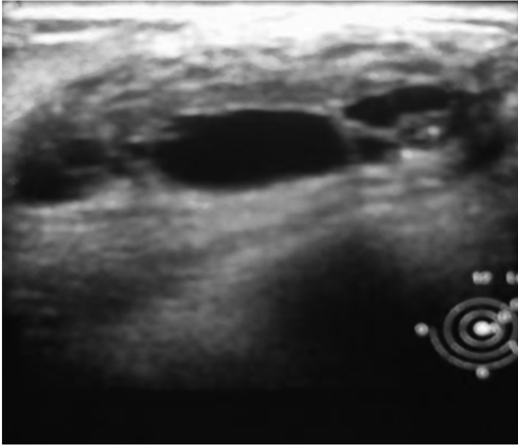

Fig. 19.9 Benign axillary lymph nodes explored by ultrasound scan

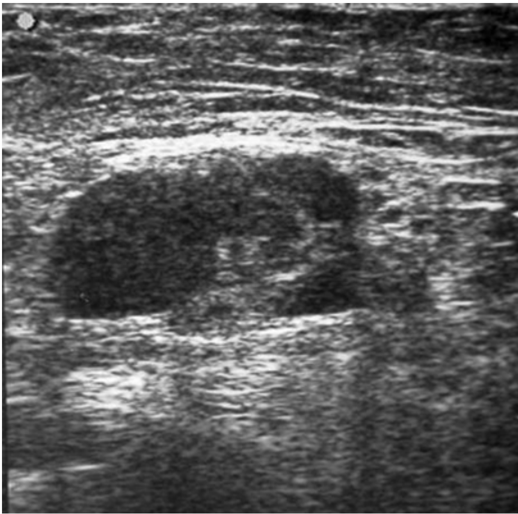

Fig. 19.10 Axillary lymph node metastasis by ultrasound scan

axillary staging and thereby transform management of the axilla in patients newly diagnosed with breast cancer [71].

Conclusion and Future Perspective

Mammography is the only screening test proven to decrease breast cancer morbidity and mortality. It meets all the criteria for a screening test. Although mammography remains the gold standard, it does have limitations, particularly in women with dense breasts. New imaging techniques are emerging to overcome these limitations and enhance cancer detection, improving patient outcome.

Ultrasound: As a breast cancer detection procedure, ultrasound cannot replace a mammogram for breast cancer screening. Over the past two decades, one of the advances in medicine and imaging research has been the marked expansion of the capabilities of breast ultrasound in the evaluation of breast disease. Breast ultrasound has become a fundamental component for the diagnosis and prognosis of breast cancer.

Magnetic Resonance Imaging: MRI is one of the most relevant diagnostic tools for breast cancer nowadays. It is used widely for screening women at increased risk of breast cancer, like BRCA-positive patients; and it is also widely used in order to select the best treatment. MRI has the highest sensitivity in breast cancer imaging, but the low specificity is still its biggest disadvantage.

Digital Mammography: Potential advantages include improvements in image contrast, post facto manipulation of the image, elimination of the problem of lost films, reduction in film library maintenance costs, and the ability to transmit the images over long distances (telemammography). Full-field digital mammography optimizes the lesion-background contrast and gives better sensitivity, and it is possible to see through the dense tissues by altering computer windows; this may be particularly useful in younger women with dense breasts. The need for repeat imaging is reduced, with the added advantage of reduced radiation dose to patients. Early experience has shown that digital mammography reduces the number of patients recalled for additional views, reduces the number of false-positive breast biopsy results, and can potentially enable detection of breast cancer at an earlier stage.

Nuclear Medicine Breast Imaging Technology: Two of the most interesting techniques offered by nuclear medicine are PET and lymphoscintigraphy.

As seen before, intense efforts are under way to improve the technological aspects of breast imaging, detection rate, and correct classification

of breast cancer. These include advances in X-ray and gamma ray detector technology, MRI techniques, tomographic image reconstruction, signal processing, and nuclear medicine techniques. Based on current understanding and results from ongoing research, it appears that high-resolution, high-contrast, anatomical X-ray imaging, either in 2D (mammography) or with added depth information, will be the primary screening modality in the next decade. Furthermore, MRI and ultrasound will have an increasingly important role for imaging high-risk patients or women with dense breasts and will help classify lesions previously seen in mammography.

On the other hand, we are likely to observe a shift in the manner in which breast cancer screening will be performed in the near future. While breast cancer screening is currently performed in a standard manner, for all women, with mammography, this level of standardization is likely to be replaced by a screening program where the selection of the image modality will depend on the individual's risk and other individual aspects.

To sum up, ongoing research and recent evidence indicate that the prospects of substantial improvements in early detection, accurate diagnosis and classification, and improved monitoring of therapeutic response of breast cancer are highly promising.

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20 Breast Cancer Biomarkers for Risk Assessment, Screening, Detection, Diagnosis, and Prognosis

Mukesh Verma and Debmalya Barh

Abstract

Breast cancer mortality can be prevented if the disease is detected early. During the past decade, progress has been made in identifying invasive and noninvasive biomarkers. Genetic biomarkers are based on mutations and single nucleotide polymorphisms (SNPs) associated with breast cancer and have potential use in screening high-risk populations to identify individuals who are likely to develop this disease. Among epigenetic biomarkers, hypermethylation of selected genes and specific microRNA (miR) profiling can be used for cancer detection, diagnosis, and prognosis. This chapter also discusses other biomarkers, such as proteomics, imaging, and glycomics, as well as the advantages of noninvasive biomarkers as compared to invasive biomarkers. Also covered are new approaches to currently available technologies and assays to make them suitable for clinical use. The ultimate goal for detection is to identify (a) biomarkers that can be assayed in samples that are collected noninvasively, (b) assays that are not expensive, and (c) biomarkers that show high sensitivity and specificity.

Keywords

Biomarker • Cancer • Chromatin • Diagnosis • Early detection • Epigenetics • Genomic instability • Histone • Methylation • MicroRNA • Prognosis • Proteomics • Surveillance • Validation

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Introduction

Mortality from breast cancer is very high worldwide [1]. More than half of breast cancer cases occur in the Western countries. The cost of treatment is higher when breast cancer is detected late in its development; therefore, detecting this cancer early is the key to success. Mammography has been successful in reducing mortality from

this cancer, but it is an expensive technique. Although men also develop breast cancer, the distribution and determinants of male breast cancer appear to be substantially different from that of women.

The occurrence of breast cancer in the general population can be explained by inherited genetic susceptibility, somatic changes, effects of endogenous and exogenous environments, and the interaction of these factors (especially gene–environment interactions). Inherited genes for breast cancer susceptibility can be low- or high-penetrance genes; the few genes with allelic variants that confer a high degree of risk to an individual are known as high-penetrance genes. Other genes confer a small to moderate degree of breast cancer risk to an individual and are known as low-penetrance genes. Relatively few individuals in the population carry risk-increasing genotypes at the loci where high-penetrance genes act; therefore, the population-attributable risk is low. On the other hand, the low-penetrance genes are not associated with syndromic or Mendelian patterns but are associated with sporadic breast cancer. The allelic variation of low-penetrance genes is relatively high, and large breast cancer populations carry low-penetrance genes. Different investigators have identified low- and high-penetrance genes in breast cancer in a number of populations.

To date, no single biomarker has demonstrated sufficient sensitivity and reproducibility for independent clinical and commercial utility [1, 2]. The background characteristics of breast cancer and the current status of biomarkers with potential for breast cancer risk assessment, screening, detection, diagnosis, and prognosis are described below.

Breast Cancer Characteristics

The presence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2 or ERBB2) is used for the clinical and pathologic classification of breast cancer [3]. Generally, ER positive (+) and PR+ are indicators of good prognosis, and Her2+ is an indicator of bad prognosis. In addition, the ER-negative (−), PR−, and Her2− (also called triple-negative) status is considered to be an indicator of poor prognosis. Basal cells exhibit triple-negative features. Additional biomarkers that are better prognostic indicators than hormone receptor status are needed, and a better understanding of the genetic characteristics of patients is needed to improve current clinical practice. On the basis of oncologic pathway activity analysis, up to 18 subtypes of breast cancer have been suggested [4]. However, the implications of this information for clinical practice remain to be determined. Furthermore, many prognostic gene expression signatures that dichotomize patient populations into treatment-responsive and nonresponsive groups lack specificity [5–8]. Ideally, a method for preoperative molecular profiling should be developed that can guide treatment strategies.

Genomic Biomarkers

BRCA1 was the first gene identified as indicating susceptibility to hereditary breast cancer. Subsequently, BRCA1 (located on 17q21) was confirmed as indicating ovarian cancer as well [9–11]. A number of cohorts with exposure data and other participant details have been used to identify breast cancer-associated genetic biomarkers [12]. One such cohort, the Collaborative Oncological Gene-environment Study (COGS), is a large-scale genotyping cohort funded by the European Commission. More than 150,000 samples have been genotyped in this study. Familial-based high-penetrance susceptibility genes were identified first, followed by low-penetrance genes by association studies [13, 14]. Carriers of such genes and single nucleotide polymorphisms (SNPs) are predisposed to breast cancer. Pharoah and Caldas found that a panel of 70 genes was able to predict breast cancer prognosis [15]. Genomic markers include SNPs, mutations, additions and deletions, recombinations, and changes in copy number [16–23].

Genome-wide association studies (GWAS) have been conducted by different groups in

different cohorts to identify breast cancer susceptibility genes that may be useful for breast cancer screening in high-risk populations [24–28]. In one such study, genotyping of 2,702 women of European ancestry with invasive breast cancer and 5,726 controls was conducted [27]. SNPs identified in this study were located primarily in the 1p11.2, 2q35, 3p, 5p12, 8q24, 10q23, 13, 14q24.1, and 16q regions. Genes affected by these SNPs are involved in actin cytoskeleton regulation, glycan degradation, alpha-linolenic acid metabolism, circadian rhythm regulation, and drug metabolism.

Epigenomic Biomarkers

In addition to genetic code, human cells contain an additional regulatory level that predominates the genetic code; this is known as the epigenetic code. The epigenetic code involves altering gene expression without changing the genomic structure. Due to different chromatin status, condensed or relaxed, the same genetic variants might be associated with different phenotypes. Chromatin status can be affected by environmental, lifestyle, and other exposures. A rapidly growing number of genes with epigenetic regulation altering their expression by chromatin remodeling (condensation and relaxation) have been identified [29–37]. Methylation of cytosines in DNA, histone modifications, and alterations of noncoding RNAs (especially miRs) are the mechanisms involved in chromatin remodeling.

The term epigenome is used to define a cell's overall epigenetic state. The basic biological properties of DNA segments, such as gene density, replication timing, and recombination, are linked to their GC content. The promoter region is rich in CpG content. A genomic region of about 0.4 kb with more than 50 % GC content is called a CpG island. In mammals, CpG islands typically are 200–300 bp long. Promoters of tissue-specific genes that are situated within CpG islands generally are unmethylated. During breast cancer development, however, these CpG sites start to become methylated. Cytosine methylation can regulate gene expression by hindering the association of some transcriptional factors with their cognate DNA recognition sequences, methyl CpG binding protein (MBP) can bind to methylated cytosines and mediate a repressive signal, or MBPs can interact with chromatin-forming proteins to modify the surrounding chromatin, thereby linking DNA methylation with chromatin modification. DNA methylation at position five of cytosine is conducted by DNA methyltransferases (DNMTs). These enzymes are needed to initiate and maintain methylation.

Alterations due to epigenetic mechanisms can be stably passed over numerous cycles of cell division, and selected epigenetic alterations can be inherited from one generation to another [38–42]. Cancer-specific methylation alterations are hallmarks of different cancers [43]. Alterations in methylation may cause genomic instability, genomic alterations, and changes in gene expression [43–45]. A systematic approach to determining epigenetic changes in tumor development may lead to identification of biomarkers for cancer diagnosis. Baylin's group suggested that integrating the genome and hypermethylome might provide insight into major cancer development pathways, which in turn might help in identifying new biomarkers of cancer diagnosis and prognosis [46]. Methylation and miR alterations are the main biomarkers that can be assayed easily and noninvasively in samples [47, 48]. The finding that monozygotic twins are epigenetically indistinguishable early in life but exhibit substantial epigenomic differences with age indicates that environmentally determined alterations in a cell's epigenetic marks are responsible [49–53]. It also is known that environmental factors influence the development of breast cancer [54–59].

When the epigenetic profiling of MCF-7, MDA-MB-231, and MDA-MB-231(S30) was followed, decreased trimethylation of H4K20 and hyperacetylation of H4 were observed. Concomitant to the decrease in trimethylation, lower levels of the Suv4-20h2 histone methyltransferase also were observed. The effect was more pronounced in MDA-MB-231 compared to other cells, which suggests that differential

expression of histone modifications could represent disease aggressiveness. In another study, HDAC6 (a histone acetyltransferase) responded to estrogen treatment [60, 61]. Retinoblastoma levels were lower whenever trimethylation of H4K20 was present. A correlation between tumor stage and grade also was established based on these histone biomarkers [62]. Another study reported on the quantitative expression of HDAC1 and its correlation with breast cancer patients' age, lymph node status, tumor size, and Her2/neu−, ER+, and PR+ status [63].

Methylation Biomarkers

Cancer cells accumulate abnormal DNA methylation patterns that result in malignant breast cancer phenotypes. The genomic distribution of methylation is not well understood, and a number of GWAS have been conducted to identify breast cancer risk-associated biomarkers [25, 64–69]. Using methylated DNA immunoprecipitation combined with high-throughput sequencing (MeDIP-seq), levels of methylation were compared in samples from normal and breast cancer cells, and global hypomethylation was observed in breast cancer samples, especially in the CpG-rich regions. The location of these CpG-rich regions was not related to the transcription start sites of various genes. Using this approach, the methylation patterns during epithelial to mesenchymal transition also were evaluated and used for disease stratification [25].

Methyl acceptance capacity in malignant breast tissues was approximately two- to three-fold greater compared with matched controls. However, there was considerable variation in methyl acceptance capacity among patients [70]. Quantitative analysis of 5meC levels showed a substantial decrease compared with normal tissues. Levels of hypomethylation in BRCA1 and BRCA2 cancers were slightly lower but significant [71]. Genome-wide hypomethylation correlated with satellite sequence hypomethylation. Definite regions (Sa2 coding) on chromosome 1 and satalpha were specifically hypomethylated [71, 72]. On chromosome 5, the region containing the coding sequence of SATr-1 also showed hypomethylation.

miR Biomarkers

miRs are the key regulators among a number of regulators of gene expression. Tissue-specific miRs have been reported by different groups [73, 74]. These RNAs are small in size and have a distinct stem-and-loop structure [75]. A number of miRs can be isolated in circulation. Because of their small size and stability (due to secondary structure), these circulating miRs provide a rich source of diagnostic biomarkers for breast cancer. In inflammatory breast cancer cells, more than 300 miRs were evaluated for their association with breast cancer [76]. The most promising miRs were miR-29a, miR-30b, miR-342-5p, and miR-520a-5p. The functional analysis of these miRs revealed their role in cell proliferation and signal transduction pathways. These markers should be useful in identifying inflammatory breast cancer cells whenever a subtyping of breast cancer cells is needed. The promoter regions of the miR coding regions were evaluated by 5-methylcytosine immunoprecipitation coupled to miR tiling microarray analysis; and several miR promoters were found to be hypermethylated, especially those of miR-31, miR-130a, miR-let7a-3/let 7-b, miR-155, and miR-137 [74]. miRs function by binding to their target mRNAs. Mitchell and colleagues demonstrated the advantage of using miRs for detecting cancer because of their stability, even in fixed tissues [75]. miR-155 predicted prognosis in triple-negative breast cancer (higher miR-155 expression correlated with higher angiogenesis and aggressiveness) [77]. In summary, miRs are useful in breast cancer screening and risk assessment before the disease has developed. Furthermore, a panel of miRs can be used for breast cancer detection and diagnosis. To follow up the treatment of breast cancer, miR profiling can be used (an application of miR biomarkers in breast cancer prognosis and survival).

Proteomic Biomarkers

Compared to transcriptomic or genomic biomarkers, protein biomarkers are more closely related to disease phenotype and more easily

targeted for therapy. Proteomics provides a powerful tool for investigating potential biomarkers in several types of cancers because of its high sensitivity, precise characterization of interaction, and ability to detect functionally significant posttranslational modifications. Proteomic biomarkers have been identified in blood (serum and plasma) as well as in breast tissue through the application of approaches such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), two-dimensional gel electrophoresis, and immunoprecipitation. In one study, investigators identified circulating proteomic biomarkers from different stages of breast cancer using an innovative strategy that employed high-sensitivity label-free proteomics. The approach was MS based, provided semiquantitative results, and could be applied in preclinical and clinical studies. A breast cancer patient serum was analyzed by two-dimensional nanoUPLC tandem nanoESI-MS to identify breast cancer biomarkers that are differentially expressed during the early stages of cancer development [78]. Higher GRHL3 expression and lower levels of TNF-alpha were reported during the early stages of the disease, whereas PMS2 expression was high in advanced stages of the disease. These results were validated in a different set of patients, although the number of participants was low. These investigators plan to evaluate the impact of such markers in determining patient survival rates and recurrence of breast cancer or other cancers.

Proteomic approaches also are useful in identifying protein–protein interactions. In one study, estrogen receptor alpha and its interactions with a number of transcription factors were characterized, resulting in clinically useful information about breast cancer therapeutics [79]. Laser capture microdissected breast cancer and normal tissue cells were analyzed by MS to identify proteomic profiles associated with breast cancer [80]. In another study, glyoxalase-1 was found to be expressed in breast cancer [81]. This protein is involved in the detoxification of methylglyoxal, a cytotoxic product of glycolysis. Further analysis of tissue microarray indicated a correlation between glyoxalase-1 and tumor grade. Based on reverse phase protein array results, a model was created to predict pathologic complete response in patients receiving neoadjuvant taxane- and anthracycline-based systemic therapies, thus indicating the translational significance of proteomic biomarkers in breast cancer [82].

Imaging Biomarkers

The clinical use of mammography for breast cancer screening has helped to reduce breast cancer mortality [83, 84]. Imaging is an enabling scientific discipline that combines advanced technology and complex computational and analytic methods to provide a unique ability to extract spatially and temporally defined information from humans [85–89]. Imaging allows us to investigate intact biological systems (without isolating samples or taking biopsies) across the spectrum, from subcellular to macroscopic, and from discovery to clinical decision-making.

Mammography is the process of using low-energy X-rays to examine the human breast and is used as a screening and diagnostic tool. Early breast cancer is detectable by this technology via characteristic masses and/or microcalcifications. Thus, mammography is considered a noninvasive biomarker for cancer diagnosis. For the average woman between the ages of 50 and 74 years, mammography is recommended every 2 years. This helps to avoid unnecessary surgery, treatment, and anxiety. It should be noted that mammography has a false-negative rate of approximately 10 % because dense tissues can obscure a cancer and the appearance of cancer on a mammogram can overlap with the appearance of normal tissues [90]. In addition, the radiation exposure associated with mammography is a potential health risk [91–95].

PET scans were used to determine the treatment response in breast cancer patients [96]. The main problem with imaging technologies is that tumor heterogeneity interferes with the interpretation of results, and a combination of other biomarkers and patient-related information is needed to infer any clinical value.

Metabolomic Biomarkers

In recent years, metabolites of biofluids have been analyzed for their potential in cancer diagnosis and treatment follow-up. For example, the use of urine analysis for the routine monitoring of metabolomic disorders has attracted interest among scientists because the procedure can be done easily, noninvasively, and repeatedly for a large number of samples with high precision. Generally, volatile organic metabolites (VOMs) get enriched in urine, and their analysis is not complicated [2]. The advantage of adopting a metabolomic approach lies in the fact that metabolites are much more stable than RNA and proteins and their levels predict pathways that are affected during disease development. In a small study of urine from controls and individuals with breast cancer, VOMs were identified that were differentially expressed in patients [97]. Higher levels of 4-carene, 3-heptanone, 1, 2,4-trimethylbenzene, 2-methoxythiophene, and phenol, and lower levels of dimethyl sulfides, were observed in breast cancer patients. Urine metabolites also have been used in diagnosing colon, lung, liver, and prostate cancers [2].

Characterizing metabolomic pathways is useful in making treatment decisions [98]. Breast cancer is stratified into a low-risk group known as the low-grade ER^+ group, an intermediate-risk group known as high-grade ER^+, and a high-risk group with triple-negative tumors (ER^-/PR^-/ and $Her2^-$). Based on molecular characterization of 149 samples from these three groups, patterns/profiles of biomarkers were identified that correlated with biologically established pathways (Myc, E2F1, Ras, beta catenin, and INF-gamma) in tumor development. The low-risk group was responsive to endocrine therapy; the intermediate- and high-risk groups were resistant to treatment, however. The new biomarker profile was named ClinicoMolecular Triad Classification (CMTC). Further studies indicated that CMTC predicted breast cancer recurrence and treatment response better than traditional clinical and pathology analyses. The new profile can be incorporated easily into current clinical practice for classification of breast cancer subtypes [98]. CMTC can be performed in needle biopsy samples collected at the time of enrollment. Prospective, randomized clinical trials may further validate these results.

Why Use Noninvasive Biomarkers?

Although noninvasive biomarkers function best in detecting breast cancer, when these biomarkers are validated in a large number of samples, some do not show reasonable sensitivity and specificity [73]. The traditional treatment options for breast cancer are radiation, chemicals, and surgery (lumpectomy, quadrantectomy, mastectomy). Surgery usually is combined with adjuvant therapy (hormonal and/or chemical therapy). The chemicals used for therapy have considerable toxicity, and hormonal treatments also have long-lasting adverse effects. Surviving patients generally have a poor quality of life. Furthermore, resistance to chemotherapy is another problem observed in breast cancer patients [99–103]. Pharmacogenomics is an area of research that may provide useful information in these cases [104–106]. By applying diagnostic tests and knowing the genetic background of an individual, personalized treatments are possible.

Noninvasive biomarkers also can be helpful in guiding the choice of therapy for breast cancer patients. In general practice, women with ductal carcinoma in situ (DCIS) are treated with tamoxifen instead of an aromatase inhibitor [107]. However, in early invasive stages as judged by a panel of biomarkers, aromatase inhibitors proved better for treatment than tamoxifen. The use of hormonal therapy varied with patient age and tumor characteristics. Most of these characteristics correlated better in early-stage carcinoma patients than in DCIS patients. This research led to the development of prevention strategies. Endocrine therapy is now used for preventing new primary breast cancers and invasive recurrence for women with DCIS or early invasive breast cancer. The dose used was higher at early stages but decreased with the patient's age.

Tissues are the best source of material in which to assay early-detection cancer biomarkers

Table 20.1 Samples used in breast cancer biomarker studies

Sample	Comments
Fine-needle aspiration biopsy (FNAB), nipple aspirate with exfoliated cells	Used to determine the receptor level and metabolomic profiling [98]
Plasma	Proteomic markers in breast cancer patients with early stages of cancer [78, 108, 109]
Serum	SELDI-TOF was used to identify proteomic biomarkers [110]; proteomic markers in breast cancer patients with early stages of cancer [78]
Tumor tissue	Proteomic biomarkers were isolated by laser capture microdissection of breast cancer tissue [80, 111]; a tissue microarray was used to identify candidate proteomic markers [81]; a reverse phase protein array was used to identify breast cancer biomarkers [82]; imaging biomarkers in the breast [91–95]

because they represent the true expression of biomarkers during cancer development. Tissue collection is an invasive procedure, however, and it is difficult to procure healthy tissue for comparison. Preferred biomarkers are those that can be assayed in samples collected noninvasively. Biofluids (urine, blood, sputum) and exfoliated cells are good examples of noninvasive sources of biomarkers for early cancer diagnosis, as shown in Table 20.1. After identifying breast cancer biomarkers, the assay and the biomarker must be approved by the U.S. Food and Drug Administration (FDA) so that these biomarkers can be assayed in clinical samples. The FDA has provided guidelines for this process. If biomarkers, assays, or devices are intended for clinical use in patient samples, they should be reviewed by the FDA's Center for Devices and Radiological Health (CDRH) for their ability to analytically measure the biomarker. Biomarkers and devices for quantification are expected to yield equivalent results. Biomarkers should have passed analytical and clinical validation tests specified by the FDA. Analytical validity in this context is defined as the ability of an assay to accurately and reliably measure the analyte in the laboratory as well as in the clinical sample. Clinical validation requires the detection or prediction of the associated disease (cancer) in specimens from targeted patients. Biomarker qualification by the FDA enables collaboration among stakeholders, reduces costs for individual stakeholders, and provides biomarkers that are useful for the general public and private entities.

Epigenomic biomarkers have enormous potential and clinical implications for cancer diagnosis and prognosis. Because of the availability of genome-wide methylation, histone, and miR analysis technologies—and our rapidly accumulating knowledge regarding the epigenome—the translation of findings discussed in this chapter may be possible in the near future. Epigenetic biomarkers also may be useful in identifying patients who will benefit from a therapy without developing a resistance to the drugs. Recently developed drugs for cancer treatment are based on specific pathways and may be useful for individuals in whom these pathways are altered. This approach can be designed for personalized medicine and precision medicine. Epigenetic biomarkers also may be useful in such approaches. Additional potential breast cancer biomarkers continue to be identified, including additional high-penetrance markers.

Breast Cancer Detection Patents

A number of patents have been issued that involve genomic, epigenomic, and proteomic biomarkers (Table 20.2). Biomarkers can be used for breast cancer diagnosis either singly or in combination. These patents were generated by investigators in the industry and academic institutes. Compared to the number of publications in the breast cancer biomarker field (Table 20.3), the number of patents is low. The reason may be that many biomarkers have not been characterized in sufficient numbers of clinical samples. It is worth noting, however, that the number of reports on genetic and imaging biomarkers exceeds those of other biomarkers, as indicated in the PubMed analysis of the field shown in Table 20.3.

Table 20.2 Patents for breast cancer diagnosis and risk assessment

Patent number	Inventor/assignee	Title and comments
US20090311671	Rado Laboratories Ltd., Co. Antrim (GB)	Diagnosis of risk of breast cancer Polymerase chain reaction (PCR) assay of samples from breast tissue
US5645995	Baylor College of Medicine, Houston, TX (USA)	Methods for diagnosing an increased risk for breast or ovarian cancer Samples used were from blood, ascites, pleural fluid, and spinal fluid Point mutations in progesterone receptor gene were assayed Sequencing, single-stranded conformation polymorphism (SSCP), heteroduplexing, and restriction mapping technologies were used
US56838885	Baylor College of Medicine, Houston, TX (USA)	Methods for diagnosing an increased risk for breast or ovarian cancer Progesterone receptor mutations were analyzed Antibody-based assay
US20100285456	Matta, Jamie (USA) Carolina, R (USA)	Method for using DNA repair capacity as a biomarker for breast cancer risk in women Measurement of DNA repair capacity of lymphocytes using the host-cell reactivation assay Blood lymphocyte biomarker to calculate the probability of having carcinoma of the breast
US7635561	Temple University of the Commonwealth System of Higher Education, Philadelphia, PA (USA)	Methods of diagnosing, prognosing, and treating breast cancer Methylation assay in breast cancer cells obtained by biopsy Presence of methylation region in the A, B, C, and E regions of the estrogen receptor alpha gene promoter
US20070141582	Li, Weiwei New York (USA) Li, Jessica New York (USA)	Method for detecting early cancer and precancer in blood or body fluid Analyzed methylation levels in a panel of genes (p16, RASSF1, APC, MGMT, GSTP1, CDH-13, MLH-1) by quantitative PCR Measured demethylation of CK7, CK20, TF-1, NKX3-1, EBV, MAT-2, PAX-1, and mammaglobin-A by quantitative PCR Assay is good for breast, lung, ovarian, colon, pancreas, liver, thyroid, and nasopharyngeal cancers
US20100221742	National Institute of Immunology, New Delhi (IN)	Novel cancer-associated antibodies and their use in cancer diagnosis Western analysis of serum
US20100221723	The Institute of Columbia University in the City of New York, NY (USA)	Early detection of cancer by methylated DNA in blood Methylation assay in DNA from blood samples (serum or plasma) from breast cancer patients and controls Methylation of RASSF1A and p16 in the promoter region
US20090035801	Power3 Medical Products, Inc., The Woodlands, TX (USA)	12 protein biomarkers for diagnosis and early detection of breast cancer Diagnostic assay for differentiating breast cancer, benign disease, and normal controls Based on a statistical analysis of the concentration in blood serum of one or more of the selected 12 proteins (inter-alpha–alpha trypsin inhibitor heavy chain (H4)-related protein, immunoglobulin lambda chain protein, alpha-1 microglobulin protein, apolipoprotein A-1, apolipoprotein E, complement C4 protein, serum albumin protein, lectin P35, transferrin protein)

US6756200	The Johns Hopkins University School of Medicine, Baltimore, MD (USA)	Aberrantly methylated genes as a marker of breast cancer malignancy Methylation of CpG islands in the promoter region of cyclin D2 by PCR-based methods of selected amplimers DNA isolated from blood, plasma, lymph, ductal cells, ductal lavage fluid, nipple aspirate, breast tissue, lymph nodes, bone marrow, or a combination of samples
US6835541	The Johns Hopkins University School of Medicine, Baltimore, MD (USA)	Aberrantly methylated genes as a marker of breast cancer malignancy Methylation of CpG islands in the promoter region of cRAR-beta by PCR-based methods of selected amplimers DNA isolated from blood, plasma, lymph, ductal cells, ductal lavage fluid, nipple aspirate, breast tissue, lymph nodes, bone marrow, or a combination of samples
US20060154245	Rigshospitalet, Copenhagen (DK) Hvidovre Hospital, Hvidovre (DK)	Method for detecting, screening, and/or monitoring a cancer in individuals Determine TIP-1 concentration from saliva samples by enzyme-linked immunosorbent assay (ELISA) and zymography Detects early-stage breast and several other cancers
US20090221010	Elting, J. J. Madison, CT (USA), Carney, W. P. North Andover, MA (USA), Hamer, P. J. Reading, MA (USA)	Methods for prediction and prognosis of cancer and monitoring cancer therapy Sandwich ELISA was applied to samples taken from blood, serum, plasma, urine, saliva, semen, breast exudate, cerebrospinal fluid, tears, sputum, mucous, lymph, cytosols, ascites, pleural effusions, amniotic fluid, bladder washes, and bronchioalveolar lavage Increasing levels of VEGF-165 in patient samples taken over time to detect early recurrence or metastasis
US7662582	Chang Gung University, Tao-Yuan (TW)	Method of identifying cancer biomarkers and cancer progression Loss or absence of pro-u-plasminogen activator indicates that a cancer is high stage, high grade, or both Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of urine samples for bladder cancer

Table 20.3 Publications of breast cancer biomarker studies indicating investigator interest in the field

Topic	Number of publications
Biomarker	593,113
Biomarker and cancer	212,324
Biomarker and breast cancer	27,988
Biomarker and breast cancer and genetics	10,514
Biomarker and breast cancer and epigenetics	45
Biomarker and breast cancer and methylation	381
Biomarker and breast cancer and histone	247
Biomarker and breast cancer and microRNA	209
Biomarker and breast cancer and proteomics	419
Biomarker and breast cancer and imaging	1,094

Note: The analysis was PubMed based, and references up to 2012 were considered

Conclusion and Future Perspective

Considerable knowledge has been gained in understanding the biology of breast cancer and identifying biomarkers that can be used in detecting breast cancer, but translation of that knowledge to the clinical setting has been challenging [1]. Clinical validation is the main hurdle in the process. In a case-control study of the Prospect-EPIC (European Prospective Investigation into Cancer and Nutrition) study in which more than 300 breast cancer patients and matched controls were tested for breast cancer over a period of 3 years using a panel of eight serum markers (osteopontin, haptoglobin, cancer antigen 15-3, carcinoembryonic antigen, cancer antigen-125, prolactin, cancer antigen 19-9, and alpha-fetoprotein), very low specificity (50 %) and sensitivity (50 %) were observed [112]. This may be due to the presence of different breast cancer subtypes in collected samples. Such epidemiologic studies should select a broader target set of potential biomarkers that could be enabled by antibody array technologies in which profiles of up to 100 antibodies can be followed simultaneously. Making different groupings based on the status of hormone receptors (estrogen and progesterone) also might be helpful.

The need to identify and characterize early cancer diagnostic biomarkers is considerable because cancer is a heterogeneous disease, and a patient's individual molecular profile, resulting from tumor microenvironment, determines disease development and response to treatment [37, 113]. Tumor microenvironment is affected by several factors, including epigenetic factors of the cell. As noted above, a number of noninvasive biomarkers for early detection of breast cancers have been identified.

In the field of cancer prevention, the National Cancer Institute (NCI) has an ongoing study, "the Study of Tamoxifen and Raloxifene (STAR)," with the objective of examining how the drug raloxifene compares with the drug tamoxifen in reducing the incidence of breast cancer in postmenopausal women who are at increased risk of developing the disease. Early results indicated that both drugs are equally effective and that about one-half of the disease incidence could be reduced. After the trial was continued for a longer time in the absence of these drugs to see whether previous prevention could be sustained, a 50 % reduction in incidence was seen with raloxifene, while a 38 % reduction was seen with tamoxifen. Both noninvasive and invasive cancers were studied in the STAR. Furthermore, participants taking raloxifene showed fewer side effects (e.g., uterine cancer, blood clots, cataracts) than those taking tamoxifen. Researchers with the National Surgical Adjuvant Breast and Bowel Project (NSABP) are conducting this study (financially supported by the NCI, USA), and more than 500 participants aged 35 years or older are involved in the study. The trial began in 1999 and stopped enrolling new patients in 2004.

The main areas in which progress and attention are needed with respect to biomarker identification are cost, high throughput, and the application of breast cancer biomarkers in clinical settings. Proper analytical and clinical validation of early markers has not been achieved. Currently, the key challenge in the field is the clinical validation of identified biomarkers. The NCI has developed guidelines for the analytical and clinical validation of biomarkers,

but no biomarkers have been validated to date [37, 113–117]. Integrating genomic and proteomic markers with epigenetic markers may be useful in subtyping different cancers and cancer stages [46, 118]. Methylation profiling results from blood and tissues often differ. Koestler and colleagues conducted a systematic epigenome-wide methylation analysis and demonstrated that shifts in leukocyte subpopulations might account for a considerable proportion of variability in these patterns [119]. Multiplexing of biomarkers may reduce false-positive results in screening studies when the intent is to identify populations who are at high risk of developing breast cancer.

Quantitative imaging data storage and maintenance present their own challenges, as discussed above. Whether miR expression is localized in a specific part of the breast tissue must be evaluated carefully. In a tissue biopsy, the local concentration (number of miRs) may be low or high. Determining the accurate level of miR concentration is critical.

Association studies are extremely powerful in identifying new low-penetrance SNPs (biomarkers) that may have therapeutic implications. Identifying common low-susceptibility alleles is useful because it provides possible insight into the mechanisms of tumor biology and identification of high-risk individuals. Because genotyping is no longer expensive, the information from such studies can be utilized in personalized medicine in the form of targeted primary and secondary prevention.

Prognostication is a promising area in the translation of experimental research into clinical practice. In this approach, patterns of altered gene expression in tumors are used to construct classifiers instead of standard indices such as Nottingham Prognostic Index, Adjuvant! Online, and PREDICT [120–122].

In conclusion, we would like to emphasize that considerable progress has been made in identifying breast cancer biomarkers that can be used in the complete spectrum of carcinogenesis, from risk assessment to survival follow-up. The information discussed in this chapter may be useful in developing new interventions and therapeutic targets.

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21 Breast Circulating Tumor Cells: Potential Biomarkers for Breast Cancer Diagnosis and Prognosis Evaluation

Phuc Van Pham

Abstract

Circulating tumor cells (CTCs) have been considered an indicator of tumor invasion. CTCs have recently been detected in breast cancer patients and have become a target for evaluating breast cancer progression, prognosis, and diagnosis. CTCs are a heterogenous population with phenotypes ranging from epithelial to mesenchymal. CTCs express various markers including epithelial cell adhesion molecule, cytokeratins, and MUC-1, depending on the stage of epithelial–mesenchymal transition. Breast CTCs are usually detected and confirmed via two steps, including enrichment and identification. These methods have become powerful tools for diagnosis and for predicting response to systemic therapies. This chapter aims to review breast CTC biology and the role of CTC detection in breast cancer prognosis and diagnosis. Recent advances in CTC research mean that CTCs are becoming a strong tool for the prognosis and diagnosis of breast cancer.

Keywords

Circulating tumor cells • Metastasis • Clinical outcome • Circulating tumor stem cells • Diagnosis • Prognosis

Introduction

Breast cancer is one of the most common cancers among women worldwide, with 1.15 million new cases each year [1], including an estimated 207,090 new cases and 39,840 deaths from metastasis in the USA in 2010 [2]. Metastasis is a major cause of death in all cancer patients, and managing metastasis is therefore an important step in treating breast cancer. Breast cancer is currently classified by TNM classification and differentiation grade, complemented by estrogen and progesterone receptor (ER/EP) status and HER2/neu expression [3–7]. Recent advances have been made in relation to the molecular characterization of tumors, and in predicting the effects of therapy, including using breast cancer gene profiling by reverse transcription-polymerase chain reaction [8–11].

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Breast Circulating Tumor Cells

Circulating tumor cells (CTCs) are biomarkers for evaluating metastasis in breast cancer. Since 2000, breast CTCs have been considered a unique target for understanding disease progression, prognosis, and treatment in breast cancer pathogenesis. CTCs are tumor cells that have been shed into the vasculature from the primary tumor and are thus present in the blood. They are found in the blood of patients with many different types of carcinomas, especially in those with metastatic disease [12, 13]. Some studies found that the presence of CTCs in breast cancer patients was associated with significantly shorter progression-free survival (PFS) and overall survival (OS) [14–17], as well as with a high risk of recurrence [18].

Disseminated tumor cells (DTCs) differ from CTCs and were a focus of earlier studies. DTCs are defined as tumor cells in the bone marrow and were present in 30 % of breast cancer patients [19]. The presence of DTCs was considered a significant predictor of outcome. However, bone marrow isolation is an invasive technique, and DTCs were only present at low levels in the bone marrow, with as few as 3 % of bone marrow aspirates containing tumor cells [20]. CTCs are therefore of more interest as targets for the diagnosis and prognosis of cancer, particularly breast cancer.

Morphology

Breast CTCs may be larger than other cells in the blood, such as leukocytes, erythrocytes, and thrombocytes. A commonly quoted range for CTCs is 12–25 μm, which is larger than 90–95 % of the largest blood cell population [21]. Size-based sorting thus represents an attractive, label-free, isolation method. Another study found similar nucleus–cytoplasm ratios in CTCs and tumor cells from a solid metastasis, suggesting that the cell populations in the two sites were similarly differentiated. However, the average size of CTCs is smaller than that of tumor cells in a solid metastasis [22], suggesting either a filtering effect of the capillary beds proximal to the site of blood collection or morphologically undetectable apoptotic effects on the circulating cells.

Immunophenotype

Epithelial to mesenchymal transition (EMT) is a process relating to a set of molecular changes, whereby carcinoma cells increase motility, invasion, and intravasation, as shown in Fig. 21.1. EMT induces proteases that trigger the degradation of the extracellular matrix, allowing carcinoma cells to be released into the blood [23, 24]. CTCs have therefore been considered as EMT-phenotype carcinoma cells in the blood. The EMT phenotype is also associated with a stem cell phenotype [25], and CTCs were demonstrated to include a small population with a cancer stem cell phenotype [26–28].

CTCs are considered as EMT forms of primary tumor cells. Carcinoma cells initially activate the trans-differentiation EMT program, during which they acquire the traits needed to execute the multiple steps of metastasis [29]. During the metastatic process, carcinoma cells in primary breast tumors gradually change from an epithelial phenotype (in the primary tumor), via EMT to a mesenchymal phenotype (in the blood), to an epithelial phenotype again (at the metastatic sites). CTCs in the blood thus exhibit the EMT phenotype.

Breast CTCs in the blood have been recognized with different phenotypes ranging from epithelial to mesenchymal, depending on the stage of EMT. CTCs usually express an epithelial phenotype during the early stage of EMT, both epithelial and mesenchymal phenotypes in the intermediate stage, and a mesenchymal phenotype in late EMT. Pecot et al. recently showed that CTC capture based on cytokeratin (CK) expression would be likely to miss populations of CTCs that had undergone EMT because CTCs in the late stage of EMT lack expression of CK markers [30].

CTCs with Epithelial Phenotypes in Early Epithelial–Mesenchymal Transition

One epithelial marker detected in CTCs is the surface epithelial cell adhesion molecule (EpCAM). EpCAM is a pan-epithelial differentiation antigen expressed on most carcinomas. CTCs express high levels of this marker. Indeed, EpCAM levels are 100- to 1,000-fold greater in primary and metastatic breast cancers than

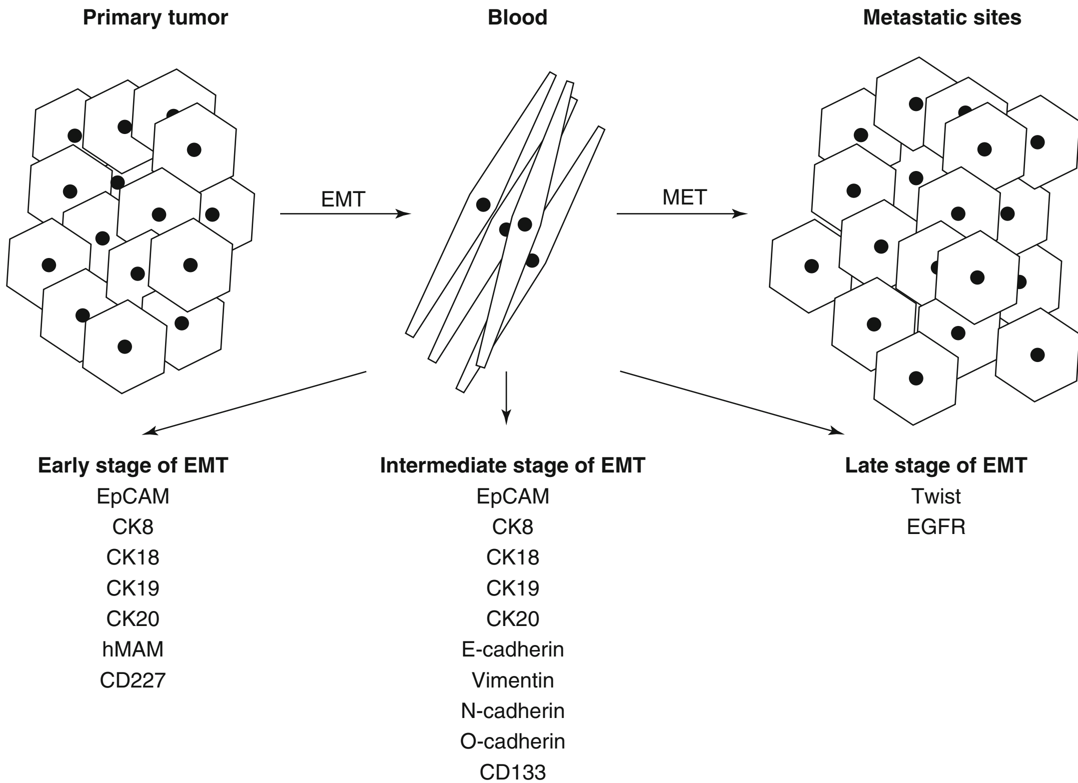

Fig. 21.1 Markers of breast CTCs depend on the EMT stage. *MET* Mesenchymal-Epithelial Transition, *EMT* Epithelial-Mesenchymal Transition

in normal epithelial cells, and this molecule is implicated in tumor invasion and migration [31]. EpCAM has been suggested as a potential therapeutic target in patients with advanced malignancies [19]. EpCAM-positive CTCs could reflect both the volume of metastatic disease in breast cancer patients and the tumor biology in terms of aggressiveness, drug resistance, and mutation.

CTCs have also been defined as mononuclear cells lacking CD45 (which is expressed in blood cells) but expressing CKs (which are strongly expressed in epithelial cells) [32]. Some specific CKs such as CK8, CK18, CK19, and CK20 have been used as markers of CTCs, included breast CTCs [33–36]. Some commercially available methods for isolating CTCs have been developed based on antibodies to these marker proteins, in combination with CD45-negative selection to eliminate white blood cells.

Zhao et al. demonstrated that EpCAM-, CK19-, and hMAM-positive cells were detected in 50 (51.0 %), 43 (43.9 %), and 68 (69.4 %) of 98 patients, respectively, and triple-marker-positive CTCs were detected in 86 of the 98 (87.8 %) patients, which was a significantly higher rate than in the control group [37]. In a recent study, Tunca et al. showed that CK20 was a novel biomarker of breast CTCs, which could be used to identify CTCs as well as to predict breast cancer progression. The CK20-positivity rate was 28.57 % (24/84) [38]. CD227 (Mucin-1 or Ca 15.3) is highly expressed by virtually all mucosal epithelial tissues and is aberrantly expressed in most human breast cancers. It is also used as marker of breast cancer cells and breast CTCs [39–41].

CTCs with Epithelial–Mesenchymal Phenotype in Intermediate Epithelial–Mesenchymal Transition

During the intermediate stage of the EMT process, CTCs exhibit both epithelial and mesenchymal traits. In patients with metastatic CTCs, more

than 80 % of CTCs co-express epithelial proteins, such as EpCAM, CKs, and E-cadherin, together with mesenchymal proteins including vimentin, N-cadherin and O-cadherin, and the stem cell marker CD133 [42]. Another study showed that 77 % of CTCs in early breast cancer patients expressed vimentin, Twist, and CK, while 100 % of CTCs expressed these proteins in patients with metastatic breast cancer. CTCs in patients with metastatic disease thus express antigens characteristic of EMT more than those with early cancer, suggesting that EMT is involved in the metastatic potential of CTCs [43].

CTCs with Mesenchymal Phenotype in Late Epithelial–Mesenchymal Transition

A recent study by Gorges et al. used the AdnaTest to detect CTCs in metastatic breast cancer patients. However, the kit failed to detect CTCs because of the downregulation of EpCAM, whereas mesenchymal markers such as Twist and epidermal growth factor receptor were upregulated, indicating that the CTCs in these patients were in the late stage of EMT. They also recognized that the late stage of EMT was associated with metastatic cancers [44].

CTCs Express Breast Cancer Stem Cell Phenotypes

Breast cancer stem cells (BCSCs) are known to have specific phenotypes, such as $EpCAM^{+}CD44^{+}CD24^{-/dim}$ or $CD44^{+}CD24^{-/dim}$. Based on the BCSC phenotype, CTCs contain 20–30 % BCSCs with the $EpCAM^{+}CD44^{+}CD24^{-/dim}CD45^{-}$ phenotype. Other CTC phenotypes are also found in breast cancer patients, including $EpCAM^{+}CD44^{-}CD24^{-/dim}CD45^{-}$, $EpCAM^{+}CD44^{+}CD24^{+}CD45^{-}$, and $EpCAM^{+}CD44^{+}CD24^{+}CD45^{dim}$ [26]. Theodoropoulos et al. detected CTCs in 66.7 % patients, with 35.2 % of CTCs positive for the BCSC phenotype $CD44^{+}CD24^{-/dim}$. CTCs also exhibited another BCSC phenotype aldehyde dehydrogenase 1 $(ALDH1)^{hi}CD24^{-/dim}$ in 17.7 % patients [27]. Like BCSCs that are the most tumorigenic cells in solid tumors, CTCs expressing BCSC markers are also known as breast circulating tumor stem cells (CTSCs). Breast CTSCs are determined as $CD45^{-}EpCAM^{+}CD44^{+}CD24^{-}$ cells in the blood. The percentage of CTSCs in CD45-negative cells detected by flow cytometry increased with increasing TNM stage (0, 0.00 ± 0.00 %; I, 0.03 ± 0.05 %; II, 0.06 ± 0.14 %; III, 0.10 ± 0.09 %; IV, 0.29 ± 0.35 %; $P = 0.034$) [28].

Notably, the expression of ALDH1 on CTCs was found to correlate with the stage of disease and with the expression of vimentin and fibronectin [45]. CTCs also express some other BCSC markers such as NOTCH1, a gene associated with self-renewing cancer stem cells [46], and almost 70 % of CTCs were found to express ALDH1 [47, 48]. Similarly to BCSCs, CTCs were also shown to be triple negative for ER, PR, and HER2 [47, 48].

Breast CTCs and HER2, ER, PR Tumor Phenotype

HER2/neu is considered as a target for breast treatment. Some studies have evaluated the correlation between HER2/neu expression in CTCs and in primary tumor cells, as well as HER2/neu expression in primary tumor cells with some specific genes in individual CTCs [48–55]. Evidence to date suggests that HER2/neu expression in CTCs does not depend strictly on HER2/neu expression in the primary tumor cells; indeed, HER2/neu-positive cells were detected in patients with HER2/neu-negative tumors [51, 54]. These results have also been confirmed by other groups [56, 57]. Punnoose et al., however, reported that there was a concordance between HER2 status in CTCs and in the primary tumor tissue in the majority of patients (89 %), though the HER2 status in CTCs differed from that in the primary tumor in a subset of patients (11 %) [58]. Thus, some patients negative for HER2/neu in the primary tumor cells may still benefit from HER-2-directed therapy [59]. Moreover, HER-2-positive CTCs were more common in women with HER-2-positive primary tumors [54].

Similarly to HER2/neu expression, ER/PR expression in CTCs does not depend on ER/PR expression in the primary tumor. Aktas et al. showed that the expression profiles of CTCs and the primary tumor differed with regard to ER/PR/HER2 positivity [47], with concordance rates of 29, 25, and 53 %, respectively [48].

Detection Methods for Breast Circulating Tumor Cells

Detection of breast CTCs is an important and essential step in their use for breast cancer diagnosis and prognosis. This step determines not only the existence of CTCs but also the number of CTCs. Like many special kinds of cells, CTCs in a heterogenous population can be detected based on their unique characteristics. However, CTCs are rare cells (one CTC/10^6–10^7 mononuclear cells), and they must therefore be enriched before detection. Hence, CTC detection methods are usually composed of two steps. The initial step enriches the CTCs using morphological and immunological techniques that are nonspecific for CTCs, while the second step involves their identification using protein and nucleic acid-based techniques that are specific to CTCs.

Based on the particular proteins or/and nucleic acids (usually RNA), CTCs can be not only detected but also quantified. CTC detection results can help in the diagnosis of breast cancer, as well as tracking cancer status or drug response during treatment. The CTC isolation and detection process therefore needs to be highly specific.

Enrichment Methods

Size-Based Enrichment

As noted above, CTCs are larger than most other blood cells, and some enrichment methods have therefore utilized this size difference. Size-based enrichment strategies for CTCs include centrifugation, microfiltration, and hydrodynamic sorting. Centrifugation is the most popular method used for cell and stem cell enrichment, while microfiltration and hydrodynamic sorting are relatively new technologies.

Centrifugation

Centrifugation uses centrifugal force to separate cells based on their density. Blood cells will separate into distinct zones in the centrifugation tube according to their different densities. CTCs, leukocytes, and thrombocytes exist in the same zone called the buffy coat layer. Density gradient centrifugation using a separating medium can then be used to further separate cells within the distinct zone. Two separating media are commonly used: Ficoll (GE Healthcare) and OncoQuick (Greiner Bio-One). OncoQuick is an improved form of Ficoll that limits cross-contamination between different zones by using a porous membrane. OncoQuick has thus been shown to produce a higher recovery [60, 61].

Microfiltration

Alternatively, CTCs can be enriched using a microfiltration device [62–66]. As noted above, CTCs are mostly epithelial cells that are larger than blood cells [62, 67, 68]. Some studies have shown that a microfiltration device with a pore size of around 8 μm in diameter is optimal for CTC retention [69]. This technique initially used polycarbonate filters fabricated using track etching [70], which generates pores at random locations. However, the low pore density and multiple pore fusion led to low CTC capture efficiency (around 50–60 %) and frequent sample clogging on the filter [67, 71]. To increase the capture efficiency, improved microfabricated filters with high-density uniform circular pores were developed as 2D [21, 68] and 3D microfiltration devices [72].

Hydrodynamic Sorting

Hydrodynamic sorting uses fluid flow in combination with microdevice geometries or parallel fluids at different flow rates to sort or separate tumor cells. The main advantage of this technique is that the cells do not pass through any physical constriction and shear forces are therefore reduced. In addition, these devices typically operate at relatively high flow rates, resulting in high throughout.

Dielectrophoresis

Dielectrophoresis (DEP) is a new technique based on differences in cell polarizability and size. When an electrical field is applied to cells, they become polarized. Cells will interact with an electric field. DEP forces can be used in two ways to separate cells: DEP migration, where different types of particles migrate to different regions based on their relative polarizabilities,

and DEP retention, where DEP forces are used in competition with fluid-flow forces. In the case of CTC isolation from blood, electrophoretic mobility distinguishes tumor cells in terms of their attraction toward the electrode, and normal blood cells migrate in the electrical field into an eluant.

Immunological-Based Methods

Immunological-based methods select cells based on their surface markers. Two selection methods are commonly used: positive selection and negative selection.

Positive Selection

Most CTCs originate from epithelial cells and thus express epithelial cell-specific markers such as EpCAM and CKs. These markers can be efficiently used to enrich CTCs. Some tumor-specific markers relating to specific cancers, such as alpha-fetoprotein, HER2/neu, MUC1/MUC2, mammaglobulin, and carcinoembryonic antigen, can also be used to enrich CTCs. Some approaches have combined popular epithelial cell markers and tumor-specific markers to improve the enrichment results. Two immunomagnetic methods can be used: (1) ferrous beads with anti-EpCAM that retain EpCAM-positive cells in magnetic columns (the so-called magnetic-activated cell sorting (MACS)) and (2) anti-EpCAM proteins bound in arrays to which EpCAM-positive cells can then attach.

However, as suggested, CTCs in the blood stream express a gradient of EMT markers, and some CTCs express low or no EpCAM. Immunomagnetic methods that positively select EpCAM-positive cells are therefore unable to detect them [73]. Indeed, a previous study considered that the evaluation of CTCs as prognostic markers should include both EpCAM-positive and EpCAM-negative cells [74].

Negative Selection

Most blood cells are positive for CD45, and anti-CD45 negative selection of leukocytes is thus used to enrich cell populations with CTCs that are negative for CD45. This selection technique uses antibodies labeled with magnetic microbeads and magnets. The most popular system used for negative selection is MACS (Miltenyi Biotec).

Many commercial systems currently exist for the enrichment and isolation of CTCs, such as MACS, CellSearch, RARE, AdnaTest, CTC chip, ELISPOT, MAINTRAC, Ikoniscope, and Ariol. The characteristics of these methods are summarized in Table 21.1. To increase the enrichment efficacy, some systems have combined selection by anti-CK and anti-EpCAM antibodies with CD45 depletion or with other markers.

Identification Methods

Identification is essential to confirm the cells as CTCs. CTC confirmation is usually based on specific markers expressed at the transcriptional or translational level. Nucleic acid-based methods are used at the transcriptional level, while antibody-based assays are used at the translational level. Recently, four methods have been used to identify and confirm the identity of breast CTCs, including (1) flow cytometry, (2) image-based approaches such as classic immunocytochemistry (CellSearch, Ariol system, laser scanning system), (3) protein-based assays such as ELISPOT that detect secreted proteins from CTCs, and (4) reverse transcription–polymerase chain reaction (RT-PCR) (qRT-PCR, multiplex RT-PCR).

Nucleic Acid-Based Methods

In most cases, RT-PCR or real-time RT-PCR is used to identify specific gene expression. However, there is currently no specific gene profile that can be used to confirm breast cancer CTCs. RT-PCR is commonly used to amplify and identify genes relating to EMT as well as breast cancer. Such genes may include CK19, mammaglobin-A (MGB1), HER2, and MUC1 [75–80].

Some commercial systems combine several markers to improve the results of RT-PCR. For example, AdnaTest BreastCancerSelect uses

Table 21.1 Summary of different CTC enrichment systems

Enrichment method		Principle of enrichment	Commercial systems
Size-based methods	Centrifugation	Difference in cell density	Ficoll, OncoQuick
	Filtration	Difference in cell shape	Nuclepore assay, Ikoniscope™, Clearbridge Biomedics
	Hydrodynamic sorting	Difference in cell density	
	Dielectrophoresis	Difference in cell hydrodynamics	ApoCell
Immunological-based methods	Positive selection with tumor-specific markers	Selection of combined MUC1- and EpCAM-expressing CTCs	AdnaTest
	Depletion of CD45⁺ cells	Leukocytes express CD45, while CTCs do not	EPISPOT, RARE
	Depletion of CD45⁺ cells and positive selection	Leukocytes express CD45, while CTCs do not, but CTCs do express CK-8,18, and 19	CellSearch™, CTC chip
	Red blood cell lysis	Eliminate red blood cells to enrich nucleated cells including CTCs and leukocytes	MAINTRAC™
	Red blood cell lysis and positive selection	Red blood cell lysis, CK- and EpCAM-antibody coupled microbeads	Ariol™
	Positive selection with epithelial specific marker	CTCs strongly express EpCAM, while leukocytes do not	CytoScale Diagnostics, Biofluidica, On-Q-ity Inc.

multiplex RT-PCR for HER2, MUC1, and EpCAM genes to confirm breast cancer CTCs [81]. Xi et al. showed that MGB2 was a marker of breast cancer CTCs [82]. The limited availability of CTCs means that single-cell PCR can be an ideal platform.

In addition to RT-PCR, fluorescence in situ hybridization (FISH) is also a valid method for CTC confirmation. Expression of HER2 on breast cancer CTCs evaluated by FISH showed 93 % concordance with the expression in primary breast cancer cells [83]. Using FISH, Hayashi et al. detected polysomy 17 in a small population CTCs in patients with metastatic breast cancer [84].

Cytometric-based Methods

At the translational level, CTCs can be confirmed using cytometric-based methods. In all cytometric-based methods, CTCs are stained with monoclonal antibodies specific for CTCs. There are three popular cytometric-based methods currently used for CTC confirmation: flow cytometry, image-based approaches (immunocytochemistry, fiber-optic array scanning technology (FAST), laser scanning cytometer (LSC)), and EPISPOT (epithelial immunospot).

Most cytometric-based methods use CKs and EpCAM as common markers to identify CTCs. Moreover, CD45 can also be used to detect leukocytes to increase the specificity. However, not all CTCs express CKs and EpCAM, and during EMT, CTCs may change from an epithelial phenotype expressing CKs or EpCAM to a mesenchymal phenotype with lower expression of these markers.

Flow Cytometry

Flow cytometry is a conventional method for identifying and counting CTCs. This technique has certain advantages compared with PCR, including high specificity, high statistical significance based on counting large numbers of cells in the blood, and analysis of multiple parameters including DNA content, cell size, and cell viability, as well as intracellular markers. However, it also has some limitations, particularly low

sensitivity (one tumor cell/10^4–10^5 blood cells) compared with RT-PCR approaches (one tumor cell/10^6 blood cells) [10, 85].

Image-Based Approaches

CTCs are usually enriched and then stained with specific markers by immunocytochemical techniques to confirm their identity. This technique has been used by pathologists for decades to identify certain types of tumor cells. However, it has some limitations, including being time-consuming, and automated systems have therefore been developed to capture the stained CTCs, such as the LSC (Compucyte Corporation, Cambridge, MA), automated cellular imaging system (ACIS, Dako, Denmark), and Ariol (Applied Imaging Corp. San Jose, CA). The LSC makes it possible to scan and relocate epithelial-positive cells immunolabeled for multiple markers such as EpCAM combined with the lymphocyte marker CD45 automatically. ACIS and Ariol allow the rapid and automatic identification of CTCs based on their morphological evaluation.

Fiber-optic array scanning technology (FAST) is another, more sensitive technique for confirming CTCs. This system is equipped with an exceptionally large (50,341 mm) field of view without sacrifice of collection efficiency, which makes it possible to locate immunofluorescently labeled CTCs on glass substrates at a scan rate 500 times faster than conventional automated digital microscopy, allowing FAST to detect CTCs without the need for an enrichment procedure [86, 87]. Moreover, the process is very rapid, with up to 300,000 cells scanned per second. However, there are currently no reports of validation studies in clinical settings.

EPISPOT

EPISPOT (epithelial immunospot) is another antibody-based approach and is an immunological assay based on enzyme-linked immunosorbent assay (ELISA) technology. EPISPOT identifies CTCs by detecting specific proteins (CKs, MUC, prostate-specific antigen) secreted by them. However, one of the features of EPISPOT is that it can only detect viable CTCs, because dying CTCs do not secrete adequate amounts of proteins [88]. The sensitivity of EPISPOT is superior to that of ELISA by two orders of magnitude in terms of detecting released CK19 from cancer cells [89]. However, validation studies in clinical settings are still awaited.

Commercial Circulating Tumor Cell Detection Systems for Breast Cancer

Significant effort has recently been devoted to the development of automated techniques for detecting CTCs, and several commercial systems now exist for detecting breast cancer CTCs, including CellSearch, CTC chip, The CTChip, MagSweeper, MAINTRAC, Ariol, and AdnaTest, which combine enrichment and confirmation steps, as shown in Table 21.2. However, only CellSearch has currently been approved by the FDA for detecting breast cancer CTCs [90–92].

CellSearch is used worldwide to detect breast CTCs and has been approved in more than 17 countries. This system comprises two steps: an initial step to enrich epithelial cells by selecting EpCAM-positive cells and a second step to identify epithelial carcinoma cells by double staining with CD45 and CK antibodies, using DAPI to visualize the cell nucleus. Analysis of cells using CellSearch is thus performed as follows: first, peripheral blood is mixed with iron particles coated with EpCAM to confer magnetic properties on all the epithelial cells, and then, anti-CK antibodies are used to identify these epithelial cells, while anti-CD45 antibodies are used to rule out lymphocytes; the nuclear dye DAPI is applied to fluorescently label cell nuclei for microscopic visualization of the enriched cell population. After incubation, washing, magnetic separation, and fixation, the immunomagnetically separated cell population can be viewed and counted by automated digital fluorescent microscopy [90]. CellSearch requires about 7.5 ml of blood for each analysis. Figure 21.2 shows some approaches in breast CTC enrichment and identification.

Table 21.2 Commercial CTC detection systems

System	Enrichment methods		Detection methods	
	Methods	Parameters	Methods	Markers
CellSearch	Immunological method, magnetic cell sorting	Positive selection of EpCAM+ cells	Cytometric-based method and immunostaining, detection by automated fluorescent microscopy	CK8,18,19+/DAPI+/CD45-
MagSweeper	Immunological method, magnetic cell sorting	Positive selection of EpCAM+ cells	Nucleic acid-based method: qRT-PCR	Gene expression profiling for *FOXC1*, *KRT18*, *PTEN*, *NPTN*, *TGFβ1*, *KRT8*, *ZEB2*, *and CXCR4*
CTC chip	Immunological method, microfluids	EpCAM-coated microspots	Cytometric-based method: immunostaining	CK+/CD45-/DAPI+
The CTChip	Immunological method, microfluids	Microfluids, enrichment based on physical properties	Cytometric-based method: immunostaining	CK+
EPISPOT assay	Immunological method, magnetic cell sorting	Depletion of CD45+ cells, enrichment of CXCR4-positive cells	ELISPOT for secreted proteins	CK19, MUC1
MAINTRAC	Red blood cell lysis		Cytometric-based method: immunostaining; laser scanning cytometry	EpCAM+/CD45-
Ariol	Red blood cell lysis, magnetic cell sorting	CK+, EpCAM+		CK8,18,19+/DAPI+/CD45-
AdnaTest	Immunological method, magnetic cell sorting	MUC1+, EpCAM+	Nucleic acid-based methods: RT-PCR	HER2, MUC1, and EpCAM
TelomeScan	Red blood cell lysis		Automated scan fluorescence microscopy	Detecting telomerase-specific replication-selective adenovirus expressing green fluorescent protein, the virus is able to replicate and incorporate the green fluorescent protein

Circulating Tumor Cells for Diagnosis and Prognosis

Many studies have demonstrated that the presence of CTCs is significantly associated with shorter PFS, disease-free survival (DFS), and OS. CTCs are considered as a stable prognosticator in patients with early-stage and metastatic breast cancer. By counting CTCs before and after treatment in patients with metastatic breast cancer, Cristofanilli et al. showed that detection of CTCs before initiation of first-line therapy in patients with metastatic breast cancer was highly predictive of PFS and OS [93].

CTCs Associate with Progression-Free, Disease-Free, and Overall Survival

Zhao et al. performed a meta-analysis of published literature to assess whether the detection of CTCs in patients diagnosed with primary breast cancer could be used as a prognostic factor. A total of 24 eligible studies with 4,013 cases and 1,333 controls were included. Meta-analyses were performed using a random-effects model, with the hazard ratio (HR) and 95 % confidence intervals (95 % CIs) as effect measures. The results showed that the positive detection of CTCs was

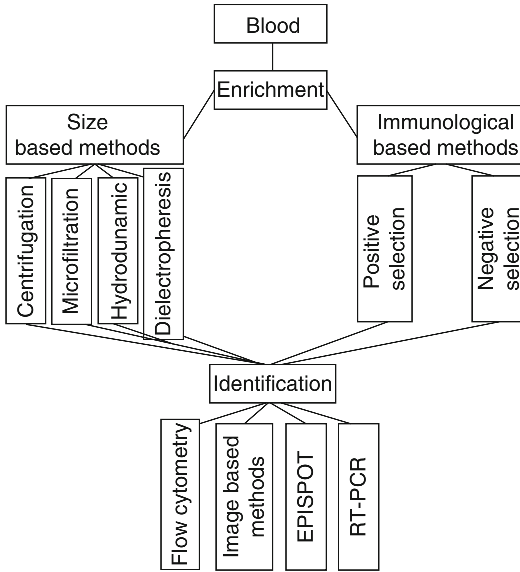

Fig. 21.2 Some approaches in breast CTC enrichment and identification

significantly associated with poor OS (HR = 3.00 [95 % CI 2.29–3.94], $n = 17$, $P < 0.0001$) and recurrence-free survival (RFS) (HR = 2.67 [95 % CI 2.09–3.42], $n = 22$, $P < 0.0001$) [94]. Another meta-analysis identified 49 eligible studies enrolling 6,825 patients and showed that the presence of CTCs was significantly associated with shorter survival in the total population. The prognostic value of CTCs was significant in both early (DFS, HR 2.86; 95 % CI 2.19–3.75; OS, HR 2.78; 95 % CI 2.22–s3.48) and metastatic breast cancer (PFS, HR 1.78; 95 % CI 1.52–2.09; OS, HR 2.33; 95 % CI 2.09–2.60) [95].

The association between CTCs and survival also depends on the number of CTCs. Patients with no CTCs at baseline had a significantly better prognosis, while an increase in number of CTCs was associated with increased risk for both PFS and OS, though the rate of increase lessened above approximately five CTCs/7.5 ml. CTCs increasing up to a maximum of five represented a prognostic factor in metastasis [96]. In another study, Hayes et al. analyzed the correlation between the number of CTCs and OS and found that the median OS for patients with <5 CTCs/7.5 ml from blood drawn at five time points was >18.5 months, while OS for patients with ≥5 CTCs/7.5 ml was significantly shorter, by 10.9, 6.3, 6.3, 6.6, and 6.7 months, respectively [15]. Liu et al. also observed shorter PFS for patients with ≥5 CTCs at 3–5 weeks and at 7–9 weeks after the start of treatment [97]. Median OS and PFS in the subgroup with ≥5 circulating tumor cells/7.5 ml of blood at baseline were significantly shorter (5 months and 3 months, respectively) compared with the subgroup with <5 circulating tumor cells (8 months and 7 months, respectively) ($P = 0.003$ and $P < 0.001$, respectively). The number of metastatic sites was significantly associated with OS and PFS and correlated with the number of CTCs [98].

In a recent study, Pierga et al. showed that ≥1 CTC/7.5 ml was a strong prognostic factor for PFS ($P < 0.0001$), while the threshold of ≥5 CTCs/7.5 ml was statistically significant for PFS and OS ($P = 0.03$) in multivariate analysis [18].

CTCs Associate with Axillary Lymph Node Metastasis

The main reason why CTCs are related with shorter OS and PFS is the high incidence of metastasis in patients with CTCs. The risk of progression for patients with ≥5 CTCs/7.5 ml was several times higher than that for patients with 0–4 CTCs/7.5 ml at the same time point. Multivariate analysis showed that axillary lymph node metastasis, serum CA15-3-positivity, and the presence of EpCAM-, CK19-, and hMAM-positive CTCs had a significant impact on PFS, while axillary lymph node metastasis and the presence of EpCAM-, CK19-, and hMAM-positive CTCs had a significant impact on OS.

CTCs and Histological Grade

CTC positivity is significantly associated with high histological grade, tumor size, and nodal status of breast cancer. Detection of CTCs in the peripheral blood indicates a poor prognosis in patients with primary breast cancer. Larger clinical studies are required to further evaluate the role of these markers in clinical practice [94].

CTCs and Clinical Treatment Response

CTC follow-up was considered as an indicator of treatment efficacy. Some studies found a correlation between a reduction of CTCs upon therapy initiation and the final tumor response [99, 100].

During chemotherapy, >6 CTCs/7.5 ml was correlated with a poorer prognosis in patients with metastatic disease. CTC levels, rather than the presence of CTCs, were associated with PFS and showed borderline significance in terms of OS. The differential prognoses and OS rates in patients with and without elevated CTCs before and at the end of chemotherapy are of particular interest in patients with no clinical evidence of metastasis [101].

Pachmann et al. evaluated the number of CTCs prior to each chemotherapy cycle and at the completion of treatment in 91 breast cancer patients [102]. CTCs were detected using the MAINTRAC method. Three groups of CTC changes were recorded: a ≥10-fold decrease, marginal change, or a ≥10-fold increase. Relapses were seen in 1/28 patients with CTC decreases, 5/30 patients with minimal change, and 14/33 with a CTC increases, demonstrating that an increase in CTC level of ≥10-fold predicted early breast cancer relapse. Similarly, Pachmann et al. showed that escalating numbers of CTCs during tamoxifen treatment were strong predictors of relapse [102].

In another study, advanced breast cancer patients were treated with docetaxel chemotherapy, and the treatment response was evaluated by [^{18}F] 3′-deoxy-3′-fluorothymidine positron emission tomography (FLT-PET) and levels of CTCs. In the individuals in whom CTCs could be detected, a decreased CTC count correlated with reduced FLT-PET signal within 2 weeks [103].

Conclusion and Future Perspective

CTCs have been shown to play prognostic and diagnostic roles and to be associated with PFS, DFS, and OS in breast cancer patients. Early breast cancer patients with CTCs are at high risk of metastasis. Recent results have also demonstrated a correlation between the presence of CTCs and poor histological grade of primary tumors. Evaluation of CTCs during treatment can provide information on treatment efficacy, as well as recurrence risk. Moreover, analysis of CTC molecular characteristics can provide information on protein targets for treatment and chemoresistant profiles. However, further progress is needed before CTCs can be applied as a powerful tool for the diagnosis and prognosis of breast cancer, including determining specific markers for breast CTCs, developing highly sensitive and specific methods to detect CTCs, and exploring the molecular characterization of CTCs, especially in terms of CTC marker profiles related to cancer progress, recurrence, and metastasis. However, rapid increases in breast CTC research will allow CTCs to become powerful tools for use in breast cancer diagnosis and prognosis in the near future.

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22 Molecular Diagnosis of Metastasizing Breast Cancer Based Upon Liquid Biopsy

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Abstract

The occurrence of distant metastases is the main cause of death for breast cancer patients. However, central factors forcing cancer cells to migrate and grow outside of the primary organ are still not well understood [1]. An association of breast cancer and bone metastasis was previously described in 1889 by Steven Paget's theory of seed and soil [2]. Rohr and Hegglin suggested the breast cancer-related metastasis in bone marrow (BM) [3] and also recognized metastatic cells in BM biopsies by hematoxylin and eosin staining. The first single disseminated tumor cells in BM smears was also screened out in nonmetastatic breast cancer patients [4], when only a few reports dealt with micrometastasis [5]. Furthermore, morphological criteria were not satisfactory to undoubtfully distinguish single epithelial tumor cells from BM cells, especially because of the extensive variety of morphologically uneven hematopoietic and mesenchymal stem as well as progenitor cells [6].

Significant progress in the field of BM micrometastasis arose from the introduction of immunocytochemical staining procedures using antibodies against epithelial-specific markers (EMA, cytokeratins) that were not

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expressed on the neighboring BM cells [7]. There is increasing evidence that the presence of disseminated and circulating tumor cells (DTCs/CTCs) and several novel molecular biomarkers is associated with an unfavorable prognosis related to metastatic progression in the bone and other organs. Using these methods and markers, it became more and more established during the last two decades that BM is a common homing and surviving organ for breast cancer cells [8]. These cells are likely to escape from the host immune system in a dormant state until internal and/or external signals might facilitate them to move and grow out to overt metastases at different organs [9].

In the present chapter, we will focus on recent advancements and investigations in the field of liquid biopsy-based biomarkers, especially DTCs and CTCs, along with the evolution of many fluid-based molecular biomarkers which have the capability to behave as potential biomarkers in metastasizing breast cancer.

Keywords
Disseminated tumor cell • Circulating tumor cell • Metastasis • Breast cancer • Molecular biomarker • Glycans • Tissue interstitial fluid

Introduction

Breast cancer results from multistep carcinogenesis. The transforming process from normal to malignant cells is linked with multiple complex factors. The existence of a specific breast cancer in a specific individual relies on complex, vibrant interaction between the tumor and the host. Breast cancer is the most frequently occurring cancer in females worldwide with an age-standardized incidence rate (ASR) of 39.0 per 100,000, and it is the most common cause of cancer mortality as it comprises 16 % of cancer deaths in adult women [10]. Incidence rates of breast cancer are increasing in most countries, and the changes are usually maximum where rates were previously low [11].

Breast cancer is the second most common cancer in all Indian women, according to current data from the Atlas of Cancer in India project—a study to assess nationwide patterns of cancer incidence across urban and rural parts of the country suggests that breast cancer is the frequent cancer in metropolitan cities and is predicted to be the most common type of cancer in the coming decade. Data from the Atlas project suggest that certain districts display even higher rates (for instance, Chandigarh 39.5 per 100,000; North Goa 36.8 per 100,000) than those reported by the population-based registry in New Delhi (28.9 per 100,000). In Bangalore, Chennai, Delhi, Mumbai, and Kolkata, the age-adjusted incidence rates are 30.9, 33.0, 31.4, 29.3, and 20.6 per 100,000, respectively [12].

A recent report by the Indian Council of Medical Research forecasts the number of breast cancer cases in India to rise to 106,124 in 2015 and to 123,634 in 2020 (Cancer Incidence Rates 1982–2005). According to the National Cancer Registry Programme projections, the number of breast cancer deaths in India will rise to 106,124 in 2015 and to 123,634 in 2020 (Cancer Incidence Rates 1982–2005).

Treatment of breast cancer depends on few well-established prognostic and predictive factors, screening, surveillance, and intervention, but many individuals will die from progressive, advanced breast cancer due to late manifestation of symptoms. Breast cancer morbidity increases

significantly if it is not detected early in its progression. Early detection of breast cancer before symptoms appear is the most effective restraint of breast cancer. It is estimated that between 15 and 25 % of women with early-stage breast cancer are currently missed by widely used diagnostic procedures such as mammography. The real challenge is to deal with the inherent limitations of breast cancer detection by identifying new breast cancer markers that can be imaged and detected in the blood by noninvasive procedures. Detection of circulating tumor cells (CTCs) in peripheral blood and disseminated tumor cells (DTCs) in bone marrow of tumor patients has become an active area of translational cancer research, with several groups developing new diagnostic assays and more than 200 clinical trials incorporating CTC counts as a biomarker in patients with various types of solid tumors. Among these activities, breast cancer has played the most prominent role as a "key player" of research on CTCs/DTCs. The clinical importance of DTCs is already well established and has been set by different large-scale studies. CTC analysis could play a role as a "liquid biopsy," which will allow physicians to follow cancer changes over time and tailor treatment, and it represents a promising new diagnostic field for advanced-stage patients; the sensitive CTC detection platforms allow monitoring of disease and treatment efficacy.

Current research on CTCs is focusing on the identification of novel diagnostic and therapeutic biomarkers expressed by these cells. However, we need to find new strategies with higher sensitivity and specificity for more accurate recognition of breast cancer. This chapter focuses on the presentation of recent data showing that CTCs/DTCs can be used as novel tumor biomarkers together with some novel robust molecular biomarkers for prognostic and predictive purposes in breast cancer.

Screening Methods of Breast Cancer

To date, a few proteins have been suggested as possible markers for the early detection of breast cancer; these include the carbohydrate antigen CA15.3 [13–18], CA 27.29 [19, 20], carcinoembryonic antigen (CEA) [13, 15, 16, 18], clusterin [21], and alpha-1-antichymotrypsin [22]. Due to lack of specificity and/or sensitivity for early disease, however, none of these markers is of value for the detection of early breast cancer [15]. Consequently, novel, highly sensitive, and specific biomarkers for the early detection of breast cancer are urgently needed.

Although the progress in screening and the treatment of breast cancer is satisfactory, about 40 % of patients still surrender to the disease. The development of distant metastases is the main cause of these deaths. Breast cancer is generally no longer curable once metastases are detected by "classical" means: clinical manifestations of the spread, imaging methods, and serum marker assays, such as those based on carcinoma antigen 15.3 (CA15.3) or carcinoembryonic antigen (CEA). According to an established hypothesis, breast cancer dissemination should involve a succession of clinical and pathological stages starting with carcinoma in situ, progressing into invasive lesion, and culminating in metastatic disease. Further, it was thought for decades that metastasizing breast cancer cells (BCC) first disseminated to the lymph nodes before reaching peripheral blood and distant locations, including the bone marrow. Sadly, it has now become clear that metastatic spreading occurs in about 50 % of cases with apparently localized breast cancer and that up to 30 % of patients with lymph node-negative disease will grow distant metastases within 5 years [23]. Hence, recurrence is most likely due to the establishment of micrometastases before primary locoregional treatment. That BCC seem rarely able to shed from the primary lesion very early in the natural history of tumors, and that a direct hematogenous dissemination route is expected to exist that bypasses the lymphogenous one, robustly supports the search for techniques and tumor markers able to unmistakably identify DTCs. This should allow examining the potential of these DTCs in predicting the development of metastases and monitoring the response of patients to various therapies.

Breast cancer screening includes three methods of early detection: (1) breast self-exams (monthly) starting when a woman is in her 20s,

(2) clinical breast exams (every 3 years) starting in a woman's 20s, and (3) mammographic screening (annually) starting at the age of 40 years. Mammography seems quite satisfactory in reducing breast cancer mortality in women who are screened annually or biannually. Consequently, mammography is currently the only accepted screening procedure to discover (measured as the sensitivity) and to exclude (measured as the specificity) the presence of breast cancer in women who are asymptomatic [24, 25]. Even though mammography has reduced breast cancer mortality significantly, it suffers from some limitations: sensitivity ranges from 90 % to as low as 75 %, it leads to overdiagnosis and overtreatment, and it is inadequate for detecting the disease at a very early stage, that is, before the tumor starts to manifest its malignant potential [26, 27]. In addition, it has low-positive prognostic usefulness in younger women [28].

When breast cancer is detected at a localized stage and has less than 10 mm in size, the 5-year survival rate is 98 %. If the lesion is larger, it has often spread to nearby lymph nodes (regional disease), and the 5-year survival rate drops to 50–80 %. If the cancer has spread (metastasized) to distant organs such as the lungs, bone marrow, or liver, the 5-year survival rate is less than 25 %. Hence, it is crucial to develop more sensitive diagnostic tools that will not only complement mammography but also enable the detection and diagnosis of breast cancer much earlier than is currently possible, allowing therapy that is less invasive, thus causing less morbidity in patients while being more effective. The ideal screening approach would involve the development of a panel of highly specific and sensitive biomarkers that can be used to screen high-risk groups, detect recurrence, and monitor treatment using a simple blood-based test that can be performed by general physicians. Currently, the development and progression in exploration of CTCs and DTCs, along with genomics-, transcriptomics-, metabolomics-, and proteomics-based biomarkers, are currently promising to be better markers in the screening of various stages of breast cancer. A fluid-based, i.e., liquid biopsy, approach which utilizes serum/plasma should be pinpointed to determine disease status and progression earlier so the management would be better for breast cancer patients. This chapter will explore the current progress and development in the field of liquid biopsy-based molecular biomarkers in metastasizing breast cancer.

Molecular Biomarker-Based Breast Cancer Classification and Characterization

Recent technological advances have allowed the simultaneous evaluation of multiple RNAs (DNA microarrays) or proteins (tissue arrays) in tumor samples. These studies have revealed that breast tumors could be categorized into very few classes characterized by the high level of expression of specific groups of genes/proteins [29, 30]. According to these studies, about two-thirds of tumors express features reminiscent of the luminal epithelial component of the breast. These lesions are often well differentiated, have a low grade, and display relatively high levels of steroid receptors; cytokeratins KRT8, KRT18, and KRT19; BCL2, CDH1, MUC1; and the transcription factors GATA3, FOXA1, XBP1 [31], TFF1, TFF3, SLC39A6, CDKN1A, CDKN1B, and CCND1. In contrast to the "luminal epithelial-like" lesions, about 15 % of tumors have a low level of the abovementioned markers, whereas they express relatively high levels of cytokeratins KRT5 and KRT17, CDH3, EGFR, FOXC1, KIT, SERPINB5, TRIM29, GABRP, MMP7, SLPI, and various proliferation markers. Most of these "basal/myoepithelial-like" tumors are poorly differentiated and have a high grade [32]. Some of them are associated with the rare medullary carcinomas [33] and mutations in the familial cancer susceptibility BRCA1 gene [34]. Tumors overexpressing ERBB2 as a consequence of gene amplification may be sorted into a separate class (ERBB2 subtype), more closely related to the basal/myoepithelial-like than to the luminal epithelial-like lesions. Of interest, the luminal epithelial-like, basal/myoepithelial-like, and

ERBB2 classes are also found in breast cancer cell lines [35], most of which are derived from DTC (obtained in most cases from pleural effusions).

It must be noted that among the markers listed above, many are relatively associated to a specific class. EGFR, SERPINB5, and GABRP are mostly expressed by basal/myoepithelial-like tumors, while high ERBB2 levels are noticeably expressed in lesions of the ERBB2 class. ESR1, TFF1, and TFF3, the expression of which is closely correlated, are found at high levels only in luminal epithelial-like tumors. Other markers related to this well-differentiated, low-grade class are the secreted proteins PIP, SCGB2A1, SCGB2A2, and SCGB2D1, as well as the mucins MUC1 and SBEM, the transcription factor SPDEF and ANKRD30A represent a stable portrait of breast cancer during progression, despite increasing genetic complexity. The existence of breast tumor classes defined by gene/protein signatures suggests that any tumor biology reflects to a large extent the biology of the cell of origin at the time of initiation. Tumors originating from more undifferentiated epithelial cells have a rapid growth pattern and more aggressive behavior and outcome compared with those beginning in a more differentiated epithelial cells. Therefore, the "portrait" of tumors seems to be stable during progression.

It is now clear—based on previous research and a number of data regarding breast cancer biology, pathology, and genetics—that during progression to metastasis, although undergoing increasing genetic alterations, most breast tumors largely maintain their portrait (luminal epithelial-like, basal-/myoepithelial-like, ERBB2). Indeed, the grade (I–III) and the expression of markers, such as ESR1, PGR, TFF1, EGFR, ERBB2, P53, and various proliferation markers, are generally concordant between primaries and metastases [36]. In fact, gene signatures underlying these portraits are preserved throughout the metastatic process of breast cancer [37]. This counters to the classical view, according to which tumor progression is commonly connected with some degree of dedifferentiation (i.e., loss of ER) and is expected to make a deep change in the biological status of cancer cells. One outcome is that DTCs are expected to express the same markers and, likely, the same properties (for instance, sensitivity or resistance to chemotherapeutic agents) than tumor cells in the corresponding primaries. While the portrait of tumors appears stable, their progression from in situ to metastasis is associated by an increasing genetic complexity. This probably results from the gathering of various minor (low-frequency) genetic or epigenetic events at many different sites of the genome, giving rise to a number of different blueprints, each restricted to a small cell subpopulation. This genetic microheterogeneity has small effects on the global portrait but will eventually modify the molecular balances controlling cell adhesion, migratory ability, proteolysis, and angiogenesis and, possibly, allow DTCs to colonize distant organs and produce secondary tumors [36].

Although genetic complexity is a hallmark of breast cancer, recent studies have allowed subclassifying tumors into a few categories, based on array-CGH analysis. Among breast tumors, DNA gains in chromosome 1q and loss in 16q appear to be the most common alterations. Some ER-positive, low-grade tumors have very few copy number alterations in addition to gain of 1q and loss of 16q and are associated with the finest patient outcome. At the other extreme of genome instability are tumors with many low-level copy number aberrations. Copy number losses involving chromosomes 3p, 4, 5q, 11p, 14q, 15q, 17q, and 18q are more ubiquitous in this group, which are composed mainly of ER-negative, high-grade lesions from patients experiencing significantly poorer outcome [38].

Fridlyand et al. have recognized an additional subgroup comprised of both ER-positive and ER-negative tumors and characterized by the presence of low-level gains and losses and recurrent amplifications [38]. The more commonly seen amplifications in this group, which occurred mostly in the ER-positive tumors, involved 8p, including FGFR1, 11q13, CCND1, and regions of 20q, including ZNF217. It is well known that specific gene amplification occurs commonly

in breast cancer. For instance, ERBB2, EGFR, MYC, CCND1, MDM2, NCOA3/AIB1, FGFR1, TOP2A, CTTN/EMS1, FGF3, AKT2, and ZNF217 are genes for which amplification has been depicted in previous breast cancer studies [39]. The amplification of some of these genes has been connected more or less clearly to the degree of tumor aggressiveness. For instance, ERBB2 and MYC amplifications have been linked to reduced survival, while ERBB2/MYC-coamplified cancers have a poorer prognosis than tumors with only one of these amplifications [39]. Therefore, a decrease of survival is observed with increasing genome instability in primary tumors, but specific DNA gains/losses combinations as well as gene amplifications appear to have more weight in this regard.

DTCs and CTCs as Important Players in Breast Cancer Biology

Metastasis is a multistage complex process that selects for CTCs that can infiltrate, survive in, and colonize distant organs [8]. Recent advances in this field are encouraging for the early dissemination model of metastasis, through the observation that DTCs isolated from the bone marrow or lymph nodes exhibit diverse changes on all levels of genomic resolution as compared to primary tumor cells [40]. Cancer cell dissemination may be followed by a dormancy period before relapse in one or more organs [41]. Research on DTCs and CTCs presents a challenge, as these cells are well-defined targets for understanding tumor biology and tumor cell dissemination in cancer patients, and will open new paths for the early detection of metastatic spread and its successful treatment. CTCs are rare, comprising a few cells per 106 hematologic cells in the blood of patients with metastasis; hence, their isolation presents a remarkable technical challenge [42]. DTCs and CTCs can now be detected and characterized at the single-cell level [43]. In Table 22.1, a comparison of CTC and DTC detection in breast cancer is represented.

Dissemination Sites: Lymph Nodes, Peripheral Blood, and Bone Marrow

Lymph Nodes (LN)

In the past, the detection of DTC is most important in pathological staging of lymph node (LN) specimens. In the last few years, the existence of DTC in bone marrow has also been shown to provide prognostic information. Promising detection strategies for DTC in peripheral blood (PB) are also being examined. Regarding LN in breast cancer, the risk of metastatic disease is classically estimated by factors such as tumor size, tumor grade, estrogen (ESR1) and progesterone (PGR) receptor status, ploidy, ERBB2 (HER2/neu), cytokines, MMPs, NF-KB overexpression, and the number of positive axillary lymph nodes (ALN). Several studies have shown that the presence of DTC in ALN is the most powerful prognostic factor, being associated with significantly poor disease-free (DFS) and overall survival [1]. During the past few years, the theory of sentinel lymph node (SLN) has emerged. SLN biopsy gears mapping of one or two LNs that primarily drain the tumor (the sentinel nodes) and therefore are most likely to harbor the metastatic disease. SLN examination is now widely performed in breast cancer, as it can provide prognostic value with minimal associated morbidity in contrast to complete ALN dissection.

The prescreening of SLN with highly sensitive detection methods for micrometastases thus represents a promising strategy. Considering that significant numbers of LN-negative patients develop metastatic disease, the dependability of current staging procedures to detect DTC in LN has been uncertain.

Peripheral Blood (PB)

Peripheral blood (PB) is historically one of the most potent diagnostic specimens. For example, circulating tumor markers have been evaluated in serum for years to give indicative values about metastatic or budding primary breast cancer. Serum markers may be good indications for tumor load, yet in most cases, they fail to provide information about minimal residual disease thus not up to mark. Technically speaking, PB appears

Table 22.1 Comparison of CTC and DTC detection in breast cancer

Detection method	Patient status	Remarks	Total no. of patients	*n* (%) DTC+, DTC+	DTC+, DTC−	DTC−, DTC+	DTC−, DTC−	Concordance (%)	Reference
Pan-CK staining	M0 and M1	–	114	26 (22.8)	2 (1.7)	41 (35.9)	45 (39.4)	62.3	[166]
Pan-CK staining	M0 and M1	–	53	3 (5.6)	2 (3.7)	6 (11.3)	42 (79.2)	84.9	[69]
RT-PCR	M0 and M1	Detection of CK19	148	8 (5.4)	14 (9.5)	34 (22.9)	92 (62.2)	67.5	[167]
CK19 or mammaglobin	M0 and M1	Detection of mammaglobin	148	11 (7.4)	18 (12.2)	19 (12.8)	100 (67.6)	75.0	[167]
CK-staining	M0	–	341	8 (2.3)	26 (7.6)	40 (11.7)	267 (78.3)	80.6	[168]
Pan-CK staining	M0 and M1	–	39	12 (30.8)	3 (7.6)	12 (30.8)	12 (30.8)	61.5	[169]
RT-PCR, CK19	M0	Patients before neoadjuvant therapy	165	88 (53.3)	3 (1.8)	7 (4.2)	67 (40.6)	93.9	[170]
		Patients after neoadjuvant therapy	84	32 (38.1)	12 (14.2)	11 (13.1)	29 (34.5)	72.6	[170]
CellSearch®, RT-PCR	M0	High-risk patients	27	16 (59.2)	4 (14.8)	3 (11.2)	4 (14.8)	74.1	[171]
Pan-CK staining, CellSearch®	M0	–	63	5 (7.9)	13 (20.6)	15 (23.8)	30 (47.6)	55.5	[172]
Average								72.8	[172]
Minimum								55.5	[172]
Maximum								93.9	[170]

as an ideal source for the monitoring of DTC. In fact, PB sampling is relatively trouble-free and can be done at frequent intervals (for instance, to permit an assessment of the patient's recovery or potential to develop metastases). Several reports have demonstrated the presence of DTC in PB of patients with early-stage cancer without overt metastases [1, 24].

Bone Marrow (BM)

Contrary to PB sampling, blood marrow (BM) aspiration during surgery appears time-consuming and uncomfortable for the patient. However, among the distant organs, BM is a normal homing site for DTCs derived from breast cancer and other primary carcinomas, even in the absence of LN metastases or clinical signs of overt distant metastases [1]. Indeed, the screening rate of DTC in BM from nonmetastatic breast cancer patients has been demonstrated to be in the range from 0 % [44] to 100 % [45], and this corresponds to the variability of results obtained by the use of different techniques or marker genes. In a recent, large (more than 3,500 cases) study of stages I through III breast cancer patients, the incidence of DTC in BM detected by immunocytochemistry (ICC) ranged from 13 to 43 % [46]. The presence of DTC in BM may be supportive not only in predicting the development of bone metastases but also in predicting the development of metastases in other remote organs, such as the lung and liver. At present, however, it remains unsolved whether BM is a reservoir that allows for DTC to adapt and disseminate later into other organs or whether the presence of DTC in BM might reflect the general tendency of these cells to disseminate and survive in organs, rather than just in the BM. Until methods are developed to detect the presence of DTC in organs, such as the lung or liver, it will not be possible to distinguish between these two possibilities. The BM could serve as a reservoir in breast cancer and is supported by the presence of epithelial (cytokeratin-positive) cells in the PB of patients with overt remote metastases years after the removal of the primary tumor. This suggests that tumor cells could break from bone metastases to recirculate and disseminate to secondary tissues [1]. This "two-step" metastasis model could explain why the DTC in patients with overt metastases closely resemble each other genetically [47].

According to Ring et al. [48], in studies using antibody-based (cytometric) assays, cells with the characteristics of tumor cells have been shown in the PB of between 0 and 100 % of patients with operable (stages I through IIIa) breast cancer and in the PB of between 3 and 100 % of patients with metastatic disease. Several reports with nucleic acid-based techniques have shown cells with the characteristics of tumor cells in the PB of 0–88 % of patients with operable (stages I through IIIa) breast cancer and in 0–100 % of patients with metastatic disease. Along the same line, in a survey on a total of more than 3,500 stages I through III breast cancer patients, the incidence of DTCs in BM detected by ICC ranged from 13 to 43 % [46]. In fact, the detection rate of DTCs in BM from nonmetastatic breast cancer patients has been reported to be in the range from 0 % [44] to 100 % [45]. The variability of results obtained in DTC detection results from dramatic variations in methodology. Factors that may influence the results as heterogeneity of the studied populations may be:

1. Stage. The number of positive patients and the absolute numbers of DTCs per patient rise as clinical stage rises [49].
2. Interval of time separating surgery from the obtaining of DTCs. Surgery may increase the number of breast cancer DTCs (from 0 to 8,000 cells/ml) in the PB, which persist for varying length of times in different patients [50].
3. Metastasis location. The separation of populations into those with early and metastatic breast cancer is probably simplistic. Moreover, metastasis sites could be missed when DTCs are obtained, leading to a misclassification of the patient in the "early breast cancer" category.

Other factors such as sample handling and preparation, delay between collection and analysis, conditions of sample storage, and contamination with normal epithelial cells may influence the results. The introduction of skin

cells into a PB sample at the time of venipuncture could lead to false-positive results. Many researchers advocate that the first few milliliters of sampled PB are discarded to avoid such contamination. It has also been suggested recently that false-positivity of SLN could result from iatrogenic displacement and transport of benign epithelial cells in patients with breast carcinoma [51]. Clearly, such epithelial cells do not represent metastasis.

Detection of DTCs/CTCs in Bone Marrow

Current models of breast cancer metastasis hold up the possibility of early dissemination of cells from primary tumors and the direct release of DTCs into the blood and BM, bypassing, in some cases, the lymphatic system. DTCs are rare with only 10–20 cells among millions of BM cells. In order to increase the opportunity to screen DTCs in this organ, procedures had to be developed for their enrichment prior to detection and further characterization. For this, different density gradient centrifugation methods such as Ficoll-based assays or the OncoQuick approach, as well as positive or negative immunomagnetic enrichment procedures and simple filtration methods separating tumor cells by their size, have been recognized [9]. Currently, there are two different methods to detect BM aspirates for DTCs/CTCs—namely, cytologic/cytometric (antibody-based) and molecular approaches and nucleic acid-based approaches. The current technologies for CTC detections are summarized in Table 22.2, and a list of commonly used markers in assays to detect disseminated tumor cells by antibody- or nucleic acid-based techniques is summarized in Table 22.3.

Antibody-Based Techniques

Approaches by fluorescence microscopy (FM), ICC, and flow cytometry (FC) analysis aim to isolate and enumerate individual tumor cells. ICC is still a gold standard for DTC detection, and most of the available clinical data have been gathered by ICC screening, especially in BM [23]. An advantage of this approach is that it may permit further characterization of the cells at a molecular level, in terms of expression of key biological markers, such as ERBB2 (ERBB2 gene amplification estimated by FISH analysis) and morphological cell investigation. However, identification of intracellular targets, such as cytokeratins, by antibodies needs cell permeabilization. As a consequence, cell viability is lost, making the important discrimination of dead and viable DTC impossible. Since only viable cells might lead to metastasis, this valuable information cannot be evaluated [23].

Like IHC, FM and ICC are labor intensive and time-consuming, making these techniques too expensive for routine implementation. When compared with conventional, essentially qualitative FM and ICC, FC offers the advantage of a fully automated technique permitting quantitative measurements with high sensitivity, good resolution, speed, reproducibility, and statistical reliability. For breast tumors, the most used targets for antibody-based techniques are the cytokeratins. ERBB2, MUC1, and TACSTD1, the latter two being known under a variety of names, have also been used as antibody targets to isolate and/or identify DTC.

Two-color ELISPOT, an immunological assay based on enzyme-linked immunosorbent assay, has been recently used to detect DTC-secreting cathepsin D (CTSD) and mucin-1 (MUC1). However, antibody-based techniques have limitations. Many of the antibodies directed at epithelial and breast cancer cells are known to also stain hematopoietic cells, including cytokeratins (KRT19), TACSTD1, and MUC1. Nonspecific staining of plasma cells can also occur due to alkaline phosphatase reaction against the k and l light chains on the cell surface [52]. According to the antibody used, a false-positive detection rate of 1–3 % can be estimated [23]. Since tumor- and epithelial-specific cell marker antigens are expressed differentially in DTCs, the use of a panel of monoclonal antibodies may help to enrich DTCs and facilitate their finding [53].

Table 22.2 Current technologies for CTC detection

Assay system	Enrichment	Detection	Comments
EPCAM-based assays			
CellSearch® system	Immunomagnetic beads EpCAM-Ab-coupled ferrofluid	Immunocytochemistry Positive for CK8, 18, 19 Negative for CD45 Nucleus positive for DAPI	Semiautomated system with FDA approval for metastatic breast, colon, and prostate cancer. CTC can be enumerated and visualized [173]
CTC-chip	Microposts: EpCAM-Ab-coupled microposts	Immunocytochemistry Positive for CK8, 18, 19 Negative for CD45 Nucleus positive for DAPI	High detection rate (approximately 100 %) even in M0 patients warrants further investigations on assay specificity; the herringbone second generation of this microchip is more specific. Needs to be validated in clinical trials [174, 175]
CTC-chip Ephesia	Column of nanobeads EpCAM-Ab-coupled ferrofluids	Immunocytochemistry Positive for CKs Negative for CD45 Nucleus positive for DAPI	Lack of validation studies in clinical settings [176]
MagSweeper	Immunomagnetic beads EpCAM-Ab-coupled ferrofluids	Microscope visualization Morphology	Isolation of CepC with a high degree of purity. Analysis of large blood volume [177]
Laser scanning cytometry Maintrac®	RBC lysis	Immunocytochemistry Positive for EpCAM Negative for CD45	High incidence of positive events up to 3 logs higher CTC counts than those obtained with other techniques warrants further investigations of assay specificity [178]
Ikoniscope® imaging system	Ficoll-isopaque or filtration with track-etched membranes	Immunocytochemistry Positive for EpCAM, CK7/8 PSA (prostate only) FISH: chromosomes 7 and 8 Nucleus positive for DAPI	Two epithelial-specific Abs and FISH to detect chromosomal abnormalities in CTCs [179]
Ariol® system	RBC lysis, then immunomagnetic beads CK-Ab-+EpCAM-Ab-coupled ferrofluids	Immunocytochemistry Positive for CK8, 18, 19 Negative for CD45 Nucleus positive for DAPI	Detection of EpCAM+ and EpCAM- CTCs [180]
AdnaTest	Immunomagnetic beads MUC1-, EpCAM-Ab-coupled microbeads	Molecular biology RT-PCR Positive for at least one of the following markers: MUC1, HER2, EpCAM	AdnaTest also does not quantify the tumor cell load, false-positive results due to unspecific amplification, no further analysis possible [181]

Functional assays			
EPISPOT assay	Rosette plus Ficoll Depletion of CD45+ cells	Secretion of proteins CK19, MUC1, Cath-D (breast); CK19 (colon); PSA (prostate); TG (thyroid)	Detection of viable epithelial secreting cells; unbiased enrichment independent of CTC/DTC phenotype [9]
Vita-Assay™ or Collagen Adhesion Matrix (CAM) technology	Invasion capacity Ingestion of fluorescent CAM fragments (CAM+)	Immunocytochemistry Positive for EpCAM, ESA, pan-CK 4, 5, 6, 8, 10, 13, and 18 Negative for CD45	Detection of CTCs with the invasive phenotype in blood [182]
Other			
ISET	Cell size	Immunocytochemistry, positive for CK nucleus: Mayer's hematoxylin	Sensitivity threshold of one carcinoma cell per milliliter of blood; HER2 amplification determined by real-time PCR on DNA extracted from CK immunostained cells (CTCs) collected by laser microdissection from selected ISET-positive filters; the possibility of false-positive diagnosis stresses the need for using ancillary methods to improve this approach [183]
FAST (fiber-optic array scanning technology)	No pre-enrichment	Immunofluorescence Positive for CK Nucleus positive for DAPI Morphology	Rare cells detected by laser scanning to almost 1,000 times faster than digital microscopy [184]
DEP-FFF (dielectrophoretic field-flow-fractionation)	Phenotype–membrane capacitance	Immunocytochemistry Wright stain	No need for labeling or modification of CTCs; PBMC/CTC ratio is enriched more than 2,000-fold; CTCs isolated by DEP are viable and suitable for a wide spectrum of analyses [185]
Versatile label-free biochip	Cell size deformability	Immunofluorescence Positive for CK Negative for CD45 Nucleus positive for DAPI Morphology	Label-free selection and CTCs are viable after blood processing [186]

Abbreviations: *Ab* antibody, *BM* bone marrow, *Cath-D* cathepsin D, *CepC* circulating epithelial cell, *CK* cytokeratin, *CTC* circulating tumor cell, *DAPI* 4′,6-diamidino-2-phenylindole, *DEP* dielectrophoresis, *DTC* disseminated tumor cell, *EpCAM* epithelial cell adhesion molecule, *EPISPOT* EPIthelial immunoSPOT, *ESA* epithelial-specific antigen, *FDA* Food and Drug Administration, *FISH* fluorescent in situ hybridization, *ISET* isolation by size of epithelial tumor cells, *MUC1* mucin 1, *NSCLC* non-small-cell lung cancer, *PBMC* peripheral blood mononuclear cells, *PSA* prostate-specific antigen, *RBC* red blood cell, *RT-PCR* reverse transcription polymerase chain reaction, *TG* thyroglobulin

Table 22.3 Markers used as assays to detect disseminated tumor cells by antibody or nucleic acid-based techniques

Marker (gene) name	Gene locus	Standard name	Other frequently used names	Reference(s) related to DTC detection
ANKRD30A	10p11.21	Ankyrin repeat domain 30A	Breast cancer antigen NY-BR-1; B726P	[117, 187]
B305D	21q11.1–q11.2	Antigen B305D	B305D; isoform A (B305D-A); B305D; isoform C (B305D-C)	[104, 187, 188]
CD44	11p13-pter	Antigen CD44	Hermes antigen, PGP1	[60]
CDH1	16q22.1	Cadherin-1 (epithelial)	E-cadherin, uvomorulin	[189]
KRT19	17q21–q22	Keratin 19	Cytokeratin 19 (CK19)	[60, 87, 97, 102–104, 107, 117, 187, 190, 191]
KRT7	12q12–q14	Keratin 7	Cytokeratin 7 (CK7), sarcolectin (SCL)	[48]
GABRP -	5q32-q33	γ-Aminobutyric acid type A receptor pi subunit	GABA receptor A, pi polypeptide (GABARAP); GABAA receptor, pi polypeptide (GABA A(pi))	[104, 187, 188]

Nucleic Acid-Based Techniques

PCR, either qualitative or quantitative, has been used to identify and characterize DTCs through the detection of genetic (allele-specific expression, microsatellite instability, loss of heterozygosity) and epigenetic alterations (methylation status) that are exclusively linked with cancer cells [54]. This includes the search for tumor-associated point mutations in oncogenes or tumor suppressors. This latter PCR approach, however, is complex by the substantial degree of genetic variability between tumors. For instance, TP53, the gene coding for p53, is mutated in about 25 % of breast tumors; however, more than 1,400 different mutations of this gene have been observed [55]. Of note, PCR has been used to screen free DNA within plasma. For instance, the analysis of DNA methylation status of specific genes (ESR1, APC, HSD17B4, HIC1, and RASSF1A) in serum of breast cancer patients has been shown to be of prognostic value [56]. The PCR-based measurement of RASSF1A methylation has been used for examining efficacy of adjuvant tamoxifen therapy [57]. However, this use of PCR is imperfect by poor specificity. This is due in part to the high stability of DNA in plasma when compared with mRNA [58]. As a result, it is unclear whether the free DNA that is amplified from plasma is from DTCs present in plasma or if the DNA is being shed from primary tumors, metastatic tumors, or from normal tissue [48]. To identify DNA gains and losses in single DTC, the technique of comparative genomic hybridization (CGH) is increasingly used [59].

Reverse transcription (RT)-PCR has been used to identify DTC through their expression of epithelial or breast cancer-associated mRNA transcripts. RT-PCR is generally more sensitive than antibody-based techniques but has also been hampered by false-positive results in samples from normal volunteers and from patients with hematological malignancies [48]. These false-positives stem from multiple sources, including issues with laboratory technique, primer selection, illegitimate expression of the target genes in normal cells, the existence of pseudogenes, or contamination (KRT19/CK19). When using assays based on RT-PCR for detection of DTCs, the balance between sensitivity and specificity must be considered. Generally, specificity decreases with the increase in sensitivity, and vice versa. One way to resolve this problem is to examine multiple tumor markers in samples. As mentioned below, multiplex RT-PCR assays have revealed a higher efficacy (in both sensitivity and specificity) in comparison with the assessment of single markers. To recover the reliability,

especially the specificity of RT-PCR assays, quantitative RT-PCR (qRT-PCR) may be used. In addition, qualitative marker information, qRT-PCR uses cutoff values of marker transcript numbers, below which transcripts can be considered as tumor cell-derived. Moreover, when compared with conventional RT-PCR, qRT-PCR relies not only on primers but also on internal probes that specifically hybridize to the amplified sequences. In addition, due to the continuous measurement of the amplified signal, false-positive results, which could produce an abnormally shaped, nonlinear amplification curve, could be easily identified and removed [23]. Variations of the RT-PCR technique, such as nested RT-PCR and competitive nested RT-PCR, have also been used [60].

Fluorescence in situ hybridization (FISH) allows the detection of gene amplifications, for instance, ERBB2 amplification in breast cancer. FISH has been used to analyze genetic aberrations in DTC in BM. Considering the importance of ERBB2 as a novel target for successful antibody-based therapy, the use of FISH to identify ERBB2 amplification in DTC appears promising [61]. Among the cytologic methods that allow isolation and enumeration of individual cells, immunocytochemistry is the most widely used approach. Because of the absence of tumor-specific target antigens—most commonly antibodies against various epithelium-specific antigens such as cytoskeleton-associated cytokeratins—surface adhesion molecules or growth factor receptors are used for the screening of carcinoma cells [62]. The main advantage of cytologic methods is the opportunity to combine immunostaining with the morphology of the cells so that cell size and shape as well as the nucleus-plasma relation might be predictable and illicit expression of the protein of interest in BM cells can be excluded.

The detection of DTCs in BM is not yet a routine part of the tumor staging in the clinical practice, but rising data anticipate a future role of DTC screening for risk estimation and therapeutic monitoring of breast cancer patients [63]. However, the detection rates of DTCs in BM from nonmetastatic breast cancer patients vary significantly [45]. This might reflect the different sensitivity, but also specificity, of the numerous detection methods and marker genes/proteins used thus far. The newly defined consensus concept for the detection of DTCs in BM, signifying enrichment of mononuclear cells from BM by Ficoll density gradient centrifugation and immunocytochemical detection of cytokeratin expression as standard procedure, should help overcome these troubles and provide the basis for future multicentric clinical trials. The researchers recommend the pan-anti-cytokeratin antibodies A45-B/B3 or AE1/AE3 against a wide spectrum of cytokeratins as standard application, thereby ensuring detection of DTCs also in cells that have downregulated the expression of individual cytokeratins in the course of epithelial–mesenchymal transition [42]. Microscopic screening of large amounts of immunostained cytologic preparations is accomplished by automatic microscopes using sophisticated imaging approaches. Criteria to examine morphology and staining results have also been defined to avoid false-positive and false-negative results [42].

Although there are existing recommendations for standard operation procedures, there are still restrictions to the standardization of immunocytochemical methods with respect to reproducibility of the staining procedure itself as well as microscopic interpretations. Therefore, both intra- and interlaboratory evaluation of the methods is required to ensure reliability of the results [64].

Besides immunocytochemical methods, very sensitive nucleic acid-based techniques now allow the detection of DTCs at the single-cell level. The main advantage of these methods is the nearly unlimited availability of primers for almost every gene of interest. Although numerous genetic alterations have been described in breast cancer cells, heterogeneity is enormous, so that at present no universally applicable DNA marker exists for the primary screening of a wide range of DTCs [9]. Further efforts have been made to detect free circulating DNA or epigenetic alterations of circulating DNA such as methylation in BM and blood plasma, but the results are still preliminary [65]. Therefore, the measurement of epithelium-specific or more organ-specific mRNA species such as cytokeratin 19 or mammaglobin mRNA by RT-PCR has been

proven to be a promising approach to detect DTCs in BM samples [66]. Because of the lack of tumor-specific markers, the main disadvantage of using surrogate tissue-specific markers is false-positive results due to illegitimate low-level transcription of epithelial or breast tissue-specific genes in normal cells [48]. Furthermore, heterogeneity in the expression of particular genes is not recognizable and the expression level of a gene of interest per cell cannot be estimated. At present, analyses are mainly performed by quantitative real-time RT-PCR, ensuring the discrimination between different levels of expression. Moreover, multimarker real-time RT-PCRs have the potential to improve the method even in the case of downregulation of the expression of a single gene [45]. However, storage and sample preparation have to be performed under conditions avoiding RNA degradation, one of the major problems of RT-PCR approaches [66].

The application of multimarker assays might also compensate for low mRNA amounts due to the low number of tumor cells. There are numerous excellent reviews listing the marker genes currently used in RT-PCR approaches to detect DTCs in BM or CTCs in blood from breast cancer patients [48]. The methods explained above are not able to discriminate between viable and apoptotic DTCs. A new technique, designated EPISPOT (epithelial immunospot), offers the advantage of detecting viable tumor cells by their ability to secrete individual proteins. In a newly published study, it was demonstrated that BM samples from metastatic and nonmetastatic breast cancer patients contain viable tumor cells which secret Muc-1 and/or cytokeratin 19 in about 90 and 50 % of cases, respectively, whereas in controls from healthy women, cells secreting these proteins could not be detected [9].

Clinical Relevance of DTCs in Bone Marrow (BM)

A large number of studies have documented DTCs in BM from patients with most types of epithelial cancers [1]. Within the last 15 years, several studies have confirmed that detection of DTCs in BM of breast cancer patients is accompanied by a substantially worse prognosis [63]. In a pooled analysis evaluating the results from 9 different European centers, including a total of 4,703 patients, Braun et al. have reported that approximately 30 % of women with primary breast cancer have DTCs in BM, and in a multivariate analysis, the 10-year follow-up of these patients revealed a significantly decreased overall survival, when compared to patients without DTCs [67]. The presence of DTCs in BM was significantly associated with higher tumor stage, worse differentiation, lymph node metastasis, and negativity in hormone receptor expression. Prognostic relevance was shown for all subgroups, even among those patients with small tumors and without lymph node metastasis. While using different antibodies and detection methods, almost all investigators participating in this pooled analysis used anti-cytokeratin antibodies to screen for DTCs in the BM [67].

Bone Marrow of DTCs Replaceable by Blood CTCs?

Aspiration of bone marrow (BM) is invasive, time-consuming, and in many cases painful or at least uncomfortable for patients, precluding repeated samplings necessary for therapy-monitoring studies. Moreover, BM aspiration is more difficult to standardize with regard to the required volume and quality. Consequently, recent efforts have concentrated on the detection of CTCs in peripheral blood (PB) of cancer patients [48], but the clinical usage of CTCs has not yet been implemented for routine clinical practice. Furthermore, there are only a limited number of studies comparing BM and PB examinations performed at the same time points, and the clinical significance of CTCs in PB is less clear than that for DTCs in BM. In all studies published thus far, there was a higher frequency of BM-positive than blood-positive samples from the same patients [68], probably due to the fact that BM might provide conditions for homing and survival of DTCs, thus contributing to their accumulation in this compartment. Although both Pierga et al. [68] and Muller et al.

reported about a significant number of patients with concordant results concerning BM and blood analysis [69], in the Pierga study, only the presence of DTCs in BM and not that of CTCs in blood had prognostic relevance for disease-free survival in nonmetastatic breast cancer patients [68]. Interestingly, the presence of both DTCs in BM and CTCs in blood in a subgroup of patients resulted in an especially poor prognosis [70]. While all studies referred above applied immunocytochemical methods, real-time RT-PCR detection of DTCs in BM also had superior prognostic significance in comparison with CTCs in patients with breast cancer. A study analyzed cytokeratin 19 and mammaglobin mRNA levels by quantitative RT-PCR [71]. Currently, the results obtained by comparative studies do not hold a replacement of BM by blood analysis, but CTC detection might have supplementary value.

There are an increasing number of studies demonstrating clinical relevance of CTCs in blood detected by real-time RT-PCR, identifying either only cytokeratin 19 mRNA or multiple markers [62]. Recently, analyzing cytokeratin 19 mRNA by real-time RT-PCR [72], they detected CTCs in 22 % of blood samples from 167 node-negative breast cancer patients as significantly associated with overall and disease-free survival. A correlation of the presence of CTCs in blood to the lymph node status was found in 2007 [73], when CTCs were detected with the help of a multimarker real-time RT-PCR in 39 of 90 (43 %) stage I through III breast cancer patients, but not in normal healthy volunteers.

Significant progress in this field arose from the development of an automated enrichment and immunocytochemical detection system for CTCs (CellSearch™) [74]. This system consists of an automated instrument for the enrichment of epithelial cells by ferrofluids coated with anti-EpCAM antibodies followed by immunostaining of captured cells with fluorescently labeled anti-cytokeratin and anti-CD45 antibodies (AutoPrep), and a semiautomated microscope for scanning and reading results (CellSpotter Analyzer). Using this system, Cristofanilli et al. [74] demonstrated in a prospective study that CTC detection provided important prognostic information for patients with metastatic breast cancer. Additionally, Hayes et al. demonstrated that CTCs at each follow-up time point during therapy of these metastatic breast cancer patients predict progression-free and overall survival. The CellSearch system has been cleared by the FDA for regular clinical use in metastatic breast cancer patients. Validation data from three independent laboratories and high interinstrument accordance confirmed the reliability of this system for CTC measurements in PB from metastatic breast cancer patients.

Also, it was shown that samples can be shipped at room temperature and CTC counts are stable for at least 72 h, which facilitates testing at central laboratories or remote sites requiring transportation [75]. There are also several reports about the detection of CTCs in patients with primary breast cancer, however, mostly with lower frequencies and varying results concerning both the number of positive patients and the number of CTCs in individual patients [76].

Molecular Characterization of DTCs in Bone Marrow and CTCs in Blood

The characterization of DTCs/CTCs is aimed to (1) provide proof for their malignant origin and (2) identify further diagnostically and therapeutically related features of these cells, which might permit a more targeted and individualized anti-metastatic therapy. This characterization is hindered by the fact that DTCs/CTCs can exhibit features distinct from the primary tumors, but on the other hand, this could help to identify cancer patients for additional targeted therapies. By multiple fluorescence in situ hybridization analysis, it was shown that the vast majority of CTCs in blood from breast cancer patients are aneusomic and derived from the primary tumor [42]. By single-cell comparative genomic hybridization, further study indicated that DTCs might be gnomically unstable and heterogeneous [77]. Moreover, research also suggests that DTCs from BM of breast cancer patients disseminate in a less progressed genomic state and might acquire genomic alterations typical for metastatic cells later [78].

In order to escape from the dormant state into the dynamic phase of metastasis arrangement, dormancy has to be disturbed by both genetic and epigenetic changes in the DTCs/CTCs as well as in the surrounding microenvironment or premetastatic niche [79]. Transcriptional analyses of EpCAM-enriched BM and blood cells resulted in gene expression profiles that may be used to differentiate normal donors from cancer patients [80]. Further studies have to reveal whether individual genes, the expression of which is changed in these cell populations, might become markers to recognize recurrence in breast cancer patients early [80].

Interestingly, TWIST1, a transcription factor that in the past has been recognized to play an important role in metastasis by accelerating epithelial–mesenchymal transition [81], was part of the gene expression signature identified in EpCAM-enriched cells from BM of breast cancer patients after chemotherapy [80]. TWIST1 expression, which was not observed in EpCAM-enriched cells of BM from healthy volunteers, linked with the occurrence of remote metastasis and local progression, even in pretreatment BM samples [80].

DTCs/CTCs seem to be heterogeneous with regard to the expression of growth factor receptors, adhesion molecules, proteases, and their inducers and receptors, major histocompatibility complex antigens, or signaling kinases [47]. Of particular attention is the epidermal growth factor receptor HER2, the expression of which in primary tumors forms the basis of Herceptin treatment decisions for breast cancer patients.

As shown by Braun et al., HER2 overexpression on DTCs in BM was predictive for a poor clinical outcome of stage I through III breast cancer patients [82]. While a study of 27 breast cancer patients showed that the HER2 status remained relatively stable between primary tumors and BM micrometastases in most cases [83], there is also increasing proof for discrepancies between the HER2 status in primary tumors and DTCs in BM [84]. They noticed HER2-positive DTCs in 12 of 20 BM samples from patients with HER2-negative primary tumors. Although HER2 expression was heterogeneous in DTCs from individual patients, HER2-positive DTCs might recognize additional patients who can benefit from Herceptin therapy. The HER2 status of CTCs from PB might also be different from that of the corresponding primary tumors as reported [85]. These authors presented a significant number of patients whose primary tumors were HER2 negative, whereas CTCs were HER2 positive before surgery. Moreover, in this study the recognition of HER2-positive CTCs correlated significantly with disease-free and overall survival [85]. It remains to be explored whether high levels of HER2-positive CTCs reflect the activity of the tumor and have predictive value for an improved response of the patients to Herceptin treatment [85]. Although Meng et al. reported a high agreement (97 %) of the HER2 status between primary tumors and CTCs in 31 cases, during tumor progression, HER2-positive CTCs could be detected in 9 of 24 breast cancer patients in spite of HER2-negative primary tumors. These CTCs might have acquired HER2 gene amplifications. Four of these patients received Herceptin therapy and three of them responded to this therapy [61].

In the study shown by Apostolaki et al., adjuvant chemotherapy eliminated HER2 mRNA-positive CTCs in 16 of 45 patients. The detection of HER2 mRNA-positive CTCs after chemotherapy was linked with a reduced disease-free survival. Moreover, in 8 of 161 patients with HER2-negative primary tumors, HER2 mRNA-positive CTCs could be noticed [86]. Therefore, the detection of HER2 mRNA-positive CTCs after adjuvant chemotherapy in the PB of stage I and II breast cancer patients might provide information about the usefulness of chemotherapy and the prognosis of the patients and identify patients in need of additional Herceptin therapy [86].

During the past few years, the number of single markers that have been assessed for DTC detection, mainly by nucleic acid-based techniques, has noticeably increased. For a detailed description of these studies, the reader is encouraged to consult the current reports published by Gilbey et al. [60] and Ring et al. [48]. In this chapter, the same name will be used for the gene and

the corresponding protein. For instance, regardless of the fact that the terms NY-BR-1 and B726P are bumped into in the literature, the name of the corresponding gene, ANKRD30A, will also preferentially be used to cite the protein. SCGB2A2 will be used instead of mammaglobin, ESR1 rather than estrogen receptor-a (ERa), etc.

An ideal marker should be universally, but exclusively, expressed on all breast cancer cells. It should be easily noticeable, with little variance, and bear clinical relevance. Since no single, precise marker that meets these criteria has been recognized, attempts are now made to develop assays with multiple tumor markers, of which some are preferably highly specific to breast tissue or breast tumors. The aim is to avoid both false-positive (detection of non-tumor cells, due to the fact that the majority of potential markers have some baseline expression in normal tissues) and false-negative (non-detection of tumor cells, due to the use of high-threshold levels for positivity) cases.

Multimarker assays have been used by various researchers [48, 60, 87] and have shown a higher efficacy (sensitivity and specificity) in comparison with the assessment of single markers. Markers with low breast (cancer) specificity cytokeratins (KRTs) regarding epithelial tumors, the cytoskeleton components KRTs have become the markers of choice for DTC recognition. They belong to a large multigene family of more than 30 identified members. They are expressed at various levels and compositions in all epithelial tumors, but hardly ever in other tissues. For antibody-based studies, most use a combination of several monoclonal antibodies that distinguish various cytokeratin antigens or a broad-spectrum anti-cytokeratin monoclonal antibody that recognizes a single epitope that is frequent to most cytokeratins [1, 48]. For nucleic acid-based studies, cytokeratin 19 (KRT19) and, to a lesser extent, cytokeratin 20 (KRT20) have been commonly used as markers. KRT19 presents an illustration of the possible sources of false-positivity in DTC detection.

Due to its high sensitivity, KRT19 is the widely used marker for finding DTCs in breast cancer patients [48, 60]. Depending on the assays, KRT19 has been discovered to be both a specific and a nonspecific marker. In fact, KRT19 is an outstanding candidate to demonstrate the potential sources of false-positivity in RT-PCR studies: illegitimate transcription, hematological disorders, the presence of pseudogenes, and sample contamination. Illegitimate transcription explains the expression in normal tissues of small amounts of mRNA by genes that have no actual physiological role in these cells. It can be estimated that every promoter could be activated by ubiquitous transcription factors, which leads to an estimated expression level of one tumor marker gene transcript in 500–1,000 non-tumor cells [23]. For hematological disorders, KRT19 expression can be induced in PB by cytokines and growth factors, which circulate at higher concentrations in inflammatory conditions and neutropenia [48]. As a consequence, false-positive results are more expected under these circumstances. The presence of pseudogenes, two KRT19 pseudogenes, KRT19a and KRT19b [88], have been identified, which have significant sequence homology to KRT19 mRNA. Consequently, attempts to identify the expression of the authentic KRT19 may result in the detection of either or both of these pseudogenes. To avoid pseudogene amplification, it is suggested to carefully design the primers used for RT-PCR analysis. Regarding contamination, it has been suggested that PB sampling for subsequent analysis could introduce contaminating epithelial cells expressing the KRT19 mRNA into the blood sample. Possible contamination could be reduced or prevented by discarding the first sample of blood taken.

In conclusion, KRT19 emerges to be a very sensitive tumor marker, whose use, however, is often held back by low specificity. It is useful in detecting disseminated epithelial cells but is not a true breast cancer marker.

KRT20

KRT20 is found in breast cancer cells [89]. However, its expression is less linked to breast tissue and more related to gastric and intestinal

epithelium, urothelium, and Merkel cells [23]. Additionally, KRT20 expression has been established in granulocytes [90]. Due to its lower specificity when compared with KRT19, the use of KRT20 is not suggested in breast cancer patients. KRT8 and KRT18 have been hardly ever used for DTC detection. In fact, the expression patterns of these epithelial cytokines are very similar to that of KRT19 and they are not expected to provide more specificity than KRT19. Of note, KRT8, KRT18, and KRT19 are expressed in the breast epithelium but at higher levels in the luminal than in the basal component. In view of recent observations that breast tumors may be classified into subtypes, or classes, including luminal epithelial-like and basal epithelial-like, one can believe that these cytokeratins will be less easily distinguished in DTCs originating from basal-like tumors.

CEACAM5

Commonly known as CEA, it functions in several biological roles, including cell–cell adhesion. It is one of the most commonly expressed markers in breast, as well as in various other, cancer cells [48, 60]. Therefore, it suffers from low specificity, as also seen with KRT19, and can likewise be induced in peripheral blood (PB) by cytokines and growth factors [48].

TACSTD1

This epithelial cell–cell adhesion protein is known under a range of names, of which GA733-2 and EpCAM are the most commonly used. Ubiquitously expressed on the surface of epithelial cells, it has been normally used as a target for positive IMS to enrich DTC for RT-PCR analysis [23]. Monoclonal antibodies against this antigen have been widely developed for diagnostic, but also therapeutic, approaches. Although highly sensitive for epithelial malignancies, including breast cancer, its use is, however, hindered by the fact that it is expressed in low amounts in PB cells [91].

MUC1

Mucin-1 is an extensive, polymorphic, and heavily glycosylated mucin. The role of mucins is mainly one of the hydrating and lubricating epithelial linings, but these proteins have also been concerned in modulating both growth factor signaling and cell adhesion. Further, it has been suggested that MUC1 expression at the surface of tumor cells could decrease cell adhesion and favor dissemination [92]. Conversely, MUC1 could play a role in the initial attachment of breast tumor cells to tissue at remote sites, facilitating establishment of metastatic sites [93]. Extensively expressed in normal epithelial tissues, MUC1 is remarkably present on the apical surfaces of breast, bronchial, pancreatic, uterine, salivary, intestinal, and other glandular tissue cells. Like TACSTD1, MUC1 has been commonly used as a target for positive IMS to enrich DTC for RT-PCR analysis [23]. Many studies have reported the expression of MUC1 in a significant proportion of healthy blood donors. Indeed, MUC1 expression has been considerably found in PB cells [23]. Although it has low specificity, the assessment of MUC1 expression in DTC is supported by the increasing interest for MUC1-based immunotherapy [94]. Although MUC1 is expressed in a majority of breast tumors, its overexpression has been associated with a lower grade and a higher ER-positive phenotype [95].

EGFR

A series of RT-PCR-based mono- or multimarker studies have assessed the relevance of this growth factor receptor for DTC detection [96, 97]. EGFR emerges as more specific but less sensitive than KRT19. Unluckily, it has also been found infrequently in the PB of healthy donors [23]. Furthermore, Weigelt et al. [97] have shown that the median expression of EGFR was higher in normal ALN than in DTC-positive ALN! Notably, EGFRvIII, a cancer-specific EGFR variant, has been now used to detect DTC in breast cancer patients. The mutant was seen in the

peripheral blood in 30 % of 33 low-risk, early-stage patients, 56 % of 18 patients chosen for neoadjuvant chemotherapy, 63.6 % of 11 patients with disseminated disease, and, remarkably, 0 of 40 control women [98].

ERBB2

Involved in signal transduction, ERBB2 participates in breast tumor biology. Yet, it is not breast specific [99], and weak ERBB2 expression has been found in the PB of healthy women in several studies [23]. However, it is overexpressed in 20–35 % of breast cancer patients, mostly as a result of gene amplification, and this forecasts for reduced survival. Furthermore, in patients with breast cancer, ERBB2 overexpression by DTC in the BM predicts poor clinical outcome [82]. This, as well as the increasing use of ERBB2 as target for immunotherapy (trastuzumab) [94], supports its assessment in DTC, at both the mRNA (RT-PCR) and the DNA (FISH) levels.

Markers with High Breast (Cancer) Specificity

Using molecular biology methods or combinations of techniques, various groups have recognized markers specifically expressed in breast and/or breast cancer tissue or cells, when compared with normal PB, BM, or other human tissues. For instance, genes profusely expressed in breast cancer tissue but absent in normal PB and BM have been identified by serial investigation of gene expression (SAGE).

By order of decreasing SAGE tag frequency, these genes are SBEM, LACRT, TFF3, COL1A1, MGP, KRT8, MUC1, KRT7, CLECSF1, IL6ST, APOC1, SCGB2A2, TFF1, TM4SF1, C6, and KRT19 [100]. A series of genes coding for secreted proteins overexpressed in breast cancer tissue when compared with corresponding normal tissue and/or other (colon, gastric, kidney, liver, lung, ovary, pancreas, prostate) normal tissues were recognized by a combination of annotation/protein sequence analysis, transcript profiling, immunohistochemistry, and immunoassay: HAPLN1, GFRA, SCGB1D2, CXCL10, CXCL11, COL11A1, E2F3, TRMT1, CHST2, SERHL2, ZNF324, SCGB2A2, COX6C, and SCGB2A1 [101]. Gene expression profiling was used to construct a site of origin classifier in order to decide the origin of cancer of unknown primary. From an analysis of 229 primary and metastatic tumors representing 14 tumor types (breast – 34 samples, colorectal, gastric, melanoma, mesothelioma, ovarian, pancreas, prostate, renal, testicular, squamous cell carcinoma, uterine, and lung), a “finest” list of 79 site-specific markers was defined. Genes linked to breast specificity were ACADSB, CCNG2, ESR1, EFHD1, GATA3, SLC39A6, MYB, SCYL3, PIK3R3, PIP, PRLR, RABEP1, TRPS1, and VAV3. Two of them, GATA3 and PIP, were recognized as appearing to be strongly and relatively consistently expressed across the range of breast tumors.

Smirnov et al. [102] achieved PB containing R100 DTC from one metastatic colorectal, one metastatic prostate, and one metastatic breast cancer patient. In a primary step, global gene expression study was performed on these samples, and a list of cancer-specific DTC genes was achieved. Among genes distinguishing between tumor (colorectal, prostate, and breast) and control patients were KRT18, KRT19, TACSTD1, TACSTD2, AGR2, TFF1, and TFF3, all genes known to be linked to the epithelial cell phenotype. Fifty-three genes distinguishing between breast tumor and controls were recognized, including ESR1 and ERBB2.

In a second step, PB samples immunomagnetically enriched for DTC from 74 metastatic patients (30 colorectal, 31 prostate, 13 metastatic breast cancer patients, and 50 normal donors) were used to confirm the DTC-specific expression of selected genes by real-time RT-PCR. The genes most restricted to breast cancer patients, when compared with normal donors and colorectal cancer and prostate cancer patients, were SCGB2A1, SCGB2A2, and PIP. Two additional genes, S100A14 and S100A16, were restricted to breast and colon cancers. Of note, two genes, KRT19 and AGR2, were expressed in the majority of metastatic samples (colorectal and prostate

and breast) and not in the control individuals. This validates the interest of KRT19 as an epithelial tumor cell marker.

Yet AGR2 expression has been less frequently examined. Smirnov et al. [102] isolated RNA from a highly metastatic SCGB2A2-overexpressing ALN (only one sample). It was diluted into a pool of normal LN RNA at various ratios. Gene expression (microarray) analysis was performed, and candidate breast cancer-associated genes were then selected based on three criteria: (a) absence of expression in a pool of four normal LN, (b) a high fluorescence signal on microarray, and (c) a fluorescence signal also present in the 1:50 dilution. The 34 genes recognized by criteria (a), (b), and (c) were specified by relative intensity of the signal in the metastatic ALN. The 14 genes were SCGB2A2, TFF1, TFF3, KRT19, SCGB1D2, S100P, FOS, SERPINA3, ESR1, TACSTD2, JUN, PGDS, KRT8, and AFP. Notably, other genes used for molecular finding of micrometastatic disease, such as PIP, SPDEF, TACSTD1, CEACAM5, and SCGB2A1, were not present among the top 15, even though their signal was observed in metastatic ALN. Real-time RT-PCR analysis of pathology-negative ALN (nZ72) demonstrated that of PIP, SCGB2A2, SPDEF, TACSTD1, and TFF1, SCGB2A2 and TFF1 had the highest evident sensitivity for the detection of micrometastatic breast cancer [103].

In a microarray approach, Backus et al. investigated RNA from samples covering normal, benign, and cancerous tissues from breast, colon, lung, ovarian, prostate, and peripheral blood leukocytes from healthy donors. By a combination of this microarray testing and database/literature searching, a series of candidate breast tissue-specific markers and candidate breast cancer status markers were recognized [104]. These potential markers were then submitted to an additional multiuse selection process: some markers were excluded for one of the following reasons: (1) their expression level in white blood cells was too high, (2) their expression in breast cancer was too low, and (3) their expression in lung, colon, and ovarian cancers was too high. The authors finally achieved 14 markers, of which 7, ANKRD30A, GABRP, KRT19, OR4K11P, PIP, SCGB2A2, and SPDEF, were further chosen (the others were CEACAM6, ERBB2, MUC1, S100A7, S100A14, SBEM, and TNNT1). The utility of these markers for identifying clinically utilizable metastases in LN was assessed through RT-PCR analysis of SLN from 254 breast cancer patients. The investigators recognized an optimal two-gene expression (KRT19 and SCGB2A2) marker set for the detection of the actionable metastasis in breast SLN [104].

A series of markers with high breast (cancer) specificity reported so far are now in details.

SCGB2A2

No breast cancer marker has been shown to be never expressed in healthy volunteers, but some markers are hardly ever found in controls. SCGB2A2 [105], widely known as mammaglobin, is one of these markers. It is a member of the secretoglobin superfamily [106], a group of small, secretory, rarely glycosylated, dimeric proteins generally expressed in mucosal tissues that could be involved in signaling, immune response, chemotaxis [107], and, probably, as a carrier for steroid hormones in humans. SCGB2A2 has become a quasi standard in breast DTC detection by RT-PCR-based methods, being the most extensively studied marker after KRT19. It has been used to identify DTC in LN, PB, BM, and even in malignant effusions. SCGB2A2 expression has been noticed, rarely and in low levels, in various normal tissues. This could restrict its prospective use as an immunotherapeutic target [108], due to concerns about autoimmune toxicity.

Zafrakas et al. have found an abundant SCGB2A2 expression in malignant and normal tissues of the breast and in the female genital tract, namely, the cervix, uterus, and ovary, while lower expression levels were hardly ever found in other tumors and normal tissues [109]. These remarks might extend the diagnostic potential of SCGB2A2 to the detection of DTC from gynecologic malignancies. While SCGB2A2 is significantly more breast cancer specific than KRT19, it is less "universal" among these tumors. Indeed,

SCGB2A2 expression level is highly changeable in breast tumors, with some of them showing no expression at all. SCGB2A2 expression, estimated at mRNA or protein level, has been reported in 61–93 % of primary and/or metastatic breast cancer biopsies [110–112]. By examining SCGB2A2 gene expression levels in 11 BCC lines, BT-474, Evsa-T, Hs578T, IBEP-1, IBEP-2, IBEP-3 [113], KPL-1, MCF-7, MDA-MB-231, MDA-MB-453, and T-47D, by microarray and RT-PCR, researchers have shown elevated SCGB2A2 mRNA level only in Evsa-T BCC, while mild expression was seen in BT-474 BCC [114]. Notably, most of these BCC lines are of metastatic origin [113].

The function of SCGB2A2 in normal breast and its promising role in breast cancer etiology are unknown. Efforts have been made to find associations between SCGB2A2 expression and various tumor features. High SCGB2A2 expression has been linked with low-grade, steroid receptor-positive tumors from postmenopausal patients [112]. O'Brien et al. [115] have shown that in breast tissue, SCGB2A2 exists in two main forms migrating with an approximate molecular mass of 18 and 25 kDa. The high molecular weight form links positively with hormone receptors and negatively with tumor grade and proliferation rate [115]. Thus, SCGB2A2 has currently the highest diagnostic accuracy for the screening of metastatic breast cancer. However, although tissue specificity is the most essential factor for a marker for circulating cells, sensitivity may not pass. Unluckily, the most aggressive, steroid receptor-negative, high-grade breast tumors and their corresponding DTCs are likely to escape detection using SCGB2A2 as a marker.

SCGB2A1

SCGB2A1 is a protein far more similar to SCGB2A2 than to other proteins, including the other members of the secretoglobin superfamily. In breast tumors, SCGB2A1 exhibits a pattern of expression similar to that of SCGB2A2 [116]. In breast cancer cell lines, SCGB2A1 is greatly expressed in MDA-MB-415 BCC, as also observed for SCGB2A2 [116]. SCGB2A1 has been detected by RT-PCR in 12 out of 30 (40.0 %) SLN from breast cancer patients [117]. Lee et al. performed a large-scale analysis of mRNA co-expression based on 60 diverse large human datasets containing a total of 62.2 million expression measurements distributed among 3,924 microarrays [118]. In line, a strong correlation between SCGB2A2 and SCGB1D2 levels has been identified in breast cancer. SCGB1D2 may bind to SCGB2A2 in an antiparallel manner forming a covalent tetrameric complex. The significance of this interaction is not known, but it appears to be the predominant form of both proteins in breast cancer cells [119].

As also observed with SCGB2A2, abundant SCGB1D2 expression has been found in malignant and normal tissues of the breast and in the female genital tract, namely, the cervix, uterus, and ovary [109]. Briefly, the secretoglobins SCGB2A1, SCGB2A2, and SCGB1D2 are expressed at variable levels in subsets of breast tumors. Despite their relatively high breast specificity, they may also be found in several other tissues, remarkably in glands and steroid-rich organs. Of these secretoglobins, SCGB2A2 has been the most used for DTC detection. Since SCGB2A1, SCGB2A2, and SCGB1D2 are often co-expressed, it is probable that in most cases, DTCs that do not express SCGB2A2 will also be negative for SCGB2A1 and SCGB1D2 expressions.

PIP

Generally known as gross cystic disease fluid protein-15, PIP has been used for years to screen breast cancer and follow breast cancer progression and metastasis. It is a small protein that is considered as a highly specific and sensitive marker of apocrine differentiation [120]. It has been identified in the majority of breast cancer biopsies [121], in correlation with steroid receptor status. In agreement, androgens, estrogens, and glucocorticoids have been found to regulate PIP expression [122]. However, as observed with SCGB2A1, PIP expression levels may

noticeably vary among breast tumors, some of them showing no expression at all. By evaluating PIP gene expression levels in 11 BCC lines (see above for SCGB2A2), researchers found elevated PIP mRNA level only in MDA-MB-453 BCC, supporting the global apocrine phenotype of these cells [114]. Therefore, PIP sensitivity in breast cancer may fail. Although being highly breast specific, PIP has also been detected, although usually at low levels, in various other tissues [121].

SBEM

Also known as BS106 [123], SBEM cDNA was identified based on its preferential illustration in libraries prepared from normal breast tissue and breast tumors. SBEM is a small secreted mucin-like protein with strong resemblance to many sialomucins [124]. In a study of 43 normal human tissues, its existence was largely restricted to the mammary and salivary glands. Concerning cancer tissues, SBEM has been identified in breast and prostate [125] MCF-7, T-47D, and ZR-75-1 BCC, but not in the poorly differentiated, ER-negative, basal epithelial-like MDA-MB-231 cells [125].

SBEM expression was noticed in 90 % of invasive ductal carcinomas, although with considerable differences in expression levels, and linked with the expression of SCGB2A2. No close connection was found between SBEM expression and steroid receptor levels or tumor grade [125].

ESR1

Although ESR1 has not been used to distinguish DTCs to date, it represents an essential marker of breast cancer. ESR1 is a transcription factor that permits regulatory functions of female sex steroids, mainly 17b-estradiol, on growth, differentiation, and function in several target tissues, including the female and male reproductive tract, mammary gland, and skeletal and cardiovascular systems. Its central role in the biology and the treatment of breast cancer is well recognized, with the mechanisms underlying its activation and function [126].

ESR1 is expressed in about two-thirds of all breast cancers. In fact, ESR1 is the main discriminator in breast tumor classifications. Its existence is characteristic of a specific class (luminal epithelial-like) of tumors with a well-differentiated, low-grade phenotype. Significant ESR1 expression has also been found in endometroid and ovarian carcinomas. TFF1 and TFF3 both are small cysteine-rich acidic-secreted proteins containing one trefoil domain that has several conserved features, including six cysteine residues with conserved spacing. Trefoil peptides function as “luminal epithelium guardians.” They are involved in the protection of luminal mucosa and mucosal restoration after damage. Rapid repair of mucous epithelia is necessary for preventing inflammation, which is a vital component of cancer progression [127]. Abnormal elevated TFF1 and TFF3 levels have been observed in various neoplastic diseases, including breast cancer. TFF3 is widely co-expressed with TFF1 in ER-positive malignant breast cancer cells [128], and both are geared up by estrogens. TFF3 is also stimulated by growth hormone.

The expression of TFF1 and TFF3 is not established in all breast tumors. Their expression pattern is close to that of ESR1, and the three genes are components of a luminal epithelial signature defining a well-differentiated, low-grade subtype that includes about 65 % of all breast cancers. Therefore, TFF1 and TFF3 may not be viewed as excellent breast tumor markers. In particular, they are unlikely to be informative in the detection of DTC from most aggressive, ER-negative, high-grade tumors.

SPDEF

SPDEF is a member of the “Ets” family. These transcription factors regulate a number of biological processes, including cell proliferation, differentiation, and invasion, and are thought to

play an important role in oncogenesis. Unlike the majority of Ets factors, SPDEF is expressed exclusively in tissues with a high epithelial content, such as the prostate and the breast [129]. Moreover, numerous studies showed SPDEF to be one of the most highly overexpressed mRNAs in human and mouse mammary tumors [129, 130]. In breast cancer cells, it has been currently shown that SPDEF could cooperate with ERBB2 to promote motility and invasion. These experimental data suggest that the coevaluation of SPDEF and ERBB2 expressions of DTC could be of high prognostic value [131].

ANKRD30A

ANKRD30A has been earlier recognized as NY-BR-1 [117] or antigen B726P [132]. The protein is regarded as an excellent transcription factor, as it contains a bipartite nuclear localization signal motif and a bZIP site (DNA-binding site followed by leucine zipper motif). Additional structural features include five tandem ankyrin repeats, implying a role for ANKRD30A in protein–protein interactions. Considering its highly restricted expression pattern, ANKRD30A may be considered as a breast differentiation antigen that could represent a suitable target for immunotherapy [133]. In fact, it was found in 80 % of breast cancer specimens, while tumors of other histological types were ANKRD30A negative. ANKRD30A expression was found in 40–50 and 60–70 % of primary and metastatic breast cancer specimens, respectively [134], which has been established by other investigators [135]. Currently, ANKRD30A expression was recognized by immunohistochemistry in breast (60 % of 124 invasive carcinoma lesions), but not in 23 other normal tissues, including prostate and testes, and in breast tumors, but not in lymphoma, seminoma, melanoma, kidney, ovarian, endometrial, prostate, and lung cancers [136].

ANKRD30A has been detected by RT-PCR in 13 out of 30 (43.3 %) SLN from breast cancer patients [117]. Therefore, even though being a highly sensitive marker, ANKRD30A is not constantly expressed by breast cancers. Furthermore, its expression has been significantly associated with the differentiation grade. For instance, in a study of 124 invasive breast carcinoma lesions, 20 out of 26 grade 1 (77 %), 24 out of 38 grade 2 (63 %), and 30 out of 60 grade 3 (50 %) samples were positive. NYBR-1 expression was also considerably associated with LN negativity, presence of ERBB2, amplification, and ER expression [136]. Therefore, ANKRD30A is likely to be detected in well-differentiated tumors and related DTCs.

SERPINB5

Generally known as maspin, it is an epithelial-specific serine protease inhibitor (serpin) that shares extensive homology to the plasminogen activator inhibitors PAI-1 (SERPINE1) and PAI-2 (SERPINB2). SERPINB5 expression has been established in the epithelium of several normal organs, including the mammary gland [137]. In breast tissue, the presence of SERPINB5 seems to be restricted to myoepithelial cells [138], when compared with the luminal epithelial ones, and it has been considered that those myoepithelial cells form a defensive barrier for the progression from ductal carcinoma in situ to more invasive carcinoma [139]. SERPINB5 has also been documented in tumors of various origins, including the breast, although, in most cases, its level was reduced when compared with normal counterparts [137].

Accumulated evidence shows that SERPINB5 may act as a tumor suppressor. Its extracellular form is enough to inhibit tumor cell motility, extracellular matrix degradation, and invasion in vitro and inhibits tumor growth and metastasis in vivo [140]. It also inhibits tumor-induced angiogenesis [141]. Intracellular SERPINB5 is accountable for an increased cellular sensitivity to apoptosis [142]. It has been formerly suggested that SERPINB5 expression in breast tumors turns down with progression and that high SERPINB5 levels were linked to low aggressiveness. For instance, a significant stepwise decrease in maspin

expression was shown to occur in the sequence ductal cancer in situ—invasive cancer—lymph node metastasis [138]. According to various studies, however, SERPINB5 overexpression has been seen only in a subset (10–35 %) of breast tumors [138]. In these studies, SERPINB5 levels in breast carcinomas have been directly linked to tumor size, high grade, high S-phase fraction, aneuploidy, positive p53 status, the presence of comedo necrosis and of lymphocyte-rich stroma, inversely correlated to the presence of steroid receptors, and recognized as a strong indicator of poor prognosis, with shorter relapse-free survival (RFS) and OS [143–145]. Therefore, in spite of its tumor-suppressor function, SERPINB5 expression seems to be a characteristic of aggressive tumors, supporting its use for DTC detection.

GABRP

The g-aminobutyric acid (GABA) receptor is a multimeric transmembrane chloride ion channel. Sixteen subtypes of GABA-receptor subunits have been classified within six structural classes (a1–6, b1–3, g1–3, g 3, q, p). These subunits are thought to assemble in different pentameric complexes. GABRP was previously identified by in silico analysis of four million ESTs as a candidate gene differentially expressed in breast cancer. It codes for the p-subunit of the GABA receptor. In a study of 23 normal human tissues, the GABRP expression level was most prominent in the breast. In breast tissue, GABRP is mainly expressed in myoepithelial/basal cells, and it is hypothesized that its function could be linked to tissue contractility. GABRP expression was established to be lower in a majority of primary breast tumors when compared with corresponding normal tissues. Along the same line, strong GABRP expression was examined in normal epithelial and benign papilloma breast cells, but no signal could be noticed in invasive ductal carcinoma, signifying that GABRP is progressively downregulated with tumor progression and that it may be valuable as a prognostic marker in breast cancer [109]. In contrast, in a study of 203 invasive breast cancers, GABRP expression was found high in a subset (16 %) of ER-negative, ERBB2-negative, high-grade tumors with basal-like (undifferentiated) phenotype [146].

Genetic Change in DTCs

There are indications that DTCs may exhibit a significant genetic diversity, reflecting the instability and microheterogeneity observed in primary tumors. Using a procedure involving whole-genome amplification and subsequent CGH of single immunostained cells, it has been observed that cytokeratin-positive DTCs in the bone marrow (BM) of breast cancer patients without clinical signs of overt metastases (stage M0) were genetically heterogeneous [47]. This heterogeneity was reduced with the emergence of clinically evident metastasis (stage M1). The fact that DTC in M1 patients closely resemble each other genetically suggests that cells could separate from lesions at secondary sites (e.g., BM) and recirculate and may cause the appearance of other metastatic sites.

As revealed earlier, it has been hypothesized that BM could serve as a “reservoir” allowing for DTC to adapt and disseminate later into other organs. Investigators using a combination of ICC and FISH found that the pattern of genetic aberrations in BM-derived DTC varied considerably among different breast cancer patients [147]. This is consistent with the CGH-based data of Klein et al. supporting a plethora of different random changes in M0 cells. Schmidt-Kittler et al. [78] also demonstrated a high genetic heterogeneity in M0 cells, although these DTCs displayed fewer chromosomal aberrations than primary tumors or cells from M1-stage patients. Numerous M0 DTCs without detectable aberration (CGH analysis) were also found by these authors.

In M0 cells, genetic aberrations appeared to be randomly generated, while characteristic chromosomal imbalances were observed in M1 cells. This suggests that in breast cancer, tumor cells may disseminate in a far less progressed genomic state than previously thought and that they acquire aberrations typical of metastatic cells thereafter.

Similarly, Gangnus et al. [148] analyzed tumor cells in BM of early-stage breast tumor patients for genomic changes by single-cell CGH. The viable disseminated cancer cells had a plethora of copy number changes in their genome. All evaluated cells showed chromosomal copy number changes with a substantial intercellular heterogeneity and differences to the matching primary tumors. The further development of M0 cells into metastasis, and hence M1 cells, apparently is a matter of mutation and selection, leading to a plausible explanation for tumor dormancy. In this interpretation, dormancy reflects the time needed for M0 cells to acquire the full capacity of unrestrained growth. This selection model is in agreement with the fact that DTCs in patients with overt metastases closely resemble each other genetically [47]. It must be noted that the genetic changes as observed in DTCs from BM [47, 148] and PB [149] confirm the tumoral nature of these DTCs. Since specific DNA gains/losses combinations and genes amplifications in primary tumors are associated with prognosis, it would be helpful to assess whether such changes are also found in DTCs, as well as the probable relationships between their presence in these cells and various parameters (survival of DTCs, time before clinical appearance of metastases, metastasis target organs). For instance, the prognostic value of genomic alterations in breast DTCs has been observed [150]. These authors found considerable correlations between genomic alterations of the DCC and ERBB2 genes in DTCs and relapse-free survival. Moreover, increasing numbers of genomic imbalances measured in DTCs were significantly associated with worse prognosis of recurrent disease. Some of the genes that are frequently amplified in breast tumors encode proteins that are or could be targeted by specific therapies. For instance, Her-2/neu, the product of ERBB2, is targeted by the antibody trastuzumab, while attempts are made to design molecules preventing the interaction between the ubiquitin ligase MDM2 and the p53 oncogene [55]. At term, the identification of specific gene amplifications in DTCs, notably by a combination of array CGH and FISH, could allow the application of specific therapies [151].

Significance of DTCs in Lymph Node, Peripheral Blood, and Bone Marrow

Prognosis and Correlations

Many studies have reported that the presence of disseminated tumor cells (DTCs) in bone marrow (BM), evaluated by ICC or RT-PCR, links strongly with an early relapse of breast cancer and decreased patient survival [71, 152]. As demonstrated by clinical follow-up data on more than 4,000 breast cancer patients studied in prospective trials by several international groups, the presence of DTCs in BM (identified by ICC at primary diagnosis) predicts the postoperative occurrence of overt metastases in bone and other organs [67]. Notably, strong correlations between the presence of BM micrometastases and poor survival have been reported in breast cancer independent from lymph node (LN) metastases [153].

Prognosis of Women with Stage IV Breast Cancer Depends on Detection of CTCs Rather than DTCs

The BM DTC detection rate is noticeably increased in the metastatic setting (59 %) compared with the 15 % detection rate in early breast cancer [154]. No significant difference in BM DTC detection rate was observed between patients in the first line (58 %, $n=110$ patients) and second (or more) line of treatment (61 %, $n=28$ patients) [155]. For CTC detection, the standard Ficoll technique used in this study was responsible for a lower blood CTC screening rate compared with an epithelial cell adhesion molecule (EpCAM) enrichment method explained previously (40 % versus 61 %) [156]. This lack of sensitivity may be counterbalanced by a higher specificity, i.e., detection of patients with high CTC count, and could explain why CTC detection represented a significant prognostic factor in several studies. Moreover, a study reported that an increased number of DTCs identified in the BM represents an independent prognostic factor in a short series of 33 metastatic breast cancer patients [157]. Further study on much larger number of patients reported that BM DTC

detection was of less clinical significance [155]. They also investigated the prognostic value of this parameter according to the two methods of analysis: presence or absence or by defining a cutoff value for the number of tumor cells. None of these analyses was statistically significant for predicting OS in these 138 patients.

Several biological studies assessing the persistence of BM DTCs after adjuvant treatments have specified a possible resistance of these cells to chemotherapy [158]. BM DTC finding has been shown to be predictive for bone metastases in the early breast cancer setting [158]. Bidard et al. showed that the strong link between BM DTC and bone metastasis was maintained after metastatic growth. They also observed a higher frequency of DTCs in patients with lobular carcinoma compared with ductal carcinoma [155]. Their observations indicate that the homing of cancer cells to bone and BM may depend on similar molecular determinants [159]. This is in accordance with the more extensive metastatic spread of lobular carcinoma previously reported by a research group [160]. In contrast, CTCs were not associated with a specific metastatic pattern. Finally, DTCs, detected in the BM (DTC) or in the blood (CTC), can be evaluated at both the early and metastatic stages of breast cancer. Thus, several researchers concluded that BM DTC detection at an early stage appears to be more closely correlated with breast cancer prognosis than CTC [69]. Clinical studies are presently ongoing to define the value of CTCs in the adjuvant setting using more sensitive and specific techniques [161]. Clinical significance of CTCs detection and overall significance in breast cancer is summarized in Table 22.4.

Potential Applications of DTCs

Since tumor cells may in some cases disseminate very early in the natural history of breast cancer, one can envisage the detection of DTCs in women apparently without cancer but who are regularly screened because they are considered at high risk. At present, the selection of patients is based on their statistical risk of developing tumor recurrence, without knowing whether they actually harbor any DTCs. This doubt may lead to overtreatment of patients with cancer with toxic agents that exert severe side effects. For example, only 20–25 % of lymph node (LN)-negative breast cancer patients undergo metastatic relapse within 10 years postsurgery, but more than 90 % of these patients are supposed to receive chemotherapy according to recommendations [162].

DTC recognition in peripheral blood (PB) or bone marrow (BM) may represent an additional clinical marker to identify those LN-negative patients who are cured by surgery alone and need no additional adjuvant systemic therapy. Monitoring the efficacy of a therapy is an important aspect; this might contribute to predicting which patients with early-stage or metastatic disease will recur. This may also possibly support the shift to another treatment, such as monitoring for recurrence after apparently successful adjuvant therapy in patients with early-stage or metastatic disease or destroying DTCs before they develop into metastases. One can consider that the observed moderate rate of response in advanced cancer patients might be caused by the fact that solid metastases form physiological barriers that prevent the access of macromolecules such as antibodies from the circulation in the metastatic lesion [163]. So, DTCs are expected to be more easily accessible for intravenously applied immunoglobulins.

Conclusion and Future Perspective

Advances in modern sciences and technology have allowed the detection of single or small groups of breast cancer cells disseminated in lymph node (LN), peripheral blood (PB), and bone marrow (BM); consequently, the screening and visibility between primary tumors and metastases has become quite easy. Current research and progress in breast tumor biology made it clear that two distinct routes may lead to tumor cell dissemination. Some cells may transit by LN before accessing the PB and BM (lymphogenous route), while other DTCs appear able to directly enter the blood stream (hematogenous route).

Table 22.4 Clinical significance of CTCs detection in breast cancer

Method	Marker	CTC detection rate	Clinical significance	Reference
Early breast cancer				
Nested RT-PCR	CK-19	44 of 148 (30 %)	DFI, $p=0.001$; OS, $p=0.014$	[192]
RT-qPCR	CK-19	Node negative 36 of 167 (21.6 %)	DFI, $p<0.001$; OS, $p=0.008$	[72]
RT-qPCR	CK-19, mammaglobin HER-12	CK-19, 72 of 145 (41 %) Mammaglobin, 14 of 175 (8 %) HER-2, 50 of 175 (29 %)	DFI, CK-19 ($p<0.001$); OS, CK-19 ($p=0.044$) DFI, mammaglobin ($p=0.011$); OS, mammaglobin ($p=0.034$) DFI: HER-2 ($p<0.001$)	[193]
RT-qPCR	CK-19, ER	181 of 444 (41 %)	DFI, CK-19 and ER- ($p=0.001$); OS, CK-19 and ER- ($p=0.001$)	[194]
RT-qPCR	CK-19	After adjuvant therapy, 179 of 437 (41 %)	DFI, $p<0.001$; OS, $p=0.003$	[195]
RT-qPCR	CK-19	Before adjuvant therapy, 91 of 165 (55.2 %) After adjuvant therapy, 79 of 162 (48.8 %)	Before adjuvant therapy: DFI, $p=0.081$; OS, $p=0.024$ After adjuvant therapy: DFI, $p=0.057$; OS, $p=0.128$	[169]
RT-qPCR	CK-19	99 of 133 (31.7 %)	DFI, $P=0.001$ and OS, $P=0.001$).	[196]
CellSearch	Pan-CK	Before and/or after neoadjuvant chemotherapy, 32 of 118 (27 %)	DFI, ps0.013	
CellSearch	Pan-CK	Before chemotherapy therapy, 95 of 115 (82.6 %) After chemotherapy, 85 of 115 (73.9 %)	Before chemotherapy: DFI, $p=0.007$; OS, $p=0.0006$ After chemotherapy: DFI, $p=0.04$; OS, $p=0.02$	[197]
CellSearch	Pan-CK	Before chemotherapy, 140 of 1,489 (9.4 %) After chemotherapy, 129 of 1,489 (8.7 %)	Before chemotherapy: DFI, $p<0.0001$; OS, $p=0.023$ After chemotherapy: DFI, $p=0.054$; OS, $p=0.154$	[198]
ICC	CK	47 of 71 (66 %)	OS, ps0.071; DFI, $p=0.052$	[199]
RT-PCR	CK-19, HER-2, P1B, PS2, epithelial glycoprotein 2	43 of 72 (60 %)	DFI, ps0.031; OS, $p=0.03$	[200]
ICC	CK and HER-2	17 of 35 (49 %)	DFI, $p<0.005$; OS, $p<0.05$	[85]
Nested RT-PCR	Mammaglobin	14 of 101 (13.9 %)	DFI, $p=0.020$; OS, $p=0.009$	[201]
Metastatic breast cancer				
CellSearch	Pan-CK	87 of 177 (49 %)	DFI, $p<0.001$; OS, $p<0.001$	[156]
CellSearch	Pan-CK	43 of 83 (52 %)	DFI, $p=0.0014$; OS, $p=0.0048$	[74]
CellSearch	Pan-CK	92 of 195 (47.2 %)	DFI, $p=0.0122$; OS, $p=0.0007$	[202]
CellSearch	Pan-CK	35 of 138 (25 %)	OS, $p<0.0001$	[203]

Abbreviations: *DFI* disease-free interval, *OS* overall survival

The mechanism leading to direct hematogenous tumor cell dissemination is not clearly recognized as yet, but it is likely favored by a high microvessel density (MVD) in the primary lesion, as this latter feature has been linked to the presence of DTCs in PB or BM [164, 165].

Screening of DTCs/CTCs according to standardized protocols and subsequent comprehensive phenotypical and molecular characterization of these cells might contribute to an improved identification of patients in need of additional systemic anticancer therapy, in accordance with their present disease status and, finally, to the development of more customized and personalized therapies for breast cancer patients. Last but not least, the various molecular biomarkers with

CTCs and DTCs, i.e., fluid biopsy-based strategies, may able to guide the path of early detection and treatment and will open new vistas in understanding the tumor biology of breast cancer; thus, the ultimate goal of better management of breast cancer patients will be possible.

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23 Exhaled Volatile Organic Compounds as Noninvasive Markers in Breast Cancer

Orna Barash and Hossam Haick

Abstract

Volatile organic compounds (VOCs) in exhaled breath are interesting candidates as breast cancer (BC) markers for malignancy, staging, histology, genotype, and distinction from other malignant and benign diseases. VOC BC markers can be derived either as BC-specific compounds by analytical chemistry or as collective breath prints by statistical treatment of the output of sensor arrays. Despite the great potential of applications in clinical diagnostics, only few studies for breath VOC BC markers have been done, and breath testing for BC has not yet left the realm of research and entered clinical practice, mainly due to lack of standardization of the experimental techniques. In this chapter, we will outline the vast potential of exhaled VOC as a novel class of molecular BC markers and describe the challenges on the way from bench to bedside. In this chapter, we provide a didactic approach to the state-of-the-art experimental techniques for breath collection, sample storage, analysis of the breath VOCs, and direct breath printing, and we present examples for applications of diagnosing BC by VOC profiling.

Keywords

Breast cancer markers • Volatile organic compounds • Breath analysis • Sensors array • Breath prints

Introduction

Breast cancer (BC) is a malignant proliferation of epithelial cells lining the ducts which transfer milk during breastfeeding (ductal cancer) or of the lobules where milk is made (lobular cancer) [1]. Human BC is a clonal disease, the product of a series of somatic or germline mutations which are eventually able to express full malignant

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potential. Thus, BC may exist for a long period as either a noninvasive disease or an invasive but nonmetastatic disease [1]. BC is the most common malignancy in Western countries in women, accounting for more than 40,000 deaths each year [2]. While it is predominantly a disease of older women, it also affects women under the age of 30.

Several well-established risk factors are associated with the development of BC: age, obesity, ionizing radiation exposure, etc. Family history is highly significant in a first-degree relative (i.e., mother, sister, and daughter), increasing the chances to have cancer by three- to four folds. Approximately 8 % of all BC cases are hereditary, and one-half of these are attributed to mutations in two BC susceptibility genes—the tumor suppressor genes BRCA1 and BRCA2 [3]. Another important gene, playing a role in about one-quarter of BC cases, is the gene-encoding epidermal growth factor receptor erbB2 (HER2/neu), which is overexpressed in these BCs due to gene amplification; this overexpression can contribute to transformation of human breast epithelium and is the target of effective systemic therapy in adjuvant and metastatic disease settings. However, if the breast tumor is detected when it is still localized, BC is also one of the most treatable malignancies, with screening mammography in asymptotic women reducing mortality by 20–30 % [1]. For example, for invasive breast cancers of size <1 cm, relative survival after 15 years ranges from 90 to 92 %; for tumors of 1–2 cm, it ranges around 75 % and continues to drop with increasing T stage [3].

An important risk factor for developing breast cancer is related to enhanced oxidative stress and induction of cytochrome p450 mixed oxidase enzymes [4]. Oxidative stress is defined as the overall balance between formation and scavenging of reactive oxygen species (ROS) and free radicals in the body. ROS are molecules or ions with an unpaired electron in the outer shell, which are constantly produced in the mitochondria as part of the cellular respiration process. They can also stem from exogenous sources, such as cigarette smoke, pollution, and radiation. When accumulating, ROS can attack intercell biological molecules such as proteins and polyunsaturated fatty acids (PUFA). ROS molecules enhance the activity of cytochrome p450, which are a large and diverse group of mixed oxidase enzymes which catalyze the oxidation of organic substances. Several studies have reported the upregulation of CYP-450 enzymes in human breast tissue [5, 6]. An important CYP-450 enzyme is aromatase, which synthesizes estrogens and is known to be overexpressed in human breast cancer tissue [7]. In normally occurring oxidative stress, reactive oxygen species leak from the mitochondria or from the peroxidate PUFA in the cell membranes and generate volatile alkanes and methylated alkanes that are excreted in the breath [8].

Conventional Imaging BC Markers for Diagnosis and Screening

Population-based screening programs with sufficient sensitivity and specificity of at-risk populations are necessary to identify presymptomatic patients with treatable BC at the earliest possible stage [9, 10]. The most widely available method for BC screening of asymptomatic women is mammographic screening combined with regular clinical breast examination. Diagnostic mammography, which may include additional tests, is done for women with signs of breast cancer (symptomatic) [3]. However, screening mammography suffers from some limitations: (1) It may detect abnormalities that will eventually turn out to be benign and false-positive results lead to additional testing, which may increase costs and unnecessary anxiety; (2) it may miss tumors at their preclinical stage—10–15 % of all breast cancers are not detectable by a mammogram [11]; and (3) the image quality depends on the breast's density, and, therefore, it is mostly suitable for postmenopausal women and not for young women who usually have dense breast tissue [12]. For maximum yield in screening programs, both physical examination and mammography are necessary, since mammography alone can

detect only 35–50 % of early breast cancers and another 40 % can be detected only by palpation. Patients with a dominant or suspicious mass on mammogram must undergo biopsy to confirm the presence of the disease. Biopsy examination of all suspicious lesions will ultimately reveal the histologic diagnosis of the disease.

A useful biopsy technique is fine-needle aspiration (FNA) cytology in which cells are aspirated with a small needle and examined cytologically. Large-needle (core needle) biopsy removes a core of tissue with a large cutting needle under local anesthesia. However, the most reliable means of diagnosis is open biopsy, and it is commonly used when uncertainty has arisen from needle biopsy or aspiration. Also, some BCs like in situ cancers are not easily diagnosed cytologically and require excisional biopsy. However, these techniques suffer from the following disadvantages: sampling problems, the requirement for pathologist skills to examine the cells/tissue, invasive and not cost-effective.

Another technique used is ultrasonography, which is performed primarily to differentiate cystic from solid lesions [13]. Ultrasonography is usually used after an inconclusive mammography or when the breast is dense. Magnetic resonance imaging (MRI) has high sensitivity to breast disease, but has significantly lower specificity than mammography, leading to false-positive diagnosis.

Volatile Organic Compounds as Potential Future Noninvasive Molecular BC Markers

Volatile organic compounds (VOCs) are emerging as potential future molecular markers of BC [14–19]. VOCs have a relatively high vapor pressure (>0.1 mmHg) and, hence, tend to be excreted with the exhaled breath after alveolar exchange from the blood. This means that VOCs could be derived noninvasively from exhaled breath. Molecularly different phenotypes and genotypes of BC are expected to generate distinguishable VOC profiles. Impressive empirical data have confirmed the potential of VOCs to serve as a basis for a noninvasive, simple, inexpensive, and easy-to-use diagnostic tool, especially in cases of lung cancer [14, 20–53]. Interestingly, pilot studies have revealed that breath VOCs can also be used to diagnose non-pulmonary diseases such as breast cancer [12, 14–17, 19]. Hence, monitoring of VOCs in the breath may soon become an interesting supplement (or even alternative) to conventional medical diagnostics and follow-up of therapeutic effects, thanks to rapid advances in the techniques for breath collection and gas analysis during the past two decades. This novel approach could revolutionize personalized BC care and management and has the potential for becoming an integral part of population-based BC screening in the future, even though the technique is not yet mature enough for imminent clinical use [54].

Volatile Organic Compounds in Exhaled Breath

Exhaled breath is potentially the most easily clinically accessible source for BC markers, allowing noninvasive sampling and even continuously online sampling [55, 56]. In addition, the matrix of exhaled breath is less complex than that of the saliva, sputum, blood, urine, stools, and tumor tissue and, hence, would be easier to analyze. Human exhaled breath is a diverse mixture of inorganic and organic molecules in the gas phase [43]. Its main constituents are nitrogen, oxygen, carbon dioxide, water, and inert gases. Exhaled breath is almost fully humidified with high and extremely variable values of relative humidity (RH ~40–80 %). The humidity in breath samples decreases with age and may vary considerably as a result of benign and malignant pulmonary diseases, as well as diet and lifestyle. In addition to the inorganic gases, thousands of VOCs may be detected at very low concentrations in the breath of different persons, in parts per billion (ppb) with respect to volume (ppbv) or even parts per trillion (pptv). The VOCs in the exhaled breath stem from blood-borne VOCs that are either generated

by the various cellular biochemical processes of the body or absorbed from the environment through ingestion, inhalation, or skin contact [39, 57]. Therefore, the VOC profiles of BC patients can be detected via the exhaled breath [12, 14–17, 19], since the changes of the blood chemistry are reflected in measurable changes of the chemical composition of the alveolar exhaled breath through exchange via the lung [24, 58]. It was found that some gases exchange in the airways, rather than the alveoli, depending on the blood/air partition coefficient, λb:a. Gases with low solubility in blood, mainly nonpolar VOCs (λb:a <10), exchange almost solely in the alveoli, while highly blood-soluble gases, mainly polar VOCs (λb:a >100), tend to exchange in the airways [59]. Furthermore, VOCs with 10< λb:a <100 interact significantly both with the airways and with the alveoli [59]. Hence, the airways may play a larger role in pulmonary gas exchange than has generally been assumed [56, 60], and the implications of pulmonary tests and breath tests might have to be reevaluated [59]. The VOC profile is also influenced by the retention of VOCs in the lungs, namely, the fraction of the molecules that remain in the respiratory tract at any time, after inhalation and exhalation, because of the blood/air partition coefficient [61]. Thus, the final partition and exhalation of the VOCs depend on their physical and chemical properties and on their interaction with the different alveolar clearance processes [61, 62].

It is important to note that several other parameters, besides diseases, may also affect the VOC concentrations in a person's breath. These include both permanent/long-term body states and short-term dynamic changes. The resulting changes of the VOC profiles are sometimes substantial and could therefore confound the disease-induced VOC profiles. Confounding factors such as age, gender, lifestyle, nutrition, medication, medical history, smoking habits, and alcohol consumption can alter the concentration of certain breath VOCs [41, 42, 63, 64]. For example, exhaled isoprene levels change with a person's age, gender, and therapeutic intervention [46].

Potential Future BC Markers Derived from Exhaled Breath VOCs

Future Clinical Potential

The disease-specific changes of a small fraction (ca. 1 %) of the exhaled VOCs could be used to derive molecular markers of BC [65]. BC-specific breath VOCs or compositional changes in VOCs that are present in everyone's breath could be products of the metabolic activity of the tumor itself or by-products of bacteria and necrotic reactions caused by local inflammation in the microenvironment of the tumor, or else they could be partially reemitted environmental toxins that were previously adsorbed into the body [54]. In addition, systemic breath VOCs could be produced or consumed because of cancer-related changes elsewhere in the body, affecting the blood chemistry, and eventually being expired via the respiratory system [54].

Breath VOCs of both exogenous and endogenous origin could be utilized for BC diagnostics and management. The following molecular BC markers could, in principle, be derived:

1. Risk markers of developing BC in healthy subjects
2. Markers indicating the presence of measurable disease in the early or more advanced stage, including staging information
3. Markers for different genotypes and phenotypes (including histology and presence of oncogenes) with prediction of prognosis and/or response to therapy
4. Markers for monitoring of the responses to therapy such as surgery, radiation therapy, immunotherapy, or chemotherapy

Breath-based BC markers have many advantages: Their noninvasive acquisition is safe and convenient for the patient; sampling and analysis are fast and could be performed in nonspecialist settings, e.g., in local GP clinics; the method could be potentially cost-effective (if sensor arrays are used); and, hence, it could be accessible also in the developing world and for use in future population-based BC screening programs.

Analysis of Separate BC-Specific VOCs Versus Direct Breath Printing by Sensor Arrays

There are two fundamentally different approaches for deriving BC markers from the exhaled breath VOCs. The first approach consists of the identification and quantification of the BC-specific VOCs, using techniques of analytical chemistry. Table 23.1 provides an overview of the strengths and weaknesses of three different analytical methods that have been used for exhaled VOC analysis, namely, gas chromatography linked with mass spectrometry (GC-MS), proton transfer reaction mass spectrometry (PTR-MS), and proton transfer reaction time-of-flight mass spectrometry (PTR-TOF-MS). In this case, the BC markers would be identical with the (concentrations of the) actual breath VOCs that characterize BC patients. However, the compositional changes of the separate VOCs are not distinct enough for reliable BC classification. Furthermore, available techniques either require additional experimental procedures (e.g., sample pre-concentration and system calibration in the case of GC-MS) or are sensitive only to specific classes of breath VOCs. The analytical techniques are described in detail in section "Chemical Analysis: Identification of Specific BC Marker VOCs."

Collective breath VOC patterns can, in principle, be derived through additional statistical analysis of the concentration profiles of preselected VOCs, but the entire process is tedious and time-consuming. To summarize, analytical chemical analysis of the exhaled breath may yield highly accurate concentration profiles of separate BC-specific VOCs that provide interesting input for studying biochemical pathways of BC. However, the experimental procedures involved would not be practical for real-world diagnostic or BC screening.

The second approach consists of the direct detection of collective breath VOC patterns (without actually identifying the constituent compounds), using arrays of broadly cross-reactive sensors [66, 67]. These patterns have been termed breath prints. Sensor arrays mimic the mammalian sense of smell and are therefore often called electronic noses. Each sensor in the array responds to all or part of the VOCs in the breath sample. Breath prints are then derived from the collective numerical output of the sensors that interact with the breath VOCs, using methods of statistical data analysis. The breath

Table 23.1 A comparison of the characteristics of the methods of analytical chemistry and the general properties of sensor arrays that have been used for studying breath VOC of BC

	GC-MS	Sensor array
Compounds	Volatile and semi-volatile compounds	Tunable through choice of sensors
Accuracy of compound identification	Very high	No compound identification
Detection limit	~ppm_v, can be improved to ~ ppb_v through sample pre-concentration	Depends on sensor type; tunable for specific VOC mixtures
Compound quantification	Requires calibration	n/a
Speed	Off-line	High
Required user skill level	High	None
Sample preparation	Pre-concentration of breath VOCs necessary	None
Possibility of direct breath sampling	No	Yes
Breath print/VOC pattern determination	Separate statistical treatment of VOC concentration profiles; requires quantification	Directly through built-in statistical treatment of the collective sensing signals
Size of equipment	Typically very large	Small, portable
Maintenance	High	Low
Consumable costs per sample	$40–$150	$1–$5

BC breast cancer, *VOC* volatile organic compound

print BC markers are dimensionless parameters. This approach avoids expensive equipment and, therefore, has realistic potential for future fast, cost-effective, and high-throughput breast cancer diagnostics. Pre-concentration is generally not necessary, because additive signals are monitored that stem from a wide range of breath VOCs, at total concentrations of at least ppmv or above. In principle, this approach can be adapted for direct sampling of patients' breath, but until now, indirect sampling of pre-collected breath samples has been more feasible, since the sensor arrays are usually operated in a research laboratory. Sensor arrays are therefore ideally suited for direct BC marker breath printing [12, 14]. Types of sensors that have been used for breath printing will be described in section "Sensor Arrays for BC Marker Breath Printing." However, breath printing is essentially a black box approach to chemical sensing, which bears the risk of over-fitting small data sets during the statistical analysis [30]. Therefore, careful validation of the study results, preferably with a blinded validation sample set, should be an integral part both of limited proof-of-concept studies and of large-scale clinical trials.

Challenges on the Way to Clinical Practice

Breath VOCs that might indicate cancer have attracted much research interest during the past decades; however, limited preliminary results on BC have been achieved both in the chemical analysis of exhaled breath and in the sensor-based breath printing (see Tables 23.2, 23.3, and 23.4). Due to the complexity of the breath collection process and analysis and the insufficient attention to confounding factors, no viable and generally accepted BC markers have yet been established, and BC breath markers are still entirely confined to research. The lack of standardization pertains to each step of the multistep process toward the establishment of reliable BC markers from exhaled VOCs. Figure 23.1 provides an overview of the breath BC marker development—from bench to bedside. Inconsistent findings of different study groups in limited pilot trials could be attributed to one or more of the following problematic aspects of current breath marker research:

1. Inconsistencies in the preselection of the rather small control groups used in the proof-of-concept clinical studies. For example, control groups might consist of healthy biopsy-proven, benign breast tumors, age-matched groups, hospital personnel, relatives and spouses of the patients, etc. Clinical studies investigating exhaled VOCs of BC, especially proof-of-concept studies of limited size, should be carefully designed to avoid biased results, by using well-matched study populations.
2. Inconsistent breath sampling and VOC pre-concentration procedures used in various studies (see section "Breath Collection, Sample Storage, and Possible Sources of Contamination"). Breath collection should be standardized, and either one universally accepted method should be adapted by all researchers in the field or a small number of well-defined techniques should be established for use. In addition, a breath collection protocol should be followed which minimizes the effect of nutrition, smoking habits, and medication.
3. Use of different analytical methods including different equipment (e.g., GC-MS (42, 48), PTR-MS (25, 37), etc.). See Tables 23.1 and 23.2 and section "Chemical Analysis: Identification of Specific BC Marker VOCs." Note that the identification of the VOCs by GC-MS or PTR-MS is not 100 % certain, even if the identification by spectral library match and retention time for GC-MS is quite reliable [22, 24, 48].
4. Use of different sensor arrays for breath print detection. Special attention must be paid to confounding factors when using sensor arrays. The response of the sensor array to the relevant confounding factors should be studied in order to exclude biased results.
5. Inconsistencies in the normalization procedures of the raw data. While part of the studies normalized the data according to the

Table 23.2 Examples for experimental studies of BC-specific VOC patterns, using different analytical techniques

Determination of VOC pattern				Study population			Obtained results				Future potential as marker for		
							Control 1	Control 2					
Analytical method	VOC as input for algorithm	Statistical algorithm	Validation	Target	Control 1 (confirmed by mammography/biopsy)	Control 2 (healthy volunteers)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Breast malignancy	Histology	Principal investigator
TD/ GC-MS	8 VOCs	Forward stepwise discriminant analysis	Cross-validation	51 BC	-	42			88.2	73.8		–	Phillips et al. [16], #144
	10 VOCs	Forward stepwise discriminant analysis	Cross-validation	52 BC	50	–	60.8	82				–	Phillips et al. [16], #144
	5 VOCs	Fuzzy logic	Prediction set	51 BC		42			93.8	84.6		–	Phillips et al. [17], #143
	10 VOCs	Weighted digital analysis (WDA)	Prediction set	54 BC	204	–	75.3	84.8	–	–		–	Phillips et al. [15], #150
	5VOCs	–	*t* test and Mann-Whitney *U* test	10 BC	–	10	–	–	n/a	n/a	x	–	Mangler et al. [19], #159
SPME/ GC-MS	5 VOCs	Forward stepwise discriminant analysis		14 BC	–	22	–	–	n/a	n/a	x	–	Peng et al. [14]

The VOC patterns were calculated from the concentration profiles of specific VOCs. The possible future potential as specific BC markers is indicated that could be highly relevant for prognosis, choice of treatment, and treatment follow-up

Table 23.3 Breath VOCs as potential BC markers

Study	Analytical method	VOC	Chemical class	Possible source	Principal investigator
1	TD/GC-MS	Nonane	Alkane	Markers of oxidative stress named: breath methylated alkane contour (BMAC)	Phillips et al. [16]
		Tridecane, 5-methyl	Methylated alkane		
		Undecane, 3-methyl	Methylated alkane		
		Pentadecane, 6-methyl	Methylated alkane		
		Propane, 2-methyl	Methylated alkane		
		Nonadecane, 3-methyl	Methylated alkane		
		Dodecane, 4-methyl	Methylated alkane		
		Octane, 2-methyl	Methylated alkane		
2		2-Propanol	Alcohol	n/a	Phillips et al. [17]
		2,3-Dihydro-1-phenyl-4(1H)-quinazolinone	Ketone	Antitumor activity	
		1-Pentyl-ethanone	Ketone	Anti-invasive activity against human MCF-7/6 mammary carcinoma cells	
		Heptanal	Aldehyde	Cancer biomarker	
		Isopropyl myristate	Ester	n/a	
3		Cyclopropane, ethylidene[a]	Diene	n/a	Phillips et al. [15]
		Cyclotetrasiloxane, octamethyl[a]	Siloxane	Exogenous	
		D-Limonene 5989-27-5[a]	Liquid alkane	Ingested from foodstuffs	
		Benzene, 1,2,4,5-tetramethyl[a]	Benzene derivative	Environmental pollutant	
		Tridacane[a]	Alkane	Oxidative stress	
		Dodecane, 2,7,10-trimethyl[a]	Methylated alkane	Oxidative stress	
		Tetradecane[a]	Alkane	Oxidative stress	
		(+)-Longifolene[a]	Oily liquid alkane	n/a	
		2-Hexyl-1-octanol[a]	Alcohol	n/a	
		2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-[a]	Ketone	n/a	
4		3-Methylhexane	Methylated alkane	Altered activity of cytochrome p450	Mangler et al. [19], #159
		Decane	Alkane	Altered activity of cytochrome p450	
		Caryophyllene	Terpenes	Altered activity of cytochrome p450	
		Naphthalene	Polycyclic aromatic hydrocarbon	Altered activity of cytochrome p450	
		Trichlorethylene	Halogenated hydrocarbon	Tumor cell metabolites	
5	SPME/ GC-MS	3,3-Dimethyl pentane	Methylated alkane	Oxidative stress	Peng et al. [14]
		2-Amino-5-isopropyl-8-methyl-1-azulenecarbonitrile	Nitrile	n/a	
		5-(2-Methylpropyl) nonane	Methylated alkane	Oxidative stress	
		2,3,4-Trimethyl decane	Methylated alkane	Oxidative stress	
		6-Ethyl-3-octyl ester 2-trifluoromethyl benzoic acid	Ester	n/a	

All listed compounds showed statistically significant differences of the average concentration between the studied BC states and control states in exhaled human breath

[a]Tentative identifications of VOCs. Only first identification is displayed

Table 23.4 Examples for experimental studies of BC breath prints, using sensor arrays

Sensor array			Study population				Sensitivity (%)	Specificity (%)	Future potential as marker for							Principal investigator
Sensor type	Sensing material	Statistical algorithm	Target	Control 1 (benign breast condition)	Control 2 (healthy volunteers)	Control 3 (clear mammography)			Breast malignancy	Distinction from other breast deficiency	Distinction from other malignancy	Staging	Histology	Treatment response	Genotype	
Chemiresistors	GNP	PCA	13	–	22	–	n/a	n/a	x	n/a	x	x	x	n/a	n/a	Peng et al. [14]
	GNP + cubic Pt NP	PCA + SVM	11	14	–	7	94	80	x	x	–	x	x	n/a	n/a	Shuster et al. [12]
	GNP + cubic Pt NP	PCA + SVM	11	–	21	–	n/a	n/a	x	–	–	x	x	n/a	n/a	

The possible future potential as specific BC markers is indicated that could be highly relevant for prognosis, choice of treatment, and treatment follow-up

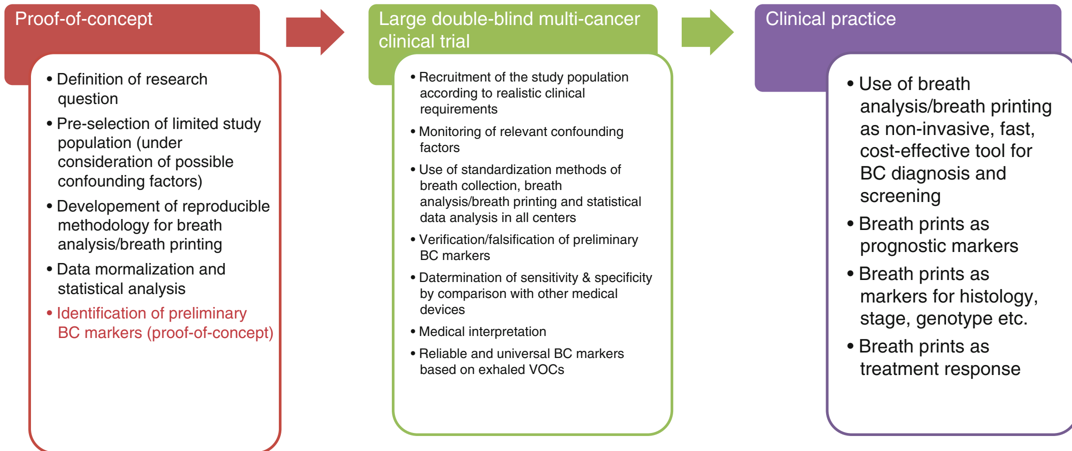


Fig. 23.1 Steps toward the development of reliable VOC-based breath BC markers for clinical practice—from bench to bedside. *BC* breast cancer, *VOC* volatile organic compound

concentration of a specific VOC in the exhaled breath [14], other studies have normalized the data according to the difference between the concentrations in the exhaled and the inhaled air [15–17]. Non-normalized data were reported as well. Normalization for sensor arrays is even more challenging, because long-term and short-term sensor drifts have to be considered [68].

6. Inconsistent data analysis. For instance, the analysis of the GC-MS raw data includes identification, separation, and area integration of the peaks in the chromatograms for each sample, as well as quantitative comparisons of the chromatogram peak areas or compound concentrations between different study groups, using statistical algorithms. Patterns distinguishing the study groups may be obtained from the collective GC-MS results through a variety of supervised or non-supervised statistical pattern recognition algorithms. For example, forward stepwise multi-linear regression, a supervised method, was used by Phillips et al. [16] in order to establish BC patterns based on (unidentified) chromatogram peaks. VOC patterns of BC were studied using non-supervised methods such as principal component analysis [12]. Also, the data analysis of the collective sensor array output involves multivariate statistical analysis of the raw data.
7. No in vitro studies have been performed. In vitro studies may serve as a way to eliminate confounding factors and, thus, will allow immediate testing to predict the clinical benefit from targeted therapy straight from the tissue. This approach could help guide treatments by tracking genetic alterations from the frozen tissue with no time or money limitations.

The following sections are designed to provide the reader with a detailed understanding of the key components of the experimental process in state-of-the-art breath BC marker research that may affect the results of experimental studies: Different methods of breath sample collection with their strength and limitations are discussed below; methods of analytical breath VOC analysis, together with selected studies, are presented in section "Chemical Analysis: Identification of Specific BC Marker VOCs;" and sensor arrays that were developed for and/or have been applied to BC marker breath printing will be described in section "Sensor Arrays for BC Marker Breath Printing."

Breath Collection, Sample Storage, and Possible Sources of Contamination

Exhaled breath samples are rather delicate. Special attention should be paid during sample collection and storage:(1) to preserve the highly volatile disease markers and (2) to avoid contamination with confounding or environmental VOCs from external sources. The study of biomarkers in exhaled breath still suffers from a lack of standardization of the breath collection and analysis. Amann and coworkers have recently proposed a standardization of the breath collection process that might be generally accepted in the future [69]. Several different procedures of direct and indirect breath collection are currently being used. Sampling procedures include, but are not limited to, mixed expiratory breath collection, end-tidal breath collected with CO2-controlled sampling [36], sampling with Tedlar or Mylar bags [41], and portable breath collection apparatus (BCA), which was developed and used by Phillips et al. [70].

Direct and Indirect Breath Sampling

During direct breath sampling, air exhaled by the subject is introduced into the measuring system without any intermediate steps [47]. This approach would be most convenient for a future clinical device. Breath print analyzers based on sensor arrays could, in principle, be adapted to direct sampling. In contrast, direct sampling cannot be used in combination with the most important method of chemical analysis (GC-MS). During indirect sampling, the exhaled air is stored on an adequate medium and analyzed later. Indirect sampling is still by far more widely used method in research settings, both for chemical analysis of breath and sensor array studies.

Several VOC collection media for indirect breath sampling are being used: bags of Tedlar, Mylar, or other (almost) chemically inert, low-emission plastic materials, empty glass vials, and stainless steel containers or glass cartridges containing adsorbent substances (so-called adsorbent traps). Tedlar bags and sorbent traps are currently most widely used [65]. Adsorbent traps are commercially available as thin glass or stainless steel tubes containing a single resin or resin mixture. Resins that can adsorb VOCs include, for example, carboxene, Tenax® TA, and Tenax® GR, which is a composite material of Tenax® TA and 30 % graphite. The selection should be made according to material parameters such as breakthrough volume and retention time [65]. Breath VOCs can be trapped at room temperature, if the retention volume for the compounds of interest is sufficiently high to prevent VOCs from being released during collection. Since the retention volume decreases strongly with temperature, the trapped VOCs can be fully and immediately thermally desorbed from the adsorbent trap at elevated temperatures around 200–300 °C, allowing for sample analysis several days or even weeks after sample collection.

Adsorbent traps offer numerous advantages over sample bags, for example, higher sample storage stability, easier transport, and preconcentration of the breath VOCs. The latter enhances the ability of the analytical equipment to detect VOCs at very low concentrations (typically of the order of magnitude of ppbv). However, while bags can be easily filled by the test person during exhalation, adsorbent traps offer such a high resistance to air transit that filling a pump device is required for pushing exhaled air into the cartridge [65]. This methodology is rather complex and may affect the overall reliability of the collection procedure. Hence, adsorbent traps are often used in combination with collection bags: The breath sample is initially collected into a bag, while the test person exhales. The content of the bag is then transferred immediately after the breath collection into the adsorbent trap, using a syringe or an electric pump. This method combines the advantages of bags and adsorbent tubes and does not require the use of specific appara-

tuses. Other methods of pre-concentrating VOCs from breath samples in collection bags are available as well, for example, solid-phase microextraction (SPME) which uses a fused silica optical fiber coated with a thin film polymeric stationary phase or a mixture of polymers and is based on the preferential partitioning of the VOCs by adsorption from the gas phase or from the solution to the stationary phase. However, the intermediate steps of sample transfer and pre-concentration may increase the risk of information loss and/or external contamination.

It is especially challenging to avoid exogenous VOCs that are exhaled by the test person during the collection process, after being adsorbed to the body via previous inhalation, ingestion, or skin contact. Exogenous VOCs or their metabolic products are exhaled either immediately (e.g., highly volatile room air contaminants in a hospital environment) or within a short period of time of 1–2 h (e.g., some ingredients of coffee, food, or cigarette smoke); or they are stored in the body's fatty tissue and are released over an extended period of weeks or even years, depending on each VOC's vapor pressure and alveolar gradient (namely, the difference between the amounts of each VOC in breath and in the room air).

Figure 23.2 shows a schematic representation of a breath collection apparatus for alveolar air that is designed to minimize sample contamination during the collection process, in which the inhaled air is cleared of such ambient contaminants that are exhaled immediately, using a so-called lung washout. During this procedure, the test person inhales repeatedly to total lung capacity for 3 min through a mouthpiece with a filter cartridge on the inspiratory port mouthpiece (can be obtained, e.g., from Eco Medics, Duerten, Switzerland). It was shown that the lung washout greatly reduces the concentration of exogenous VOCs [14, 32]. Following the lung washout, subjects inhaled to full lung capacity and exhaled slowly through the mouthpiece into a separate exhalation port against 10–15 cm H_2O pressure. This ensures the closure of the vellum in order to exclude contamination through nasal entrainment. Exhaled breath consists of respiratory dead space air that is exhaled first (i.e., the volume of air which is inhaled, but does not take part in gas exchange region of the lung), followed by the alveolar air from the lungs. The collection apparatus in Fig. 23.2 automatically fills the dead space into a designated dead space bag that can later be removed. Although the dead space air is usually not analyzed for VOCs, it should be taken into consideration that certain gases exchange in the airways, rather than the alveoli, depending on the blood/air partition coefficient, λb:a. For example, highly

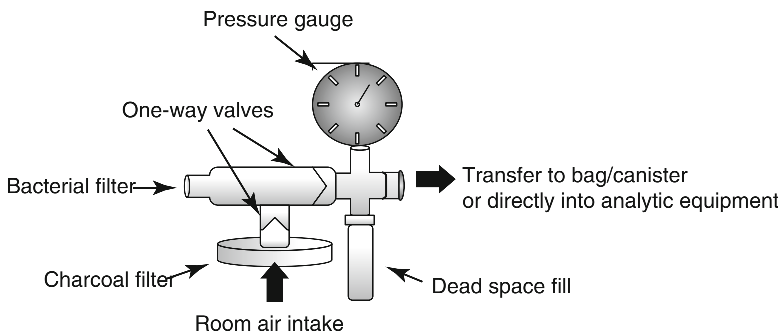


Fig. 23.2 Example of a breath collection apparatus. The system allows performing a lung washout from highly volatile room air contaminants prior to the collection of the breath sample, whereby the test person inhales several times through a charcoal filter. Thereafter, the test person exhales through a bacterial filter. The system automatically separates alveolar air and air from the respiratory dead space. A pressure gauge ensures closure of the vellum. The sampled alveolar air can be collected in an inert bag and canister or transferred directly into the instrument. The system was used in several studies [12, 14]

blood-soluble, polar VOCs tend to exchange in the airways [59]. The alveolar breath from the end of the exhalation can be sampled indirectly into an inert bag or canister or directly into the analyzing equipment.

Exogenous contaminants with intermediate release times from nutrition, smoking, medication, or body care products can be minimized by following a breath collection protocol. For example, the test persons can be instructed to fast for 1–2 h prior to the breath collection, to refrain from smoking, drinking coffee, taking medication, using perfume, etc. However, some contaminants with longer release times, mainly those originating in cigarette smoke or from continuous uptake of a certain VOC through long-term occupational exposure, cannot be avoided altogether [8]. Indeed, some of these VOCs are known carcinogens and may be utilized, for example, as exogenous markers of developing BC.

Chemical Analysis: Identification of Specific BC Marker VOCs

Over the past three decades, hundreds of studies have addressed the identification and quantification of a wide variety of separate breath and headspace VOCs. GC-MS is the gold standard for determining the composition of breath samples and has been used in most studies on BC marker VOCs [14–17, 19]. The gas chromatograph separates the VOCs according to their volatility: The sample is carried in a helium stream through a long, heated capillary column, whereby more volatile compounds travel faster than less volatile ones. The separating ability of the GC depends on the column's dimensions (length, diameter, film thickness). The retention time in the column is a measure for the volatility. The mass spectrometer determines molecular mass and chemical structure of the breath VOCs, after they have been broken up into characteristic fragments and ionized. In the mass analyzer, the ions are filtered by an electric field according to their mass charge ratio (*m*/*e*). The range of masses can be adjusted to the compounds of interest or, on the other hand, known contaminants can be excluded. The compounds are identified according to the masses of their fragments and their retention times in the GC column.

Combining GC and MS reduces the possibility of error considerably, as it is extremely unlikely that two different molecules have the same mass and the same retention time. Tentative compound identification can be achieved through spectral library match, using tabulated values from the literature. However, verification of compound identity can only be achieved experimentally, through calibration of the actual GC-MS instrument for each compound of interest, using highly pure laboratory standards.

Although GC-MS yields highly accurate results and presents a wealth of information for basic research, the method has several prominent disadvantages for use as a clinical point-of-care application (see Table 23.1). First and foremost, it still requires sophisticated, expensive, equipment that would only be available in large, well-equipped laboratories. The first GC-MS instruments were slow and bulky, but speed and sensitivity have been greatly improved during the past decades, and miniaturized equipment for limited, well-defined applications will most probably become available during the next decade [31]. The second setback of GC-MS lies in the high expertise that is required to interpret the GC-MS raw data. Third, the analysis of breath VOCs at ppbv/pptv concentrations requires pre-concentration prior to GC-MS—for example, onto solid-phase microextraction fibers or to other suitable absorption media [31] or by cryo-focusing [53], as described earlier in the section on breath collection. The pre-concentration methods complicate the overall experimental procedure. Furthermore, they selectively enhance the signals of certain VOCs, while potentially missing others. All in all, the method would be too time-consuming and expensive for clinical application in high-throughput LC diagnosis and screening and does not allow direct breath sampling.

Empirical data from several GC-MS studies on BC marker VOCs in exhaled breath have been reported [15–19]. Figure 23.3 shows the results of such a study in form of surface plots [16].

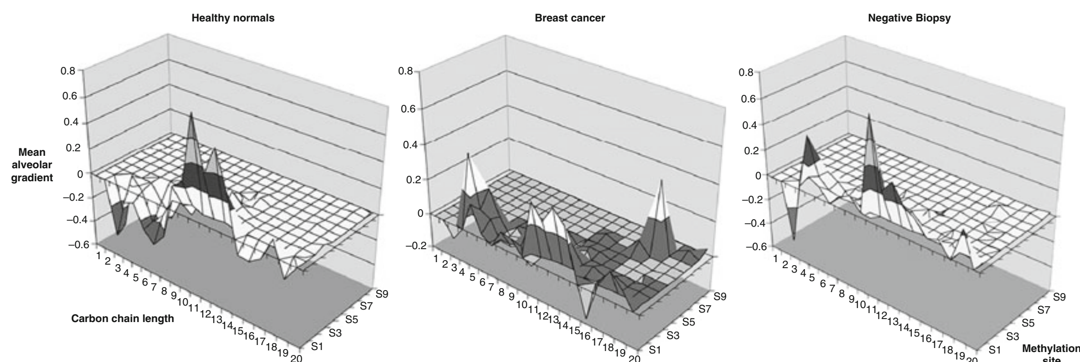


Fig. 23.3 Surface plots of VOCs in exhaled breath. Surface plots showing the distribution of BMAC VOCs in exhaled breath of three investigated groups: 51 women with biopsy-proven breast cancer, 50 age-matched women with no histologic evidence of breast cancer in a breast biopsy, and a third group of 42 healthy volunteers. The *x*-axis represents carbon chain lengths, the mean alveolar gradients (abundance in breath minus abundance in room air) of C4–C20 alkanes, and their monomethylated derivatives are represented in the *y*-axis and the methylation site is shown on the *z*-axis. Eight compounds were able to identify women with breast cancer with sensitivity of 88.2 % and specificity of 73.8 % after cross-validation, whereas the diagnostic cutoff was designed. *BMAC* breath methylated alkane contour, *VOC* volatile organic compound (Reproduced with permission from Phillips et al. [16]. Copyright © 2003, John Wiley and Sons)

Breath samples were analyzed by GC-MS in order to determine the distribution of breath methylated alkane contour (BMAC) in three investigated groups: 51 women with biopsy-proven breast cancer, 50 age-matched women with no histologic evidence of breast cancer in a breast biopsy, and a group of healthy volunteers. Data on smoking status and tumor histology is also available. The x-axis represents carbon chain lengths, the mean alveolar gradients (abundance in breath minus abundance in room air) of C4–C20 alkanes and their monomethylated derivatives are represented in the *y*-axis, and the methylation site is shown on the *z*-axis. Table 23.3 lists the compounds shown to either increase or decrease when comparing the age-matched healthy volunteers to the groups with breast cancer. The designed model exhibits sensitivity of 88.2 % and specificity of 73.8 % after cross-validation, whereas the diagnostic cutoff was designed as the point where the sum of sensitivity and specificity was maximal. When employing statistical analysis to distinguish between women with breast cancer and women without evidence of breast cancer based on mammographic screening, the results were less robust. The model comprised of 10 VOCs exhibits sensitivity of 60.8 % and specificity of 82 % after cross-validation. The expected NPV of the screening breath test was superior to that of a screening mammogram—an important achievement, implying that additional screening mammograms for women with negative breath tests may not provide any additional clinical effect [16].

The biochemical pathways leading to the release of these VOCs in exhaled breath, as understood based on the GC-MS results, are mainly related to oxidative stress. The VOCs tested in this study are reported to be markers of oxidative stress in several studies [8, 16, 18, 71], and since BC is known to be related to oxidative stress and induction of polymorphic cytochrome P450 mixed oxidase enzymes (CYP), these markers may serve as potential volatile markers for the diagnosis of breast cancer. However, this is still subject to controversy until more studies are established and a larger population is tested. Nevertheless, the optimal approach to determine the biochemical pathways for the production of the BC VOCs would be to compare VOC profiles from different sources (organs or clinical samples) in the same BC patient and/or the same animal model. However, many technical challenges have been hindering the implementation of such an approach. Despite the inconsistency in experimental methods, interesting conclusions

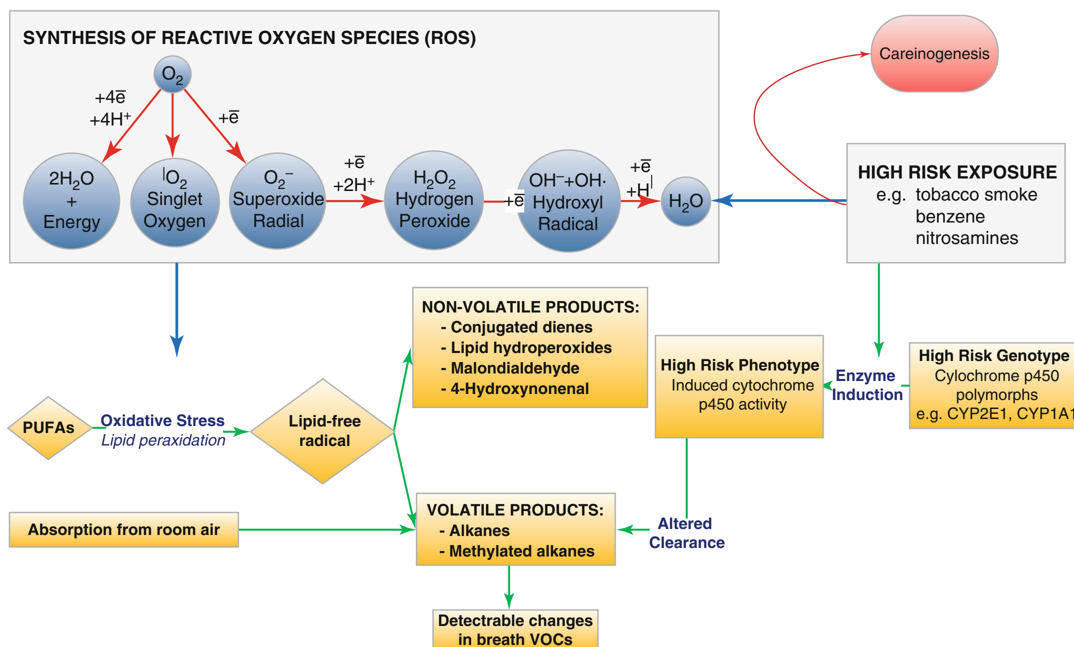


Fig. 23.4 Hypothetical biochemical origin of the exhaled BC marker VOCs: BC may result from the interaction of hereditary and environmental factors. Several cytochrome p450 mixed oxidases are activated by exposure to environmental toxins such as tobacco smoke and radiation. The induced phenotype may increase the BC risk due to increased conversion of precursors to carcinogens. An altered pattern of cytochrome p450 mixed oxidase activity could potentially modulate catabolism of endogenous VOC products of oxidative stress and generate an altered pattern of breath VOCs. *BC* breast cancer, *VOC* volatile organic compound (Reprinted with permission from Hakim et al. [8]. Copyright © 2012 American Chemical Society)

could be drawn from the meta-study about possible biochemical pathways like what was recently published for lung cancer (see Fig. 23.4) [8] Furthermore, statistically significant differences between the VOCs of some alkanes, alkenes, and aromatic compounds were observed when directly compared with the breath of BC patients and the breath of patients with other cancer diseases (namely, lung, colorectal, and prostate cancer) [14].

Sensor Arrays for BC Marker Breath Printing

Direct breath printing using sensor arrays is better suited for clinical applications than breath analysis. Implementations of sensor arrays for breath printing can be quite diverse. The sensors should meet the following requirements: Since the sensor arrays would be exposed directly to the breath samples in an anticipated future clinical application, the constituent sensors should be sensitive to very low concentrations of the VOCs in exhaled breath in the presence of water vapor, because breath samples are fully humidified. Secondly, each sensor should respond rapidly to small changes in the concentrations of the BC-specific breath VOCs, so that the sensor array output is specific to a given disease state. Ideally, the sensors should relax rapidly to their baseline states when removed from the breath sample. Alternatively, disposable sensor arrays could be used, if the device fabrication is reproducible and simple enough that large quantities of identical units could be manufactured at acceptable costs.

Studies of BC breath prints have used chemiresistor sensors with electronic transduction mechanisms (see Table 23.4). Chemiresistors are simple electronic devices that consist of a chemiresistive material between two metal electrodes (see Fig. 23.5 in Table 23.4). The electric

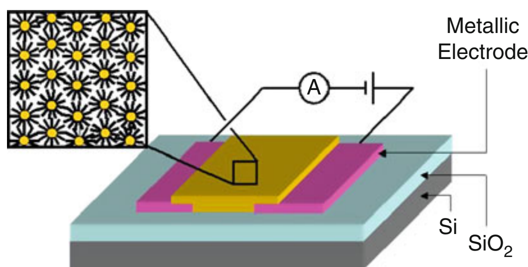


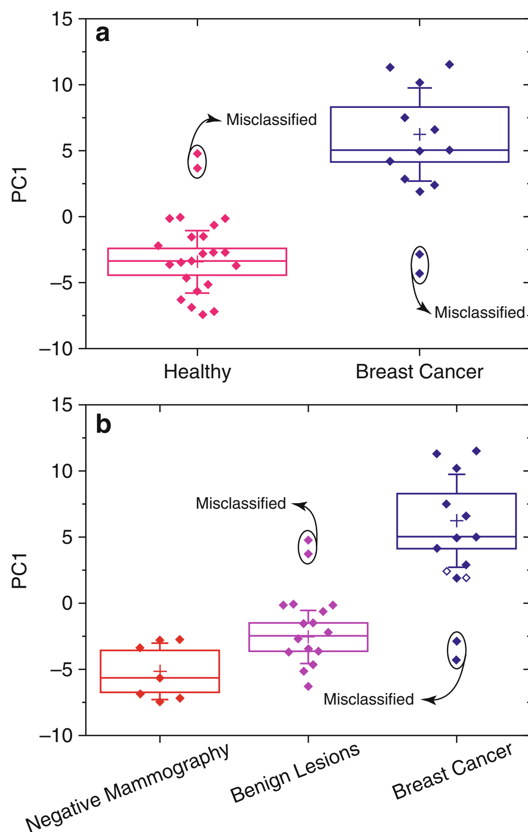
Fig. 23.5 Example of a chemiresistor consisting of a chemiresistive material between two metal electrodes

resistance of a chemiresistor varies when its active material interacts with the breath VOCs. The change of the resistance upon exposure to the breath sample can easily be probed, either by applying a constant DC bias, V, and monitoring the current change, ΔI, or, alternatively, by supplying a constant current, I, and measuring the change in voltage drop, ΔV, across the chemiresistor. Chemiresistors are very attractive for breath printing applications, because of their simplicity, ease of fabrication and use, small size and weight, fast response, and reliability. Furthermore, automatic packaging of sensor arrays at wafer level, on-chip integration, and mass production of portable systems with integrated read-out electronics are easily possible at low cost [30, 72].

Incorporating nanomaterials into chemiresistors may help to overcome many limitations of bulk sensing layers. Nanomaterials offer several important advantages for sensing applications. Most importantly, their small characteristic dimensions (1–100 nm) increase the active surface-to-volume ratio and generate novel interfaces, yielding excellent sensitivity as well as rapid response and recovery times. In addition, nanomaterials offer high flexibility in their chemical and physical properties, which can be tailored to achieve unusual target-binding properties, including a reduced sensitivity to water molecules. This is especially attractive for the sensing of breath VOCs. This section focuses on sensors comprising layers of gold or platinum nanoparticles (GNPs and PtNPs, respectively; see Fig. 23.5). In these films, the inorganic nanomaterials provide the electric conductivity, and the organic film component provides sites for the sorption of VOCs. Gold and platinum are preferred choices of NP metal cores because of their chemical inertness.

GNP and PtNP films exhibit two countering effects during the adsorption of VOCs: a three-dimensional swelling of the film, which increases the interparticle tunneling distance for charge carriers and, hence, the film resistance, and an increase in the permittivity of the organic matrix around the metal cores which decreases the potential barriers between the metal cores and, consequently, the film resistance. These two mechanisms enable the metal NP layer to sense the breath VOCs for BC marker breath printing [12, 14]. The sensitivity of the sensor array can be tuned depending on the choice of the organic ligand through the variety of available ones (e.g., alkylthiols, alkylamines, para-thiophenols, carboxylates, organodithiols, etc.). Different combinations of GNP and GNP+PtNP sensors have been used to derive breath prints for breast malignancy, distinction from other cancers, and benign breast deficiency (as shown in Table 23.4), with impressive values for sensitivities and specificities (in Table 23.4 and references [12] and [14]). Figure 23.6a shows an example for breath print BC markers for correctly classifying breast cancer patients (malignant lesions) and healthy controls (including negative mammography and benign conditions). The sensor arrays could also distinguish patterns of breast malignancy individuals among patients with benign breast conditions and negative mammography healthy controls (see Fig. 23.6b). Furthermore, Fig. 23.7 shows that the collective response of GNP sensor array is also able to successfully map distinct patterns for breast cancer patients from other malignancies (lung, colon, and prostate).

All GNP and PtNP sensors in these studies have been tested under laboratory conditions prior to their application in clinical studies to ensure that they have sufficient detection limits, sensitivities, resolutions, and dynamic ranges for the very low concentrations of the VOCs in the breath of patients with lung cancer. The sensors



Figs. 23.6 Examples for breath print BC markers for diagnosing cancer malignancy from benign and healthy states (derived from exhaled VOC patterns, using sensor arrays comprising GNP chemiresistors). Graphical representation of the PC1 values from the six-sensor NA-NOSE for (**a**) healthy controls and breast cancer individuals, (**b**) for the healthy subpopulations with negative mammography and with benign breast conditions, and the breast cancer subgroups with DCIS (*open symbols*) and IDC (*full symbols*). Each point represents one patient. The positions of the PC1 mean values are marked with, the boxes correspond to their 95 % confidence limits, and the error bars corresponds to the standard deviation of PC1. The four misclassified individuals that are marked in the graphs were not considered for the statistical analysis. *BC* breast cancer, *VOC* volatile organic compound, *GNP* gold nanoparticle, *PC* principal component, *NA-NOSE* nanoscale artificial NOSE, *DCIS* ductal carcinoma in situ, *IDC* infiltrating ductal carcinoma (With kind permission from Springer Science + Business Media: Shuster et al. [12]. Copyright © Springer Science + Business Media, LLC)

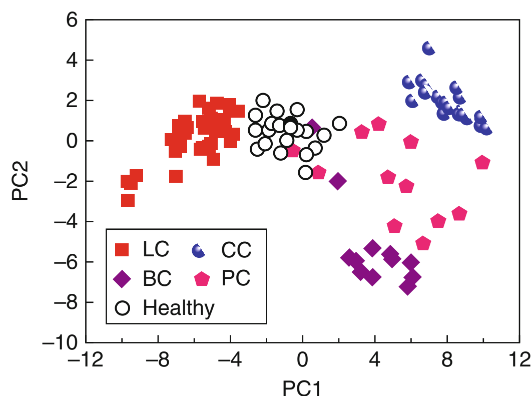


Fig. 23.7 Examples for breath print BC markers for diagnosing breast cancer malignancy from other malignancies (derived from exhaled VOC patterns, using sensor arrays comprising GNP chemiresistors). Graphical representation of the PC1 and PC2 values from the six-sensor NA-NOSE for all malignancies and healthy state. Each point represents one patient. The first two principal components depicted contained >88 % of the total variance in the data. *NA-NOSE* nanoscale artificial NOSE, *PC* principal component (Reprinted by permission from Macmillan Publishers Ltd. on behalf of Cancer Research UK: by Peng et al. [35]. Copyright © 2010 Nature Publishing Group)

all had low, well-defined responses to water vapor. It is particularly relevant that the same breath samples were analyzed both with GC-MS and with sensor arrays [14]. The breath VOCs identified by GC-MS were used to optimize the sensor arrays and validated the sensor arrays' results by considerably reducing the risk of false-positive sample identification.

Finally, arrays that incorporate GNPs and PtNPs can be designed in such a way that they are insensitive to important confounding factors, which could be relevant to future diagnostic breath printing. Figure 23.8 illustrates the stability of the breath prints against some important confounding factors, including age, gender, family cancer history, place of birth, ethnicity, smoking habits, work pollution, and consumption of food additives, among a population of 52 healthy subjects.

Nanomaterial-based chemiresistors seem to have a realistic potential of becoming the preferred choice in future BC breath printing, based on their excellent performance in laboratory settings that were demonstrated by the presented pilot studies.

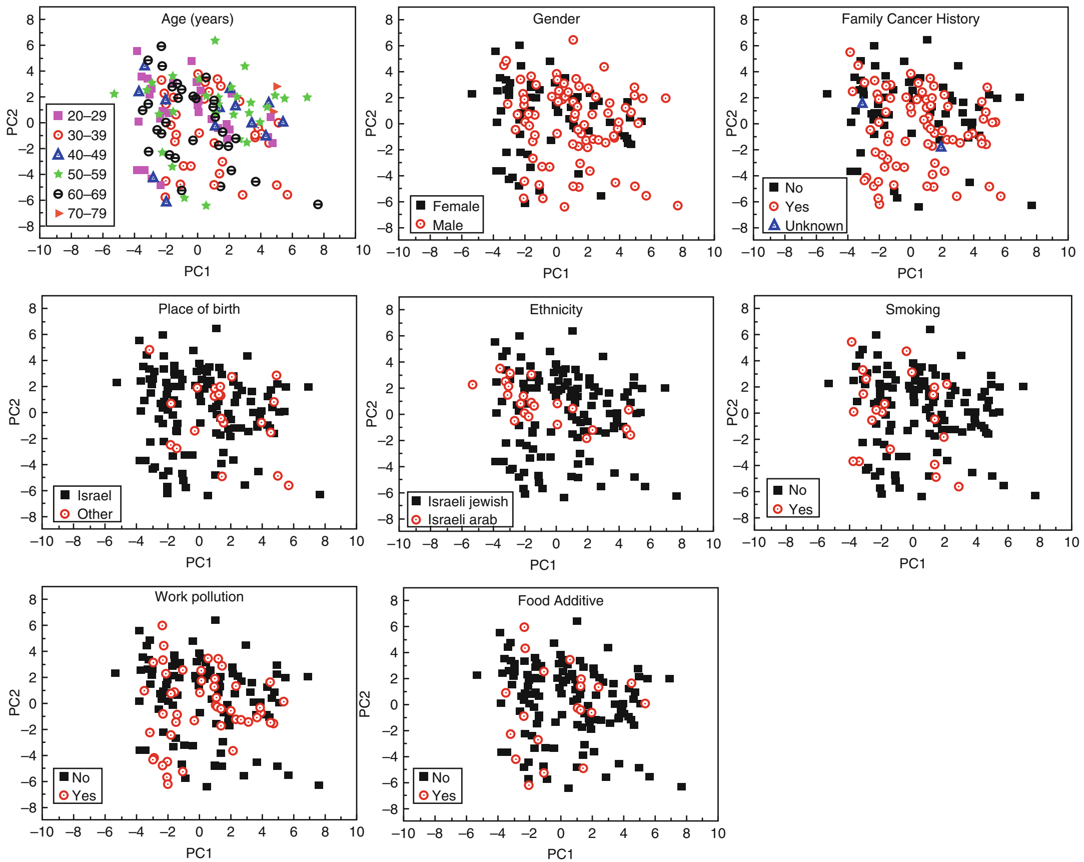


Fig. 23.8 Stability of the breath prints obtained by sensor arrays against confounding factors. PCA plots of a sensor array comprising GNP chemiresistors, exposed to VOCs in the breath of 52 healthy subjects. Two breath samples were analyzed per test person; each point in the plot represents one breath sample. The plots were analyzed according to important confounding factors that could be relevant to future diagnostic breath testing: age, gender, family cancer history, place of birth, ethnicity, smoking habits, work pollution, and consumption of food additives. *GNP* gold nanoparticle, *PCA* principal component analysis, *VOC* volatile organic compound (Reprinted by permission from Macmillan Publishers Ltd. on behalf of Cancer Research UK: by Hakim et al. [51]. Copyright © 2011 Nature Publishing Group)

Conclusion and Future Perspective

Exhaled breath VOCs hold great potential for providing a new class of molecular BC markers. Either BC-specific concentration profiles of specific breath VOCs or breath prints from collective VOC patterns can be used to establish different markers for early BC, for different pheno- and genotypes, for BC risk in healthy individuals, and for BC treatment response. BC-specific VOC profiles may represent products of metabolic tumor activity or by-products of local inflammation in and around the tumor. Those VOC profiles could also contain partially reemitted environmental toxins and/or could be of systemic origin. Both breath prints and individual VOCs have been studied as markers of BC and of different BC phenotypes in numerous proof-of-concept studies. However, this research effort has not yet resulted in the establishment of reliable BC markers for clinical use. Insufficient attention to confounding factors in the preselected study population of small studies and inconsistencies in the experimental techniques used for breath collection, sample storage, breath gas analysis, and sensor-based breath printing, as well as disparities in the statistical

analysis of the experimental data, have impeded any clinical impact of the BC breath VOC marker research. Issues of standardization have to be prioritized in order to progress the field.

Breath printing by sensor arrays is better suited than breath gas analysis for real-world clinical applications because it is fast and potentially cost-effective and allows direct breath sampling with online results. Arrays of nanomaterial-based chemiresistors and colorimetric sensor arrays are best suited for breath printing. This noninvasive approach has a realistic potential for becoming an integral part of population-based breast cancer screening, posttreatment follow-up, and personalized breast cancer management in the near future, even though the technique is not yet mature enough for imminent clinical use.

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24 Breast Cancer Therapy–Classical Therapy, Drug Targets, and Targeted Therapy

Eugenia Ch Yiannakopoulou

Abstract

Therapeutic options for breast cancer treatment include chemotherapy, endocrine therapy, and novel targeted therapy. In the adjuvant setting, chemotherapy is indicated for early breast cancer treatment based on tumor stage, histological grading, estrogen and progesterone receptor, proliferative index, and human epidermal growth factor receptor. In the metastatic setting, based on National Comprehensive Cancer Network (NCCN) guidelines, systemic chemotherapy is recommended for patients with symptomatic visceral involvement due to metastatic disease. Currently, the taxanes and anthracyclines represent the most potent drugs for use in breast cancer, including adjuvant, neoadjuvant, and metastatic settings. Endocrine treatment is indicated for the treatment of women with estrogen receptor-positive breast cancer and includes tamoxifen, aromatase inhibitors, fulvestrant, LHRH agonists, and progestins. The selective estrogen receptor modulator tamoxifen is indicated for the treatment of both premenopausal and postmenopausal women, for the treatment of male breast cancer, and for the chemoprophylaxis of high-risk women with breast cancer. Third-generation aromatase inhibitors exemestane, anastrozole, and letrozole are indicated for the treatment of postmenopausal women. Fulvestrant, a selective estrogen receptor downregulator that behaves as a complete antagonist, is indicated for the treatment of metastatic breast cancer. LHRH agonists goserelin and triptorelin are indicated for premenopausal women with hormone-positive breast cancer. Progestins megestrol acetate and medroxyprogesterone acetate are indicated for the treatment of metastatic breast cancer.

Novel-targeted treatment includes HER2 inhibitors, antiangiogenetic agents, and mTOR inhibitors. Trastuzumab is a humanized anti-ErbB2 monoclonal antibody that has been approved for the treatment of breast

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cancer with ErbB2 overexpression, or ErbB2 gene amplification, in the adjuvant and the metastatic setting. Lapatinib is a small molecule and a potent, reversible, dual inhibitor of the tyrosine kinase domains of both EGFR and ErbB2 currently approved for use in combination with capecitabine in the treatment of advanced breast cancer overexpressing HER2. Bevacizumab, the first approved antiangiogenetic agent for the treatment of human cancer, in combination with chemotherapy has provided substantial benefit for the treatment of metastatic breast cancer in terms of improving progression-free survival and objective response rate. Oral everolimus (Afinitor®) in combination with exemestane is indicated for the treatment of hormone receptor-positive, human epidermal growth factor receptor (HER)2-negative advanced breast cancer in postmenopausal women after the failure of treatment with letrozole or anastrozole (in the USA) or after recurrence of progression following a nonsteroidal aromatase inhibitor in women without symptomatic visceral disease (in the EU).

Keywords
Breast cancer • Chemotherapy • Endocrine therapy • Tamoxifen • Aromatase inhibitors • Targeted therapy

Introduction

Breast cancer is one of the leading causes of cancer death for women. It is estimated that 1.2 million new breast cancer cases are diagnosed annually worldwide. Undoubtedly, breast cancer treatment has been improved since the last decades. However, despite advances in breast cancer treatment, mortality from breast cancer is still high. Breast cancer treatment includes surgery, medical therapy, and radiotherapy. Medical therapy is indicated in the neoadjuvant, adjuvant, and metastatic settings. Medical therapy for breast cancer treatment includes chemotherapy, endocrine therapy, and novel targeted therapy. The taxanes and anthracyclines represent the most potent drugs for use in breast cancer, including adjuvant, neoadjuvant, and metastatic settings. Endocrine treatment is indicated for the treatment of women with estrogen receptor-positive breast cancer and includes tamoxifen, aromatase inhibitors, fulvestrant, LHRH agonists, and progestins. Novel targeted treatment includes HER2 inhibitors, antiangiogenetic agents, and mTOR inhibitors.

Chemotherapy

Anthracyclines, capecitabine, taxanes, and epothilones are the main chemotherapeutic agents used in the treatment of breast cancer (Table 24.1). Combination therapy using regimens such as CMF (cyclophosphamide, methotrexate, 5-FU), CEF (cyclophosphamide, epirubicin, 5-FU), or CA(F) (cyclophosphamide, doxorubicin, [5-FU]), or regimens including vinorelbine or a taxane, is commonly used as a first-line adjuvant breast cancer treatment. However, currently, the taxanes and anthracyclines represent the most potent drugs for use in breast cancer, including adjuvant, neoadjuvant, and metastatic settings. The addition of adjuvant chemotherapy in early breast cancer improves overall survival by approximately 10 % [1]. In early breast cancer, the molecular subtypes luminal A and B and basal like are important for planning adjuvant systemic therapy. Prognostic and predictive markers, such as hormone receptor status, HER2, Ki-67, uPA/PAI-1, or multiple gene tests, such as Oncotype DX®, currently allow avoidance of an over-therapy or

Table 24.1 Breast cancer chemotherapy: drug classes and agents

Drug class	Agents
Alkylating agents	Cyclophosphamide
Antimetabolites	5-Fluouracil (5-FU), methotrexate, capecitabine, gemcitabine
Platinum salts	Cisplatin, carboplatin, oxaliplatin
Anthracyclines	Daunorubicin, doxorubicin, idarubicin, epirubicin, mitoxantrone
Topoisomerase I inhibitors	Irinotecan
Topoisomerase II inhibitors	Etoposide
Vinca alkaloids	Vinorelbine
Taxanes	Docetaxel, paclitaxel
Epothilones	Ixabepilone

under-therapy [2]. According to St. Gallen guidelines, luminal B (triple positive), HER2, and triple-negative subtypes should receive both anthracyclines and taxanes. Thus, recommendations favor the use of anthracyclines and taxanes in patients with luminal B disease (triple positive), while the use of an anthracycline, taxane, and alkylating agent is recommended in triple-negative disease [1] Molecular subtype luminal A (ER/PR positive, HER2 negative) is the least sensitive in adjuvant chemotherapy. According to international guidelines, in general, adjuvant chemotherapy is not recommended for patients with pT1, pN0, ER-positive, low proliferation index, HER2-negative breast cancer [3]. On the contrary, it is particularly indicated in at least one of the following biological conditions: ER negative, HER2 positive, high proliferative index (>30 %), and grading 3 [4]. However, it remains unclear which is the role of adjuvant chemotherapy in endocrine-sensitive breast cancer with intermediate recurrence risk (tumor diameter of 2 cm or more or node positive from one to three lymph nodes), grading 2, and a proliferation index below 30 % [4].

In the metastatic setting, based on National Comprehensive Cancer Network (NCCN) guidelines, systemic chemotherapy is recommended for patients with symptomatic visceral involvement due to metastatic disease such as the liver, lung, and bone marrow. However, toxicity remains a limitation of breast cancer treatment, as shown in Table 24.2.

Table 24.2 Breast cancer chemotherapy and systemic toxicities

Agent	Main toxicities
Anthracyclines	Cardiotoxicity, myelosuppression
Capecitabine	Hyperbilirubinemia, diarrhea, hand-foot syndrome
Taxanes	Neurotoxicity, neutropenia
Epothilones	Neutropenia, peripheral neuropathy

Anthracyclines

Anthracyclines include daunorubicin, doxorubicin, idarubicin, epirubicin, and mitoxantrone. The anthracyclines commonly used in treatment of breast cancer are either epirubicin or doxorubicin. Epirubicin is an epimer of doxorubicin with an important role in the chemotherapy treatment of both early and metastatic breast cancer [5]. The efficacy of epirubicin is similar to doxorubicin, while epirubicin has a different toxicity profile, particularly in regard to cardiotoxicity. The major side effects of anthracyclines are cardiotoxicity and myelosuppression. Doxorubicin is the most extensively studied anthracycline regarding cardiotoxicity [6]. Reported rare cardiac complications of doxorubicin treatment include acute reversible arrhythmias, subacute toxic myocarditis, and pericarditis. The most thoroughly evaluated cardiotoxicity of doxorubicin is cumulative progressive damage that leads to clinical events ranging from light reduction of left ventricular ejection fraction to irreversible life-threatening congestive cardiac failure [6]. Congestive cardiac failure due to anthracycline appears early, i.e., several days to months after treatment, peaking at 3 months following the last dose, or late appearing up to 10–12 years after the last anthracycline dose. Risk factors for anthracycline cardiotoxicity include cumulative dose (the strongest risk factor), age, prior irradiation, concomitant administration of other drugs, and previous history of drug disease. A maximum cumulative tolerant dose of 450–500 mg/m^2 has been recommended.

Since the maximum cumulative dose in most breast cancer chemotherapeutic regimens ranges from 240 to 300 mg/m^2, breast cancer patients seem not to be affected by doxorubicin cardiotoxicity. Liposomal doxorubicin has been shown to have less cardiotoxicity. Epirubicin has been reported to have lower cardiotoxicity than doxorubicin, and a cumulative tolerable dose of 900–1,000 mg/m^2 has been suggested [6].

Epirubicin has been incorporated into most of the anthracycline-containing chemotherapy combinations in well-conducted clinical trials involving large numbers of patients. It has also been investigated in studies involving the administration of epirubicin in dose-dense chemotherapy schedules. Short-term follow-up of dose-dense clinical trials demonstrated safety comparable to that of doxorubicin.

Capecitabine

Capecitabine is an oral prodrug of 5′-deoxy-5-fluorouridine (5′-DFUR). It is converted to 5-FU preferentially in tumor tissue through exploitation of high intratumoral concentrations of thymidine phosphorylase. After oral administration, capecitabine passes intact through the intestine, thus avoiding the direct release of 5-FU within the gastrointestinal tract. Capecitabine subsequently undergoes a three-step enzymatic conversion, the final stage of which relies on thymidine phosphorylase. This enzyme is overexpressed in a proportion of tumor tissues compared with normal tissues, thus resulting in the generation of 5-FU preferentially at the tumor site and decreasing systemic exposure to 5-FU. Bioavailability after oral administration is close to 100 % [7].

Capecitabine is effective and adequately tolerated, both as a monotherapy and as an addition to intravenous polychemotherapeutic to treat several types of cancers. Furthermore, the combination of capecitabine with taxanes is appropriate, because taxanes show synergistic effects with capecitabine. Capecitabine is effective for salvage treatment of patients with metastatic breast cancer. Thus, according to the Breast Cancer Guidelines Committee of the NCCN, capecitabine/docetaxel is considered a preferred combined chemotherapy regimen for recurrent or metastatic breast cancer after failure of anthracycline-based chemotherapy. The drug has also been approved for use as a single agent in metastatic breast cancer patients who are resistant to both anthracycline- and paclitaxel-based regimens or in whom further anthracycline treatment is contraindicated.

In addition, capecitabine has also been used in neoadjuvant breast cancer chemotherapy, with conflicting results. A very recently published meta-analysis including five trials of 3,257 patients with early or operable breast cancer without distant metastasis, treated in the neoadjuvant setting with capecitabine and anthracycline- and/or taxane-based therapy, found that adding capecitabine to neoadjuvant chemotherapy regimens is unlikely to improve outcomes in breast cancer patients without distant metastasis [8].

The most common dose-limiting adverse effects associated with capecitabine monotherapy are hyperbilirubinemia, diarrhea, and hand-foot syndrome. Myelosuppression, fatigue and weakness, abdominal pain, and nausea have also been reported. The dose of capecitabine approved by the US Food and Drug Administration (FDA) for both metastatic colorectal and breast cancer is 1,250 mg/m^2 given orally twice per day, usually separated by 12 h for the first 2 weeks of every 3-week cycle.

Taxanes

The taxanes docetaxel and paclitaxel were initially introduced in metastatic breast cancer treatment. The taxanes exert their cytotoxicity through tubulin stabilization and cell cycle arrest [9]. They have also been shown to promote apoptosis, inhibit angiogenesis, and induce genes that mediate diverse cellular processes. Although in general the mode of action is similar, docetaxel has a longer plasma half-life and longer intracellular retention, in addition to greater potency in promotion of tubulin assembly and microtubule stabilization compared with paclitaxel. The major

toxicities include neurotoxicity for paclitaxel, peripheral neuropathy with docetaxel, and neutropenia, which appears to be more prevalent with docetaxel than with paclitaxel. Other important toxicities include hypersensitivity reactions for paclitaxel and fluid retention and gastroenteric toxicity for docetaxel. In addition, asthenia, alopecia, and mouth ulcers are common adverse events with both agents. Taxane resistance, defined as progression while on therapy or ≤12 months in the adjuvant setting or 4 months in the metastatic setting, is a common problem.

Epothilones

Epothilones are cytotoxic macrolides with a similar mechanism of action to paclitaxel but with the potential advantage of activity in taxane-resistant settings in preclinical models. The antineoplastic activity of epothilones has been attributed to stabilization of microtubules, which results in mitotic arrest at the G2/M transition.

Ixabepilone is a semisynthetic analog of epothilone B designed to optimize the characteristics of its natural precursor. It is characterized by low susceptibility to common mechanisms of tumor resistance, including those mediated by the multidrug resistance protein, P-glycoprotein [10, 11]. In addition, microtubule-stabilizing agents such as ixabepilone prolong activation of the spindle assembly checkpoint, which may promote cancer cell death in mitosis or following mitotic exit. Furthermore, epothilone B analog, ixabepilone, has also been shown to induce apoptosis via a Bcl-2-suppressible pathway that controls a conformational change of the proapoptotic Bax protein. Ixabepilone is metabolized in the liver, and caution should be used when considering patients with liver impairment for therapy with this agent. Ixabepilone exposure is greater in patients with hepatic impairment and those receiving concomitant strong cytochrome P-450 CYP3A4 inhibitors.

In October 2007, ixabepilone was approved by the FDA for the treatment of locally advanced and metastatic breast cancer. Ixabepilone is indicated as monotherapy for the treatment of metastatic or locally advanced breast cancer in patients whose tumors are resistant or refractory to anthracyclines, taxanes, and capecitabine. Ixabepilone is also indicated in combination with capecitabine for the treatment of patients with metastatic or locally advanced breast cancer resistant to treatment with an anthracycline and a taxane or whose cancer is taxane resistant and for whom further anthracycline therapy is contraindicated.

Hematologic toxicities associated with ixabepilone use include neutropenia, leukopenia, anemia, and thrombocytopenia. Neutropenia is the most common toxicity of ixabepilone monotherapy. Dose reductions are recommended in patients who experience severe neutropenia or thrombocytopenia. Ixabepilone is contraindicated in patients with a neutrophil count <1,500 cells/mm^3. Symptoms of peripheral neuropathy such as burning sensation, hyperesthesia, hypoesthesia, paresthesia, discomfort, or neuropathic pain have been reported in clinical trials of ixabepilone. Hypersensitivity reactions to ixabepilone treatment have also been reported. Although ixabepilone is not considered cardiotoxic, caution is recommended in patients with a history of cardiac disease, and ixabepilone should be discontinued in patients who develop cardiac ischemia or impaired cardiac function while on therapy.

Chemotherapy of Metastatic Breast Cancer

Anthracycline- and taxane-based regimens are recommended as first-line treatment of metastatic breast cancer (Table 24.3). However, there are few options for the treatment of patients with anthracycline- and taxane-resistant or taxane-refractory metastatic breast cancer [12]. Single-agent capecitabine is approved for the treatment of patients after failure of anthracyclines and taxanes. Ixabepilone has demonstrated efficacy in patients with metastatic breast cancer resistant to multiple chemotherapeutic agents and is the only agent approved by the FDA as a monotherapy for anthracycline-, taxane-, and capecitabine-resistant metastatic breast cancer [12].

Endocrine Treatment

Endocrine treatment is the oldest targeted treatment of breast cancer. Selective estrogen receptor modulators (SERMs), such as tamoxifen, aromatase inhibitors, and GnRH agonists, are the drugs of choice. GnRH agonists suppress ovarian function, inducing a menopause-like condition in premenopausal women [13]. Fulvestrant and progestins are used in the treatment of metastatic breast cancer (Table 24.4).

Selective Estrogen Receptor Modulators (SERMS)

SERMS are a structurally diverse group of compounds that bind to estrogen α (ERα) and estrogen β (ERβ) receptors and produce estrogen agonist effects in some tissues and estrogen antagonist effects in others. The tissue specificity of SERMS is determined in part by the formation of estrogen receptor-SERM complexes that vary in their ability to activate genes when bound to ERα or ERβ.

Tamoxifen, a synthetic nonsteroidal antiestrogen, is a classical partial agonist and exhibits both species and tissues specificity for inducing either an agonist or antagonist response. In the mouse, tamoxifen is an agonist. In rats and humans, it exhibits partial agonism, i.e., producing antagonist effects in the breast but agonist effects in the vagina and endometrium [14]. Long-term tamoxifen use is associated with a reduced incidence of contralateral breast cancer (antagonist), a reduced incidence of primary breast cancer in high-risk women (antagonist), maintenance of bone density (agonist), and increased risk of endometrial carcinomas (agonist).

The parent drug has weak affinity for the estrogen receptors but undergoes excessive biotransformation catalyzed by phase I and II enzymes into active and inactive metabolites. N-dimethyl-tamoxifen is the primary metabolite formed via CYP3A4/5. N-dimethyl-tamoxifen is a weak antiestrogen, but it is subsequently metabolized into a-hydroxy-tamoxifen, N-didesmethyl-tamoxifen, and 4-hydroxy-N-desmethyl-tamoxifen (known as endoxifen). 4-hydroxy-tamoxifen is a minor primary metabolite whose production is catalyzed by multiple enzymes including CYP2D6. Endoxifen and 4-hydroxy-tamoxifen each have at least tenfold higher affinity for estrogen receptors than tamoxifen and are associated with equivalent antiestrogenic potency. In patients receiving chronic tamoxifen therapy, endoxifen is found in serum concentration 6- to 12-fold higher than 4-hydroxy-tamoxifen. Thus, it is suggested that endoxifen is

Table 24.3 Metastatic breast cancer—possible chemotherapeutic options

Combined chemotherapy	Monochemotherapy
Taxanes–anthracyclines	Anthracyclines
Oxaliplatin–gemcitabine	Taxanes
Docetaxel–vinorelbine	Eribulin
Docetaxel–capecitabine	Vinorelbine
Vinorelbine–capecitabine	Capecitabine
Paclitaxel–gemcitabine	Platinum salts
	Cyclophosphamide
	5-FU

Table 24.4 Breast cancer hormonotherapy—drug classes other than aromatase inhibitors

Drug class	Agent	Indication
SERM	Tamoxifen	Breast cancer adjuvant and metastatic, male breast cancer, chemoprophylaxis of high-risk women
SERD	Fulvestrant	Metastatic breast cancer
LHRH agonists	Goserelin, triptorelin	Premenopausal women with hormone-positive breast cancer
Progestins	Megestrol acetate, medroxyprogesterone acetate	Metastatic breast cancer

the most important metabolite required for tamoxifen treatment. Tamoxifen administered as a single oral dose of 20 mg is rapidly absorbed and reaches its peak concentration in about 5 h. The terminal elimination half-life is about 5–7 days. Steady-state concentrations in plasma are reached after 4 weeks of tamoxifen treatment. About 65 % of the administered dose of tamoxifen is excreted over 2 weeks, primarily by fecal excretion.

Tamoxifen is approved for the adjuvant and metastatic treatment of estrogen receptor-positive breast cancer. Currently, tamoxifen is the preferred treatment for premenopausal women with estrogen receptor-positive breast cancer. In addition, postmenopausal women with intolerance to aromatase inhibitors could change to tamoxifen after at least 2 years of treatment with aromatase inhibitors. Furthermore, tamoxifen is approved for the hormonal treatment of male breast cancer. Finally, tamoxifen is the only drug approved for breast cancer chemoprophylaxis of high-risk women.

Clinical trials in women older than 50 years diagnosed with breast cancer have shown treatment benefit in overall survival. In women undergoing surgery for node-negative breast cancer, tamoxifen therapy was associated with significant prolongation of disease-free survival in comparison with the placebo-treated women. Tamoxifen reduced the rate of treatment failure at local and distant sites, tumors in the opposite breast, and the incidence of tumor recurrence after lumpectomy and radiation. The Early Breast Cancer Trialist Collaborative Group performed a meta-analysis of 55 clinical trials including more than 37,000 women and concluded that tamoxifen therapy was associated with a significant reduction in recurrence (26 %) and death (14 %) compared with placebo after a median follow-up of 10 years. Additionally, it was shown that women with estrogen receptor-positive tumors and those treated for at least 5 years had substantially greater benefit than the women with estrogen receptor-negative tumors or those treated for a time period shorter than 5 years. Specifically, in women with estrogen receptor-positive disease, 5 years of tamoxifen reduced the annual breast cancer death rate by 31 % irrespective of age, administration of adjuvant chemotherapy, progesterone receptor status, or other tumor characteristics [14–16].

The efficacy of tamoxifen treatment in estrogen receptor-negative tumors remains controversial. Regarding the effect of the duration of tamoxifen therapy on recurrence and death, it has been demonstrated that in women with estrogen receptor-positive tumors, 5 years of adjuvant tamoxifen treatment was superior than 1 or 2 years [15]. Furthermore, it has been shown that tamoxifen therapy for a period greater than 5 years confers no additional benefit [15].

In addition, tamoxifen has been proved effective in the treatment of metastatic breast cancer. Tamoxifen still remains the initial treatment of choice for women who present with relapse of disease ≥6 months after discontinuation of tamoxifen treatment.

On the other hand, tamoxifen treatment has been associated with a number of benign lesions in the endometrium, including endometrial thickening, endometrial polyps, and endometrial cystic atrophy, as noted in Table 24.5. However, the most serious uterine condition associated with tamoxifen use is endometrial cancer, which is increased by twofold to fourfold relative to placebo. The most common adverse effects of tamoxifen are menopausal symptoms including hot flashes and atrophic vaginitis. Vaginal discharge and irregular menses have also been reported in postmenopausal women. In addition, retinopathy has been reported in women taking higher doses of tamoxifen. However, vision-threatening ocular toxicity has rarely been reported. Tamoxifen has been associated with a slightly increased incidence of cataracts. Tamoxifen increases the risk of thromboembolic events, including deep venous thrombosis and

Table 24.5 Breast cancer hormonal therapy—adverse events

Tamoxifen	Aromatase inhibitors
Thromboembolic events	Bone fractures
Benign endometrial lesions	Arthralgias
Endometrial cancer	Hypercholesterolemia
Retinopathy–cataracts	Cardiovascular events

Table 24.6 Aromatase inhibitors

Drug class	Agents	Indication
1st class	Aminoglutethimide	Metastatic breast cancer
2nd class	Fadrozole, rogletimide, formestane	Metastatic breast cancer
3rd class	Exemestane, anastrozole, letrozole	Postmenopausal women with hormone receptor-positive breast cancer in the adjuvant and metastatic settings

pulmonary embolism. Estimates of the risk ratio (RR) of thromboembolic events range from 1.3 to 7.0 [15, 17].

Aromatase Inhibitors

Aromatase inhibitors block the aromatase enzyme in the final step of estrogen synthesis, thus lowering circulating estrogen levels and depriving the estrogen receptor of its substrate. The suppression of circulating estrogen is profound, approximately 95–98 %, with all the third-generation aromatase inhibitors. Aromatase inhibitors are classified as nonsteroidal, such as anastrozole and letrozole (reversible type II), and steroidal aromatase inhibitors (irreversible type I), such as exemestane (see Table 24.6). Aromatase inhibitors are now part of the standard treatment for most postmenopausal women with estrogen receptor (ER)- and/or progesterone receptor (PgR)-positive invasive breast cancer. These agents are given either alone or in sequence before or after tamoxifen. Postmenopausal women with estrogen receptor-positive breast cancer should be treated with aromatase inhibitors for 5 years. For perimenopausal women who have been initiated with tamoxifen, switching to an aromatase inhibitor to complete 5 years of treatment is recommended after 2–3 years of tamoxifen treatment, in case of definitive amenorrhea. In addition, according to the results of the extended adjuvant therapy studies in the postmenopausal women after completion of 5 years of standard tamoxifen treatment, continuation with aromatase inhibitors for at least 2–5 years could be an option, especially for node-positive women or for women with risk prognostic factors. Aromatase inhibitors are contraindicated in premenopausal and perimenopausal women [16].

Two large randomized trials have compared tamoxifen with aromatase inhibitors as initial adjuvant treatment. The Arimidex (anastrozole), Tamoxifen Alone or in Combination (ATAC) trial randomized 9,366 women with estrogen receptor-positive or unknown invasive breast cancer to 5 years of adjuvant tamoxifen, anastrozole, or both. There was no difference in the disease-free survival between the combination arm and the tamoxifen arm, and anastrozole was superior to both. In addition, at a median follow-up of 120 months, the study demonstrated a longer disease-free survival in the anastrozole group, significantly lower risk for recurrence (hazard ratio [HR], 0.87; 95 % confidence interval [CI], 0.78–0.97; $p=.01$), and longer time to recurrence (HR, 0.79; 95 % CI, 0.70–0.90; $p=.005$) for anastrozole versus tamoxifen given for 5 years [18]. Anastrozole also resulted in significantly less distant metastasis (HR, 0.86; 95 % CI, 0.74–0.99; $p=.04$) and significantly fewer contralateral breast cancers (42 % less; 95% CI, 12–62 %; $p=.01$). However, thus far, there have been no differences in the rates of death from any cause (HR, 0.97; 95% CI, 0.85–1.12; $p=.7$), and few breast cancer-related deaths have occurred [19–22].

The second large, upfront aromatase inhibitor trial, the Breast International Group (BIG) 1–98 ($n=8{,}010$) study, compared letrozole with tamoxifen for 5 years. That trial had four treatment arms: (1) letrozole for 5 years; (2) tamoxifen for 5 years; (3) tamoxifen for 2 years, then letrozole for 3 years; (4) and letrozole for 2 years, then tamoxifen for 3 years. The published analysis compared the two groups assigned to initially receive letrozole with the two groups assigned to

initially receive tamoxifen. The primary analysis, with a median follow-up of 25.8 months, showed that letrozole treatment resulted in a significantly lower risk for recurrence (HR, 0.81; 95 % CI, 0.70–0.93; $p = .003$), with 5-year disease-free survival rate estimates of 84.0 % for the letrozole group and 81.4 % for the tamoxifen group. Letrozole resulted in significantly fewer recurrences at distant sites (HR, 0.73; 95 % CI, 0.60–0.88; $p = .001$). However, overall survival did not differ significantly between the two groups (HR, 0.86; 95 % CI, 0.70–1.06; $p = .16$) [18, 23, 24].

Nowadays aromatase inhibitors have mostly replaced tamoxifen as the treatment of choice for hormone-responsive breast cancer in postmenopausal women because of better relapse-free survival, although no significant overall survival benefits have been reported. However, aromatase inhibitors can result in musculoskeletal pain that could lead 10–20 % of patients to termination of treatment. In addition, adverse events of aromatase inhibitors include bone fractures, hypercholesterolemia, and cardiovascular events [25–28], as outlined in Table 24.5. Due to the mechanism of action of aromatase inhibitors that prevent peripheral estrogen production, circulating blood estrogens are suppressed to levels lower than those achieved by natural menopause. Indeed, aromatase inhibitor-associated bone loss occurs at more than twice the rate of physiologic postmenopausal bone mass loss. Negative effects on cardiovascular function with subsequent elevation of cardiovascular risk are also expected [29, 30].

Hormonal Treatment of Metastatic Breast Cancer

In postmenopausal women with hormone-positive breast cancer, aromatase inhibitors are the first line of treatment for untreated patients or for those who had prior AI treatment and progress after 12 months of adjuvant therapy. A longer disease-free interval and absence of visceral disease is associated with a better response. If the disease recurs in less than 12 months, treatment initiation with tamoxifen or fulvestrant is recommended. In the second-line setting, the best option after progression is the administration of either tamoxifen or fulvestrant. In the third-line setting, treatment with an aromatase inhibitor is considered an acceptable option [31].

In premenopausal women who have progressed after 12 months following adjuvant treatment, it is recommended to initiate therapy with a combination of tamoxifen and a luteinizing hormone-releasing hormone analog. If there is treatment failure with the use of this combination, megestrol acetate or an LHRH agonist plus an aromatase inhibitor could be an option.

Selective Estrogen Receptor Downregulators

Fulvestrant is a 7α-alkylsulphinyl analog of 17β-estradiol, which is distinctly different in chemical structure from the nonsteroidal chemical structures of tamoxifen, raloxifene, and other selective estrogen receptor modulators. Fulvestrant is a selective estrogen receptor downregulator that behaves as a complete antagonist, competitively inhibiting binding of estradiol to the estrogen receptor, with a binding affinity that is 89 % that of estradiol. Fulvestrant binds to the estrogen receptor but, due to its steroidal structure and long side chain, induces a different conformational shape with the receptor to that achieved by the nonsteroidal antiestrogen tamoxifen. As a result of this, fulvestrant prevents estrogen receptor dimerization and leads to the rapid degradation of the fulvestrant–estrogen receptor complex, producing the loss of cellular estrogen receptor.

The drug is intramuscularly administered and is devoid of all known estrogen receptor agonist effects. Fulvestrant has been approved as a second-line treatment for postmenopausal women with estrogen receptor-positive advanced breast cancer who have shown disease progression following prior endocrine therapy [32]. Phase III studies have shown that fulvestrant is at least as effective as the third-generation aromatase inhibitor anastrozole in patients whose disease has relapsed or progressed on prior endocrine

therapy. However, in a phase III trial comparing fulvestrant versus tamoxifen for the first-line therapy of advanced breast cancer, fulvestrant did not attain the requirements for equivalence to tamoxifen. Because of its different mode of action than that of other hormonal agents, fulvestrant is effective in the treatment of tamoxifen-resistant disease and, unlike tamoxifen, has no known estrogen agonist effects. In fact, the optimal position of fulvestrant in the sequence of endocrine therapies for postmenopausal women and its role in combination regimens are not yet resolved [33, 34].

Drug Targets

Tyrosine Kinase Receptors

Tyrosine kinases contain transmembrane growth factor receptors such as insulin-like growth factor 1 receptor (IGF-1R), fibroblast growth factor receptors family (FGFRs), and epidermal growth factor receptor family (EGFRs) and are the upstream of intracellular signaling pathways.

ErbB2 (HER2/new)

The ErbB family of type I tyrosine kinase receptors includes ErbB1 (also known as the epidermal growth factor receptor (EGFR or HER1)), ErbB2 (also known as Her2), ErbB3, and ErbB4. All HER receptors have a similar structure including an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and a cytoplasmic tyrosine kinase domain. These receptor tyrosine kinases are widely expressed in epithelial, mesenchymal, and neuronal tissues where they play a role in regulating cell proliferation, survival, and differentiation. Epidermal growth factor (EGF) and transforming growth factor α (TGFα) bind to EGFR and activate it, while heregulin binds to ErB3 and ErB4, leading to EGFR phosphorylation and subsequent formation of homodimers and heterodimers. ErB2 forms heterodimers with EGFR, ErB3, and ErB4 [74]. No ligand has been identified for ErB2. Available data suggest that ErB2 is transactivated following heterodimerization. EGFR and Her2 contain multiple tyrosine phosphorylation sites, and autophosphorylation of specific tyrosine residues takes place within the highly conserved catalytic kinase domains of ErbB1 and ErbB2. Subsequently, the phosphorylated tyrosine residues located within the carboxyl terminus of the receptors recruit mediators and activate signaling pathways that result in cell proliferation (mitogen-activated protein kinase or MAPK pathway) and survival (phosphatidylinositol-3-kinase or PI3K pathway).

ErbB2 is amplified in approximately 20–25 % of metastatic breast cancer tumors. Increased expression of ErbB2 has been correlated with poor outcome in breast cancer patients. ErbB2 confers resistance to some chemotherapy and hormone therapy. In addition, ErbB2 confers an aggressive form of disease with significantly shortened disease-free survival and overall survival. ErbB receptor inhibition may suppress cell growth, enhance cell death, and improve response to other cancer therapy in some tumors. Inhibiting ErbB receptors may more selectively target cancer cells and spare normal cells, thus reducing unwanted adverse events of therapy.

VEGF

Vascular endothelial growth factor (VEGF) is a key mediator involved in the angiogenesis switch, which processes the development of a high-density blood vessel network connecting the primary tumor to the host circulation, as well as a premature vascularization characterized by high permeability status. The elevated expression of VEGF is an independent prognosis predictor that has been observed in both early and late-stage breast cancer and has been related to advanced stage of the disease, poor prognosis, and decreased response to chemotherapy or endocrine therapy. The overexpression of VEGF is closely linked to the loss of tumor suppressor p53 and the amplification of oncogene HER2.

PI3K/Akt/mTOR pathway

The intercellular signal pathway involving phosphatidylinositol 3-kinase (PI3K), protein kinase B/PKB (Akt), and mammalian target of rapamycin (mTOR) regulates several cellular functions, such as cell growth, survival, and proliferation, which are essential for tumorigenesis and progression. Following the activation of membrane receptors by extracellular signals, PI3K and Akt can be activated by phosphorylation cascades and eventually activate their downstream substrates, including mTOR, a serine/threonine kinase. mTOR is a central regulator of protein translation, which can phosphorylate and activate the eukaryotic translation initiation factor eIF4E-binding proteins (4E-BP1) and the 70kD ribosomal protein S6 kinase (p70S6K). Furthermore, mTOR can induce a positive feedback effect to phosphorylate Akt and enhance the signal transduction of this pathway. High activation level of PIEK/Akt/mTOR pathway has been associated with resistance to conventional breast cancer treatment, resistance to endocrine treatment, and increased risk of metastasis.

Targeted Therapy

HER2 Inhibitors

Trastuzumab (Herceptin™) is a humanized anti-ErbB2 monoclonal antibody that has been approved for the treatment in the adjuvant and the metastatic setting of breast cancer that either overexpresses ErbB2 or demonstrates ErbB2 gene amplification [35]. Treatment duration is 1 year in the adjuvant setting. Trastuzumab binds to the extracellular domain of the ErbB2 receptor and has been reported to exert its antitumor effects through several mechanisms, including inhibition of tyrosine kinase activation, induction of receptor endocytosis and degradation, inhibition of extracellular domain cleavage, decreased DNA repair, decreased intracellular signal transduction and antiangiogenic effects, and induction of immune-mediated cytotoxicity. In addition, in ErbB2-overexpressing cells, trastuzumab has been reported to downregulate ErbB2 expression.

Herceptin adjuvant (HERA) trial is an international, multicenter, randomized, open-label, phase III trial comparing treatment with trastuzumab for 1 and 2 years with observation after standard neoadjuvant or adjuvant chemotherapy, or both, in patients with HER2-positive early breast cancer [36]. According to the HERA trial and NSABPB/31NCCTG N9831, 1 year of adjuvant Herceptin after chemotherapy reduces the risk of breast cancer recurrence by 50 %. The primary endpoint was disease-free survival. After a positive first interim analysis at a median follow-up of 1 year for the comparison of treatment with trastuzumab for 1 year with observation, event-free patients in the observation group were allowed to cross over to receive trastuzumab. Trial outcomes for the 1-year trastuzumab and observation groups at a median follow-up of 48.4 months have been reported indicating that treatment with adjuvant trastuzumab for 1 year after chemotherapy is associated with significant clinical benefit at a 4-year median follow-up [37].

Thus, currently, trastuzumab in combination with chemotherapy is standard of care for patients with early HER2-positive cancers larger than 1 cm. However, there are also patients who may not need or simply may not want to receive chemotherapy. For example, patients with small (<1 cm) tumors with node-negative HER2-positive disease have been largely excluded from the large randomized adjuvant trastuzumab trials on the basis of perceived excellent prognosis. In neoadjuvant trials, the combination of trastuzumab and pertuzumab without chemotherapy has achieved pathological complete remission rates in a significant minority of patients, suggesting that a subgroup exists for whom anti-HER2 therapy alone may be as effective as with additional chemotherapy. Trials and prospective studies are needed to investigate further the issue of trastuzumab therapy without chemotherapy in selected patients. In the meantime, it can be suggested that there is already enough evidence to justify anti-HER2 therapy alone in select patients for whom chemotherapy is contraindicated [38].

Serious adverse events of trastuzumab treatment include hypersensitivity reactions with first infusions and congestive heart failure. The risk of

congestive heart failure is increased when trastuzumab is co-administered with anthracycline. An increase by a factor of 4–5 in the rate of congestive heart failure has been noted when adjuvant trastuzumab was used with anthracyclines, and an even larger proportion of patients had subclinical loss of left ventricular function. The majority of trastuzumab-related cardiac events observed have been asymptomatic declines in left ventricular ejection fraction. The incidence of severe congestive heart failure and cardiac death observed in the large adjuvant trastuzumab trials ranges from 0.6 to 4 %. Both symptomatic and asymptomatic events have been reported to be reversible and manageable; however, little is known about the significance of asymptomatic left ventricular ejection fraction decline, and longer cardiac follow-up is needed. Close cardiac monitoring must be performed for all patients receiving anti-HER2 agents currently in the clinic or in development [39].

Trastuzumab should not be administered during pregnancy. However, for women who become accidentally pregnant during trastuzumab administration and wish to continue pregnancy, trastuzumab should be stopped and pregnancy could be allowed to continue.

Approximately 30 % of patients with metastatic cancer who test positive for HER2, either by immunohistochemistry or FISH, exhibit an objective clinical response to trastuzumab alone, and about 50 % responds to trastuzumab plus chemotherapy. A percentage of the remaining patients may still derive clinical benefit without an objective response. However, there is also a significant proportion of patients who exhibit primary resistance to trastuzumab. In addition, many patients who do benefit initially in the metastatic setting eventually progress while on trastuzumab treatment (acquired resistance).

There are several potential mechanisms for trastuzumab resistance. These include inactivation or loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and activation of other tyrosine kinase receptors, including the insulin-like growth factor receptor (IGF-1R). Another potential mechanism of resistance is the accumulation of truncated forms of the HER2 receptor that lack the extracellular trastuzumab-binding domain.

Tyrosine Kinase Inhibitors

Lapatinib

Lapatinib is a small-molecule, potent, reversible, dual inhibitor of the tyrosine kinase domains of both EGFR and ErbB2 currently approved for use in combination with capecitabine in the treatment of advanced breast cancer overexpressing HER2 (HER2+) [40]. Unlike trastuzumab, lapatinib enters the cell and binds to the intracellular domain of the tyrosine kinase receptor, allowing for complete blockage of the autophosphorylation reaction and a complete halt to the downstream cascade of events. Preclinical studies demonstrated potent antitumor effects in HER2-overexpressing models, including cell lines with acquired trastuzumab resistance. Lapatinib has been well tolerated in a phase II monotherapy trial in patients with advanced breast cancer; however, the response was minimal in HER2+ patients, and no HER2- patients achieved an objective tumor response. A phase II trial of lapatinib monotherapy in 39 HER2+ patients with breast cancer and brain metastases yielded 1 partial response, although 15.4 % of patients had stable disease for > or =16 weeks. In a phase III trial comparing lapatinib plus capecitabine with capecitabine alone in HER2+ patients with advanced breast cancer refractory to anthracycline, taxane, and trastuzumab regimens, the median time to progression was 8.4 months with combination therapy, compared with 4.4 months with capecitabine alone ($p<0.001$). There were no significant differences between combination therapy and capecitabine alone in terms of the overall response rate or overall survival. Unlike trastuzumab, lapatinib is an orally acting agent. The most frequently reported adverse events in patients receiving combination therapy with lapatinib and capecitabine were diarrhea and hand-foot syndrome.

Novel Anti-HER Drugs

Like trastuzumab, pertuzumab targets the HER2 extracellular domain but at a different epitope, resulting in inhibited dimerization of HER2 with other HER family receptors. Trastuzumab-DM1 is a novel chemistry-driven conjugated HER2

monoclonal antibody in which the trastuzumab is conjugated with a fungal toxin DM1 (maytansine). This compound was designed to overcome trastuzumab resistance. Maytansine is an antimicrotubule agent that inhibits the assembly of cellular microtubules. In vitro studies showed that the cytotoxicity of maytansine is more than 1,000 times that of any other chemotherapeutic agent. In trastuzumab-DM1, trastuzumab mainly works as a carrier that delivers DM1 to the tumor cells labeled with HER2. Trastuzumab-DM1's mechanism of action is independent of functional HER2 signaling. Transtuzumab-DM1 can be active if a high expression level of HER2 exists on the cellular surface. Therefore, trastuzumab-DM1 can successfully overcome several trastuzumab-resistance mechanisms related to HER2 downstream signaling. In the metastatic setting, the response rate for trastuzumab-DM1 has been reported to be 26–64 %, comparable to those obtained for capecitabine plus lapatinib (48 %), continuing trastuzumab in combination with capecitabine (48 %), pertuzumab plus trastuzumab (24 %), and neratinib (24 %). In fact, trastuzumab-DM1 represents a major shift in the treatment of patients with breast cancer as it replaces traditional nontargeted chemotherapy with a "smart" medication that directs the cytotoxic therapy to cancer cells by using a known biomarker [41–43].

Neratinib is an oral small-molecule pan-HER tyrosine kinase inhibitor that irreversibly inhibits HER1 and HER2. In preclinical HER2 models, antiproliferative effects of neratinib were accompanied by G1 cell cycle arrest and decreased downstream signal transduction. In general, treatment strategies combining multiple HER2-directed therapies might yield additive or synergistic effects and lead to improved outcome. The future challenges include understanding HER2 functions, designing rational combinations, and optimal selection of patients [44, 45].

Antiangiogenetic Agents

Bevacizumab is the first approved antiangiogenic agent for human cancers. Numerous clinical trials have been conducted to test the efficacy of bevacizumab in metastatic breast cancer, especially in a form combining it with first-line chemotherapy. A recently published meta-analysis study summarized the available randomized trials using bevacizumab in addition to chemotherapy in metastatic breast cancer patients [46]. Their results concluded that regimens combining bevacizumab with chemotherapy provided substantial benefit for the treatment of metastatic breast cancer in terms of improving progression-free survival and objective response rate. However, there was no significant difference in overall survival.

Sorafenib and sunitinib are both novel multi-targeted tyrosine kinase inhibitors that inhibit several proangiogenic tyrosine kinase receptors, including VEGFRs. Several phase I and II studies are ongoing currently to evaluate the safety and efficacy of these tyrosine kinase inhibitors as combined with chemotherapy in treating metastatic breast cancers.

mTOR Inhibitors

Rapamycin is an mTOR inhibitor with antifungal activity and immunosuppressive effect, approved as an immunosuppressive in organ transplantation. Temsirolimus and everolimus are ester derivative analogs of rapamycin. Temsirolimus was approved by the FDA in 2007 for the intravenous treatment of metastatic renal cell carcinoma. Everolimus was initially developed as an immunosuppressant for renal and heart transplant patients. Both temsirolimus and everolimus display anticancer effects and inhibit mTOR by binding to the FK506-binding protein. Preclinical in vitro and in vivo studies have demonstrated that both rapamycin analogs were capable of inhibiting the proliferation of multiple breast cancer cell lines which were ER positive and with the overexpressed HER2 or the loss of PTEN function, when administered either alone or in combination with chemotherapeutic agents, endocrinal drugs, other targeted substances, or radiotherapy. The first randomized phase I study of everolimus plus tamoxifen versus tamoxifen alone demonstrated improved 6-month

benefit for the combination of everolimus with tamoxifen as compared with tamoxifen alone in hormone receptor-positive/HER2-negative metastatic breast cancer patients. The first large phase III study comparing temsirolimus plus letrozole versus letrozole alone in postmenopausal women with advanced or metastatic breast cancer was terminated prematurely because of high grade 3 toxicities without any significant clinical benefit in the combination treatment of temsirolimus plus letrozole over letrozole alone. Currently, oral everolimus (Afinitor®) in combination with exemestane is indicated for the treatment of hormone receptor-positive, human epidermal growth factor receptor (HER)2-negative advanced breast cancer in postmenopausal women after failure of treatment with letrozole or anastrozole (in the USA) or after recurrence of progression following a nonsteroidal aromatase inhibitor (AI) in women without symptomatic visceral disease (in the EU). In the well-designed BOLERO-2 study, the addition of everolimus to exemestane was shown to significantly prolong progression-free survival of postmenopausal women with hormone receptor-positive, human epidermal growth factor receptor (HER)2-negative advanced breast cancer after recurrence of progression following a nonsteroidal aromatase inhibitor (AI) in women without symptomatic visceral disease [47].

Conclusion and Future Perspective

Therapeutic armamentarium for breast cancer includes chemotherapy, hormonal therapy, and novel targeted therapies. Undoubtedly, further research is needed for the treatment of estrogen receptor-negative breast cancer as well as for the treatment of metastatic breast cancer. In addition, further research is needed for biomarkers that will make feasible the goal of a patient-oriented treatment.

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25 Pharmacogenomics–Pharmacoepigenomics of Breast Cancer Therapy: Clinical Implications

Eugenia Ch Yiannakopoulou and Debmalya Barh

Abstract

Breast cancer is the most common type of cancer and one of the leading causes of death for women. Therapeutic options for breast cancer include tamoxifen, aromatase inhibitors, fulvestrant, chemotherapy, monoclonal antibodies, tyrosine-kinase inhibitors, mTOR inhibitors, and VEGF inhibitors. Thus, endocrine therapy reduces the risk of recurrence and improves survival among women with hormone receptor-positive breast cancer. However, a significant percentage of women who receive therapy in the adjuvant or metastatic setting do not benefit from this therapy, while a number of women who respond will eventually develop disease progression and relapse while on therapy. For example, 30 % of early breast cancer patients treated with tamoxifen acquire tamoxifen resistance and relapse. The observed variability in treatment response to targeted breast cancer treatment could be partly explained by pharmacogenomics–pharmacoepigenomics, i.e., the study of genetic variation in drug response. At the nucleotide level, genetic variation is due to polymorphisms, large insertions, deletions, and duplications. Polymorphisms represent common variations in the DNA sequence that may lead to reduced activity of the encoded gene but, in some cases, to increased activities. Polymorphisms include single-nucleotide polymorphisms (SNPs), microsatellites, and mini-satellites. Pharmacoepigenetics is a novel field of research, with possible relevance in breast cancer treatment.

Epigenomics is another aspect of genetic variation that may affect drug response. The term epigenomics refers to heritable traits in the cells and organisms that do not involve changes to the underlying DNA sequence, i.e., changes in gene expression, caused commonly by environmental factors. These changes may persist through cell division and for the

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remainder of the organism's life. Epigenetic processes include methylation of DNA, acetylation, phosphorylation, ubiquitylation and sumoylation of histones, histone modifications, and noncoding RNA-mediated regulation of gene expression. This chapter will review data on pharmacogenomics–pharmacoepigenomics of breast cancer treatment, focusing on clinical implications for drug efficacy and drug safety.

Keywords
Breast cancer • Pharmacogenomics • Tamoxifen • Aromatase inhibitors • CYP2D6 • Clinical implications

Introduction

Adjuvant endocrine therapy, i.e., tamoxifen and aromatase inhibitors, reduces the risk of recurrence and improves survival among women with hormone receptor-positive breast cancer. Other forms of breast cancer targeted by therapy such as trastuzumab and bevacizumab contribute to the increase in survival of women suffering from breast cancer. However, a significant percentage of women who receive targeted therapy in the adjuvant or metastatic setting do not benefit from this therapy, while a number of women who respond will eventually develop disease progression and relapse while on therapy [1]. For example, about 30 % of early breast cancer patients treated with tamoxifen acquire tamoxifen resistance over the 5-year treatment period and relapse [2]. The observed variability in treatment response to targeted breast cancer treatment could be partly explained by pharmacogenomics, i.e., the study of genetic variation in drug response [3]. DNA sequence variation is due to copy-number variation, i.e., deletions and duplications of genomic DNA segments that are at least one kilobase long, single-nucleotide polymorphisms (SNPs), and microsatellite and minisatellite repeats [4–7]. In the case of tumors, both pharmacogenomics of the host and pharmacogenomics of the tumor tissue itself could affect response to drug treatment. Pharmacogenomics–pharmacogenetics of breast cancer-targeted therapy is anticipated to contribute to personalized medicine with the choice of the appropriate drug for the appropriate patient.

Metabolism of Tamoxifen

Tamoxifen can be considered a prodrug. The parent drug has weak affinity for the estrogen receptors but undergoes excessive biotransformation catalyzed by phase I and II enzymes into active and inactive metabolites. CYP3A4 is the main CYP450 enzyme involved in the metabolism of tamoxifen to a-hydroxytamoxifen and to N-desmethyltamoxifen [8–11]. CYP2D6 is the major metabolite involved in the hydroxylation of trans-tamoxifen to trans-4-OH-tamoxifen [11]. Endoxifen and 4-hydroxytamoxifen each have at least tenfold higher affinity for estrogen receptors than tamoxifen and are associated with equivalent antiestrogenic potency [11–14]. In patients receiving chronic tamoxifen therapy, endoxifen is found in serum concentration 6- to 12-fold higher than 4-hydroxytamoxifen. Thus, in vitro and in vivo studies suggest that endoxifen is the most important metabolite required for tamoxifen treatment. However, clinical correlative studies have produced mixed results [15].

CYP2D6 Pharmacogenomics and Tamoxifen

Pharmacogenetics of CYP2D6 is expected to affect endoxifen concentrations and possibly tamoxifen-associated long-term outcomes [16]. Over 80 allelic variants and a series of subvariants of CYP2D6 have been identified and characterized [17]. They include fully functional alleles, alleles with reduced function, and nonfunctional

alleles which convey a wide range of enzyme activity ranging from no activity to ultrarapid metabolism of substrates. Most inactivating mutations in CYP2D6 are point mutations resulting in splicing defects or deletions that result in a truncated protein or no protein at all [18]. Thus, adverse effects or lack of effect may occur if standard doses of a drug are applied [18–20].

The CYP2D6 activity ranges considerably within a population and includes extensive metabolizers, intermediate metabolizers, poor metabolizers, and ultrarapid metabolizers, shown in Table 25.1 [18–20]. Extensive metabolizer phenotype occurs when there is at least one functional wild-type allele at the relevant gene locus. Extensive metabolizers have normal responses to the standard doses of a particular drug [21]. Many individuals are characterized as intermediate metabolizers. Intermediate metabolizers are heterozygous for one of the inactivating mutations or homozygous for alleles associated with impaired metabolism [21]. In European populations, two alleles associated with impaired metabolism, CYP2D6*9 and CYP2D6*41, are quite common [22]. CYP2D6*9 encodes a protein with deletion of one amino acid. CYP2D6*41 includes a number of different polymorphisms including two nonsynonymous mutations that are also seen in CYP2D6*2 allele, an upstream polymorphism at position −1584 and a base substitution in intron 6. The nonsynonymous mutations do not appear to alter the enzyme activity, but the intron 6 polymorphism is associated with altered RNA splicing, resulting in lower levels of protein.

The poor metabolizer phenotype occurs when both alleles carry inactivating mutations and give rise to synthesis of enzyme with impaired activity or no synthesis of enzyme at all. About 2–10 % of the population are poor metabolizers. In Caucasians, about 8 % of the population are poor metabolizers of CYP2D6. It is estimated that 95 % of European poor metabolizers have two copies of any combination of four alleles termed CYP2D6*3, CYP2D6*4, CYP2D6*5, and CYP2D6*6, with each encoding defective forms of CYP2D6 [19]. The remaining 5 % of poor metabolizers are homozygous or heterozygous for a range of relatively rare loss-of-function alleles including CYP2D6*7, CYP2D6*8, CYP2D6*11, CYP2D6*12, CYP2D6*13, CYP2D6*14, CYP2D6*15, CYP2D6*16, CYP2D6*18, CYP2D6*19, CYP2D6*20, CYP2D6*21, CYP2D6*38, CYP2D6*40, CYP2D6*42, CYP2D6*44, CYP2D6*56, and CYP2D6*62

Table 25.1 CYP2D6 alleles

Normal-function alleles	Reduced-function alleles	Null-function alleles	Increased-function alleles
CYP2D6*2	CYP2D6*3	CYP2D6*8	CYP2D6*1xN
–	CYP2D6*4	CYP2D6*11	CYP2D6*2xN
–	CYP2D6*5	CYP2D6*12	–
–	CYP2D6*6	CYP2D6*13	–
–	CYP2D6*9	CYP2D6*14	–
–	CYP2D6*10	CYP2D6*15	–
–	CYP2D6*41	CYP2D6*16	–
–	–	CYP2D6*17	–
–	–	CYP2D6*18	–
–	–	CYP2D6*19	–
–	–	CYP2D6*20	–
–	–	CYP2D6*21	–
–	–	CYP2D6*38	–
–	–	CYP2D6*40	–
–	–	CYP2D6*42	–
–	–	CYP2D6*44	–
–	–	CYP2D6*56	–
–	–	CYP2D6*62	–

[19]. On the other hand, ultrarapid metabolizer phenotype, due to a gene amplification, gives rise to ultrarapid metabolism of drugs metabolized by CYP2D6. There have been reports of up to 12 copies at the same gene locus [19].

The most relevant clinical question is if CYP2D6 genotyping affects patient outcome. In that aspect, based on the abovementioned data, it was hypothesized that patients who are poor metabolizers of CYP2D6 and therefore are expected to have low plasma concentration of endoxifen might have inferior outcomes taking tamoxifen than do extensive metabolizers [23].

Is there an association between CYP2D6 polymorphisms and endoxifen plasma concentrations? Prospective cohort studies of adjuvant tamoxifen treatment have shown extensive interindividual variation in plasma concentrations of active metabolites tamoxifen and 4-hydroxytamoxifen in breast cancer patients carrying CYP2D6 polymorphisms, shown in Tables 25.2, 25.3, and 25.4 [23–37]. In particular, the patients designated as poor metabolizers show four times lower plasma concentration of endoxifen compared with extensive metabolizers.

Goetz et al. [24] were the first to show evidence that genetic variability in CYP2D6 may affect the treatment outcome of patients receiving tamoxifen. The authors performed a retrospective analysis of a prospective adjuvant tamoxifen trial (NCCTG 89-30-52) in postmenopausal women with surgically resected ER-positive breast cancer (stages I to III) to determine the role of genetic variation in CYP2D6 on patient outcome. Because of the difficulty in amplifying DNA from formalin-fixed paraffin-embedded tissue, only the CYP2D6*4 that is the most common null allele contributing to the poor metabolizer phenotype and the CYP2D6*6, an infrequent null allele, were studied. No *6 variants were detected. Women with the CYP2D6*4/*4 genotype had shorter relapse-free time and worse disease-free survival compared to women with either one or no *4 alleles. Subsequent retrospective studies verified that the CYP2D6 genotype plays a role in tamoxifen treatment outcome [25].

Schroth et al. [29] investigated the association between metabolic phenotypes of CYP2D6 and outcome of adjuvant tamoxifen treatment in estrogen receptor-positive patients suffering from

Table 25.2 Studies demonstrating a positive association between CYP2D6 polymorphisms and treatment outcome of breast cancer patients treated with tamoxifen in the adjuvant setting

Allele	Outcome	Trial reference
CYP2D6*4	Women with the CYP2D6*4/*4 genotype had shorter relapse-free time and worse disease-free survival compared to women with either one or no *4 alleles	[24]
CYP2D6*4 or drug-induced impaired CYP2D6 metabolism	Patients with decreased CYP2D6 metabolism had significantly shorter time to recurrence and worse relapse-free survival	[25]
CYP2D6*10, CYP2D6*41, CYP2D6*4, CYP2D6*5	Tamoxifen-treated patients carrying at least one of the CYP2D6 alleles *4, *5, *10, and *41 presented with impaired formation of antiestrogenic metabolites had significantly more recurrences of breast cancer, shorter relapse-free periods, and worse event-free survival rates	[26]
CYP2D6*3, CYP2D6*4, CYP2D6*5, and CYP2D6*41 in patients with familial breast cancer	Patients carrying impaired alleles had worse overall survival	[29]
CYP2D6*10	Women with the CYP2D6 *10 T/T genotype variant had worse disease-free survival	[28]
CYP2D6*10, CYP2D6*41, CYP2D6*3, CYP2D6*4, CYP2D6*5	The presence of 2 functional CYP2D6 alleles was associated with better clinical outcomes and the presence of nonfunctional or reduced-function alleles with worse outcomes	[29]

Table 25.3 Studies demonstrating lack of any association between CYP2D6 polymorphisms and treatment outcome of breast cancer patients treated with tamoxifen in the adjuvant setting

Allele	Outcome	Trial reference
CYP2D6 *1, *2, *2 L, *3, *4, *5, *10B (*10), *14, *18, *21, *41, *49, *52, and *60 alleles	CYP2D6 polymorphisms were not associated with treatment outcome in the multivariate analysis	[30]
CYP2D6*1; CYP2D6*4; CYP2D6*5; CYP2D6*6b/c; CYP2D6*9; CYP2D6*10; CYP2D6*41; CYP2D6*UM	CYP2D6*6 may affect breast cancer-specific survival in breast cancer patients treated with tamoxifen. However, no association between more frequent variants like CYP2D6*4 and breast cancer-specific survival was identified	[23]
27 alleles via AmpliChip CYP450	No significant effect of CYP2D6 genotyping on the risk of recurrence in early breast cancer patients treated with tamoxifen in the adjuvant setting	[31]
CYP2D6*10, CYP2D*10, CYP2D6*41	No association was demonstrated between any of the investigated CYP2D6 variants and disease-free survival	[32]
9 CYP2D6 single-nucleotide polymorphisms; genotype combinations were used to categorize CYP2D6 metabolism phenotypes as poor, intermediate, and extensive	CYP2D6 phenotypes of reduced enzyme activity were not associated with worse disease control	[33]

Table 25.4 Effect of CYP2D6 polymorphisms on tamoxifen treatment outcome: tamoxifen indications other than adjuvant breast cancer treatment

Indication	Allele	Outcome	Trial reference
Breast cancer chemoprevention	CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6, CYP2D6*10, CYP2D6*17, CYP2D6*41, CYP2D6*1xN, CYP2D6*1xN	No association was demonstrated between CYP2D6 polymorphisms and breast cancer occurrence	[34]
Breast cancer chemoprevention	33 alleles via AmpliChip CYP450	CYP2D6 poor metabolizers showed increased risk of breast cancer occurrence. Increased efficacy of tamoxifen in patients carrying the CYP2D6*2A allele was suggested from the data of an exploratory analysis	[35]
Metastatic breast cancer	CYP2D6*10	Patients carrying the CYP2D6*10/CYP2D6*10 genotype had significantly shorter time to disease progression	[36]
Metastatic breast cancer	CYP2D6*3, CYP2D6*4, CYP2D6*5, CYP2D6*6, CYP2D6*10, and CYP2D6*41	Overall survival was significantly shorter in women with a CYP2D6 poor metabolizer phenotype	[37]

early breast cancer. After a follow-up period of 9 years, it was demonstrated that the risk of recurrence was higher in patients who carried either the extensive/intermediate or the poor genotype of CYP2D6, while there was no significant difference in overall survival. CYP2D6 genotype was associated with an increase in the odds of breast cancer recurrence in the Austrian Breast and Colorectal Cancer Study Group (ABCCSG) trial, association observed only in patients receiving tamoxifen monotherapy, and not in those receiving anastrozole, an active drug not metabolized by CYP2D6, following tamoxifen.

Other investigators, focusing on the CYP2D6*4 allele, the most common poor metabolizer allele in Caucasians, have established a

model using data from Goetz's study to estimate whether women with wild-type CYP2D6 have longer disease-free survival if they receive tamoxifen rather than an aromatase inhibitor. By applying the model, the investigators proposed that women with wild-type CYP2D6 had a similar or lower rate of relapse when treated with tamoxifen compared with aromatase inhibitor treatment.

However, there are also a number of relevant negative or conflicting studies [23, 30, 38–41], with a few studies suggesting an inverse association between CYP2D6 genotype and tamoxifen response [42]. Retrospective analysis from two large randomized clinical trials (ATAC and BIG), comparing tamoxifen with aromatase inhibitors as treatment for postmenopausal women with early breast cancer in the adjuvant setting, demonstrated no association between CYP2D6 genotype and outcome in patients taking tamoxifen [43, 44]. In a well-designed case control study, Morrow et al. [31] investigated the effect of CYP2D6 polymorphisms of breast cancer recurrence. The investigators performed CYP2D6 genotyping from whole-blood and fresh frozen tumor samples using the AmpliChip CYP450 Test from patients treated with tamoxifen for early breast cancer in the adjuvant setting. Patients experiencing breast cancer recurrence were matched by date of diagnosis, menopausal status, and clinical stage to patients without recurrence. This study demonstrated no significant effect of CYP2D6 genotyping on the risk of recurrence in early breast cancer patients treated with tamoxifen in the adjuvant setting. The inconsistency can be attributed to the retrospective design of the trials, since only a few trials have used specimens collected and archived from prospective clinical trials and no trial has been designed for the investigation of the issue as a primary objective [31].

CYP2C19 and Other CYP Enzyme Polymorphisms and Tamoxifen Pharmacogenomics

A potential effect of CYP2C19 polymorphism on breast cancer risk prolapse during tamoxifen therapy has also been suggested. CYP2C9 and CYP2C19 seem to play a minor role in metabolic pathways of tamoxifen (Table 25.5). A large number of CYP2C19 polymorphisms (CYP2C19*2, CYP2C19*3, CYP2C19*4, CYP2C19*5, CYP2C19*6, CYP2C19*7, CYP2C19*8) are associated with reduced enzyme activity [45]. CYP2C19*17 is associated with ultrarapid metabolism [46]. Limited data exist on the effect of these polymorphisms on the outcome of women treated with tamoxifen, as shown in Table 25.6 [26, 33, 47, 48].

Table 25.5 Phase II metabolizing enzymes and transporters involved in tamoxifen disposition

CYP 450 enzymes	Phase II metabolizing enzymes	Drug transporters
CYP3A4	UGT1A8	ABCB1
CYP3A5	UGT1A4	–
CYP2D6	UGT1A10	–
CYP2C9	UGT12B7	–
CYP2C19	UGT2B15	–
CYP2D9	UGT2B17	–
CYP2C8	SULT1A1	–

In one retrospective study of 621 patients with breast cancer treated with adjuvant tamoxifen, archived specimens were analyzed for various genotypes. Among the tamoxifen-treated women, the presence of one or two CYP2C19*17 alleles was associated with more favorable relapse-free survival compared with CYP2C19*1, CYP2C19*2, and CYP2C19*3 carriers [26]. On the other hand, in a Japanese study, 173 patients with ER- and/or PR-positive breast cancer treated with adjuvant tamoxifen had peripheral blood analyzed for CYP2C19 genotype. Genotypes associated with reduced enzyme activity were not associated with any difference in disease-free survival compared with wild-type genotype [47]. Thus, currently, there is no clinical reason for testing CYP2C19 genotype in breast cancer patients treated with tamoxifen.

Other CYP enzymes involved in tamoxifen metabolism are CYP3A4 and CYP3A5. CYP3A4 is the human enzyme known to be involved in the metabolism of the largest number of medications. It has been estimated that CYP3A4 is responsible for approximately 50 %

Table 25.6 Effect of CYP2C19 polymorphisms on tamoxifen treatment outcome

Tamoxifen indication	Allele	Outcome	Trial reference
Adjuvant breast cancer treatment	CYP2C19*1, CYP2C19*2, CYP2C19*3, CYP2C19*17	CYP2C19*7 high enzyme activity variant identified patients likely to benefit from tamoxifen	[26]
Adjuvant breast cancer treatment	CYP2C19*2, CYP2C19*3	The investigated genotypes were not associated with any difference in disease-free survival compared with wild-type genotype. The investigated alleles were also not associated with endometrial cancer nor with bone mineral density	[47]
Adjuvant advanced breast cancer treatment	CYP2C19*2, CYP2C19*17	CYP2C19*2 was independently associated with time to treatment failure. CY2C19*17 was independently associated with a longer disease-free survival	[48]
Breast cancer chemoprophylaxis	CYP2C19*1, CYP2C19*2, CYP2C19*3, CYP2C19*17	CYP2C19 polymorphisms did not show any correlation with tamoxifen efficacy	[35]

of all cytochrome P450-mediated reactions of prescribed drugs. There is interindividual variation in overall levels of activity of CYP3A4 and several polymorphisms of this gene have been described, but the allelic frequencies are low. The association of CYP3A5 polymorphisms with plasma concentrations of tamoxifen metabolites or with treatment outcome of tamoxifen therapy has also been investigated, but no clinically significant association has been identified. A CYP3A4 promoter variant has been identified, CYP3A4*1B, with no studies linking CYP3A4*1B with altered tamoxifen metabolism [1]. However, it has been reported that CYP3A4*1B confers a threefold increased risk of endometrial carcinoma in tamoxifen-treated women [49].

The most frequent and functionally relevant polymorphism in the CYP3A5 gene consists of an A6986 gene transition with intron 3 (CYP3A5*3). This polymorphism creates an alternative splice site, resulting in a frame shift and truncation of the protein [50]. CYP3A5*3 results in decreased CYP3A5 activity. However, relevant studies have shown conflicting results regarding the association of CYP3A5*3 with tamoxifen metabolism and/or breast cancer outcome [24, 51, 52]. Goetz and colleagues [24] investigated the effect of CYP3A5*3 polymorphism on outcome of tamoxifen-treated women enrolled in a North Central Cancer Treatment Group. CYP3A5*3 genotype was determined from paraffin-embedded tumor samples and buccal cells (living patients). No effect of CYP3A5*3 polymorphism was displayed on patient outcome, disease-free survival, or overall survival [24]. Tucker et al. [51] investigated whether polymorphisms in CYP3A5 were associated with altered metabolism of tamoxifen; they found no difference in tamoxifen or metabolite concentration by CYP3A5*3 status in breast cancer-treated patients [51]. However, Wegman et al. [52] in a study investigating the influence of genetic variants of CYP3A5, CYP2D6, SULT1A1, and UGT2B15 on patient outcome found that postmenopausal women homozygous for the CYP3A5*3C variant, treated with adjuvant tamoxifen, displayed significantly improved recurrence-free survival [52]. Yet, as the authors point out, this was an unexpected finding, as CYP3A5*3 genotype represents an inactive form of the enzyme. Other investigators have suggested that CYP3A5 could be a minor contributor to the overall metabolism of CYP3A5 [53]. In addition, since there is overlap in substrate specificity between CYP3A4 and CYP3A5, the contribution of each to total CYP3A activity could depend on the drug and the individual treated with this drug [25]. Current evidence, although

limited, suggests that there is no clinically meaningful need in genotyping CYP3A in breast cancer patients treated with tamoxifen.

Phase II Metabolizing Enzyme and Drug Transporters, Pharmacogenomics, and Tamoxifen

Apart from CYP450-mediated pathways, other non-CYP450-mediated pathways seem to be important in terms of overall metabolism and activity profile of tamoxifen. At the level of phase II tamoxifen metabolism, sulfation and glucuronidation are the major mechanisms [54, 55].

Glucuronosyltransferases (UGTs) catalyze the glucuronidation of many lipophilic xenobiotics and endobiotics to make them more water soluble and therefore enhance their elimination. The human UGT superfamily has been classified into the UGT1 and UGT2 families, further classified into three subfamilies, UGT1A, UGT2A, and UGT2B. Overall, 30 UGT isoforms with overlapping specificities have been identified. Functional polymorphisms have been described for UGT1A1, UGT1A6, UGT1A7, UGT2B4, UGT2B7, and UGT2B15. Overall, polymorphisms of UGTs have not been investigated adequately due to the overlapping activities of the UGTs and the lack of selective probes, due to the complexity of the glucuronidation cycle and due to the difficulty of developing analytic methods to measure glucuronides [56]. Variation in drug metabolism due to altered UGT activity as a consequence of polymorphisms has been described for UGT1A1 and UGT2B7 [57–59].

It seems that the most important route of elimination of tamoxifen and its metabolites is via glucuronidation by the uridine diphosphate (UDP)–glucuronosyltransferases (UGTs), UGT1A8, 1A10, 2B7, 2B15, and 2B17 isoforms, with UGT2B7 being the most active hepatic UGT [60, 61]. UGTs catalyze the addition of glucuronide moieties to 4-OH-tamoxifen and endoxifen, which negate their antiestrogenic properties and promote their excretion [60, 61]. Tamoxifen is excreted predominantly through the bile, a process that is facilitated by tamoxifen conjugation to glucuronic acid during the glucuronidation process [62]. Missense polymorphisms have been identified in the UGTs active against metabolites of tamoxifen including nonsynonymous SNPs at codons 24 and 48 of the UGT1A4 gene, at codon 268 of the UGT2B7 gene, at codon 139 SNP of the UGT1A10 gene, and at codons 173 and 277 of the UGT1A8 gene [63].

In vitro studies suggested that UGT1A4 variant Leu48Val shows increased glucuronidation activity against tamoxifen and its metabolites. However, the clinical significance of this finding was not further explored [64]. The effect of a nonsynonymous polymorphism in UGTB215 has been investigated in breast cancer patients treated with adjuvant tamoxifen, and it was found that patients with SULT1A1*2*2 and either UGT2B15*1*2 or UGT2B15*2*2 had significantly reduced 5-year survival [65]. In addition, it has been demonstrated that O-glucuronidation of both trans-4-OH-TAM and trans-endoxifen in human liver tissue specimens was significantly associated with UGT2B7 genotype and with lower activities correlating with increasing numbers of the UGT2B7*2 (UGT2B7268268 Tyr) alleles [66]. The UGT2B7*2 allele is present in about 50 % of Caucasians and Asians.

Sulfotransferases are highly polymorphic. SULT1A1 is regarded to be the primary SULT responsible for the sulfation of 4-hydroxytamoxifen and endoxifen. Large individual variation has been demonstrated in the activity of SULT1A1 in humans. This variability has been attributed to a single-nucleotide polymorphism (G638A) in the coding region of the gene, to single-nucleotide polymorphisms in the 3-untranslated region and in the 3-flanking region of the gene, to gene deletion, and to gene duplication. The association of SULT1A1 polymorphisms with tamoxifen metabolism or with treatment outcome of tamoxifen therapy has been investigated, but the findings are discordant [67, 68].

Transporters can be classified as influx or efflux transporters, which are located either at the basolateral or apical membrane in polarized cells [69]. Efflux transporters include ATP-binding cassette transporter family (ABC) and multidrug toxin extrusion proteins (MATES) [70]. Influx

transporters are the organic anion transporters (OATs and OATPs), organic cation transporters (OATPs), and oligopeptide transporters [56].

ABC transporters are classified into seven families (ABCA to ABCG) based on the nucleotide-binding domain and the transmembrane domains. There are at least 49 ABC transporter genes, but mainly ABCB1, ABCC1, ABCC2, ABCC3, and ABCG2 are involved in drug transport [71]. More than 50 SNPs and other polymorphisms affect the function of the well-characterized ABCB1 gene encoding the transporter P-glycoprotein (P-gp) [56]. Data from P-glycoprotein knockout mice have suggested that P-glycoprotein is involved in the transport of tamoxifen metabolites endoxifen and 4-hydroxytamoxifen [72]. ABCB1 polymorphisms have been reported to correlate with the treatment outcome of tamoxifen therapy [73, 74]. In addition, Kiyotani et al. [75] demonstrated that the allele of *ABCC2*rs3740065 has an additive effect on recurrence-free survival outcome of adjuvant tamoxifen therapy for breast cancer patients, although no association was found between this polymorphism and plasma concentrations of tamoxifen metabolites [75].

While the majority of studies focus on the effect of CYP2D6 polymorphisms on the outcome of adjuvant tamoxifen treatment, CYP2D6 polymorphisms could also affect the outcome of chemoprophylaxis with tamoxifen. In that context, in a very recently published study, a nested case control study in the context of NSABP-1 and NSABP-2 was performed to investigate the impact of CYP2D6 genotype and CYP2D6 inhibitor use, as well as metabolizer status (CYP2D6 genotype combined with CYP2D6 use), on breast cancer events. Cases were women who developed breast cancer while on tamoxifen or raloxifene chemoprophylaxis for at least 5 years and controls were women free of breast cancer. CYP2D6 genotyping was performed for alleles associated with reduced, absent, or increased enzyme activity. The authors concluded that there was no effect of CYP2D6 metabolism on the efficacy of tamoxifen or raloxifene in breast cancer chemoprophylaxis [34].

Undoubtedly, further research is needed for predicting clinical outcome to tamoxifen treatment based on pharmacogenomics. Further research efforts should focus on prediction based on genetic variations of a number of genes, breast tumor genomics, epigenetics, and micro-RNA pharmacogenomics.

Pharmacogenomics and Aromatase Inhibitors

Exemestane is a noncompetitive third-generation aromatase inhibitor used in the treatment of breast cancer in postmenopausal women. A major pathway for exemestane metabolism is the reduction of the 17-keto group by aldo-keto reductase to form 17-dihydroexemestane [76]. Existing limited data suggest that 17-dihydroxyexemestane exhibits similar anti-aromatase activity with exemestane, implying that 17-dihydroxyexemestane may significantly contribute to the anti-aromatase activity of exemestane in vivo [77]. In this regard, it has been shown that the absolute concentration of 17-dihydroxyexemestane accounts for about 10–15 % of total exemestane concentration in the plasma of patients treated with exemestane [76, 78]. According to another study, the level of 17-hydroxyexemestane accounts for 35–40 % of exemestane in the plasma of patients treated with exemestane [79].

Another major pathway of exemestane metabolism is the subsequent 17-dihydroxyexemestane glucuronidation to exemestane-17-O-glucuronide [80]. Four UGT enzymes have been shown to exhibit activity against 17-dihydroxyexemestane in vivo: the hepatic UGT1A4 and UGT2B17 and the extrahepatic UGT1A8 and UGT1A10, with UGT2B17 being the major active enzyme responsible for the glucuronidation of 17-dihydroxyexemestane [81].

Literature data suggest that UGT2B17 deletion polymorphism might play a role in exemestane metabolism, with higher levels of 17-dihydroxyexemestane being associated with UGT2B17 deletion [81]. A recent patent, WO2011017696A2, provides methods for aiding in determining therapeutic efficacy of the aromatase inhibitor exemestane [82]. According to the present invention, UDP glucuronosyltransferase 2 family

polypeptide B 17 (UGT2B17) has activity to modify at least one metabolite of exemestane by glucuronidation, particularly 17-dihydroexemestane. Individuals having increased or decreased glucuronidation of at least one metabolite of exemestane, particularly 17-dihydroexemestane due to UGT2B17 polymorphism, have correspondingly decreased or increased therapeutic efficacy of exemestane treatment. Therefore, determination of UGT2B17 gene deletion, mRNA, and/or protein expression and/or enzyme activity in an individual subject aids in determining therapeutic efficacy of exemestane treatment in the individual [82].

In addition, pharmacogenomics of the host aromatase enzyme may account for variability in response to aromatase inhibitors. Eighty-eight polymorphisms accounting for 44 haplotypes have been identified for aromatase (cytochrome P450 19, CYP19 gene), a critical enzyme for estrogen biosynthesis [83]. In a relevant study, Colomer et al. [84] investigated the association of 3 SNPs in the CYP19 gene with the outcome of letrozole adjuvant treatment in postmenopausal women with hormone receptor-positive advanced breast carcinoma. The investigated SNPs were rs10046 and rs4646, located in the 3′ UTR, and rs727479, located in the intron of the CYP19 gene. Letrozole treatment was continued until disease progression or unacceptable toxicity occurred. No association was demonstrated between time to disease progression and rs10046 or rs727479 polymorphisms. In addition, it was shown that time to disease progression was significantly improved in patients with the rs4646 variant, compared with the wild-type gene (17.2 versus 6.4 months; $P=0.02$) [84]. However, in another study, it was shown that the polymorphism in rs4646 in the 3′UTR in the aromatase CYP19 gene was associated with poor response after 4 months of letrozole treatment, especially in elderly women [85].

CYP2A6 polymorphisms have also been implicated in letrozole pharmacokinetics. In a recent trial, Desta et al. investigated the associations between plasma letrozole concentrations and CYP2A6 and CYP3A5 genetic variants in a multicenter open-label prospective trial in women randomized to receive either 2 years of oral letrozole or exemestane [86]. A significant association was noted between plasma letrozole concentration and CYP2A6 variants, while no association was demonstrated between plasma letrozole concentration and CYP3A5 variants [86]. These data are in agreement with a population pharmacokinetic study in Japanese population that concluded that CYP2A6 genetic variants are causes of ethnic differences in pharmacokinetics of letrozole [87]. However, the authors noted that dose adjustment is not necessary due to the wide therapeutic range of letrozole.

Apart from prediction of treatment efficacy, pharmacogenomics also contribute to the prediction of adverse events associated with aromatase inhibitor-treated patients. Aromatase inhibitors can result in musculoskeletal pain that could lead to 10–20 % of patients to terminate treatment. In a genome-wide association study that used DNA samples from a large clinical trial of aromatase inhibitor treatment in breast cancer patients, there was an association between musculoskeletal pain and variants in the gene cluster encoding T-cell leukemia–lymphoma proteins (TCL) [88].

Pharmacogenomics and Fulvestrant

Fulvestrant is a selective estrogen receptor downregulator that behaves as a complete antagonist, competitively inhibiting binding of estradiol to the estrogen receptor, with a binding affinity that is 89 % that of estradiol [89, 90]. Fulvestrant is a 7α-alkylsulphinyl analog of 17β-estradiol, which is distinctly different in chemical structure from the nonsteroidal chemical structures of tamoxifen, raloxifene, and other selective estrogen receptor modulators. The drug is intramuscularly administered and is devoid of all known estrogen receptor agonist effects [89, 90]. Fulvestrant has been approved as second-line treatment for postmenopausal women with estrogen receptor-positive advanced breast cancer who have shown disease progression following prior endocrine therapy [91]. Up to now, the effect of genetic variation on the treatment outcome of fulvestrant has not been investigated. Regarding metabolism of fulvestrant, evidence suggests that cytochrome P450 enzymes, UDP-glucuronosyltransferases, and sulfotransferases are involved in the metabolism of fulvestrant [91].

Pharmacogenomics of Estrogen Receptors

The physiological effects of estrogen in the breast are mediated by estrogen receptors that are expressed as two structurally related subtypes, estrogen receptors α (ERα) and β (ERβ) [92]. ERα is the predominant receptor isoform expressed in breast cancer cells, and approximately 70 % of breast cancer patients score positive for ERα upon diagnosis [93]. Tamoxifen acts via modulation of estrogen receptors. De novo resistance to tamoxifen treatment derives primarily from loss of ERα expression.

ER polymorphisms might also account for the lack of response of breast cancer patients to adjuvant endocrine treatment. SNPs have been identified for both ERS1 and ESR2, the genes that encode for ERα and Erβ, respectively. For instance, differential expression of the exon 5 deletion splice variant of the ER (del5-ER) has been proposed to account for acquired resistance in tamoxifen treatment [94]. In addition, two rare point mutations in the ER, Asp351Tyr and Tyr537Asn, have been shown to be associated with variant response to estradiol and antiestrogens [95]. An association between ER polymorphisms and change in bone mineral density in women treated with selective estrogen receptor modulators has been suggested [96, 97]. However, a recent study found no association between the ERα SNPs (*Xba*I = rs 9340799 and *Pvu*II = rs 2234693) and the ERβ SNPs (ESR2_01 = rs 1256049 and ESR2_02 = rs 4986938) and the bone mineral density of women treated with tamoxifen [98]. In addition, evidence suggests a possible role of tandem repeats of ERα gene in tamoxifen response of breast cancer patients [99].

Clinical Implications of Pharmacogenomics of Breast Cancer Endocrine Treatment

In clinical practice, the interest focuses on the clinical implications of pharmacogenomics of breast cancer endocrine treatment. Undoubtedly, the most thoroughly investigated issue is the association between CYP2D6 polymorphisms and treatment of breast cancer in the adjuvant setting. In that context, an interesting issue is whether CYP2D6 genotyping could guide tamoxifen dosing, which has been suggested in a few recent trials investigating this issue [100–102]. Thus, in a recently published prospective study, Irvin et al. [100] investigated whether CYP2D6 genotyping could determine optimal tamoxifen dose. CYP2D6 genotype and tamoxifen metabolite concentrations were assessed in breast cancer patients taking adjuvant tamoxifen for at least 4 months. Extensive metabolizers continued with 20 mg daily, while the dosage was increased to 40 mg daily for poor and intermediate metabolizers. Four months later, tamoxifen metabolites were assessed again. Mean endoxifen measurements increased in both poor and intermediate metabolizers. Thus, the authors concluded that CYP2D6 genotyping could guide tamoxifen dosing [100].

In another recent trial, Kiyotani et al. [101] enrolled 98 Japanese women with breast cancer who had been treated with 20 mg of tamoxifen daily in an adjuvant setting. Dosages of tamoxifen were increased to 30 and 40 mg daily for the patients who had one or no normal allele of CYP2D6, respectively. The plasma concentrations of tamoxifen and its metabolites were measured at 8 weeks after dose adjustment using liquid chromatography-tandem mass spectrometry. In the patients with CYP2D6*1/*10 and CYP2D6*10/*10, the mean plasma endoxifen and 4-hydroxytamoxifen levels after dose increase were significantly increased and at similar levels as those of breast cancer patients with the genotype CYP2D6*1/*1 receiving 20 mg/day of tamoxifen. Dose adjustment did not seem to affect the incidence of adverse events [101].

Breast cancer patients taking adjuvant tamoxifen treatment are often co-prescribed selective serotonin reuptake inhibitors (SSRIs) to alleviate menopausal symptoms, as shown in Table 25.7 [12, 103–105]. However, given that SSRIs are strong CYP2D6 inhibitors, there is growing interest on the effect of SSRI co-prescription on endoxifen levels. Moderate/weak CYP2D6 inhibitors are cimetidine, amiodarone, and haloperidol. A retrospective study investigated the effect of co-prescription of CYP2D6 inhibitors

Table 25.7 Trials investigating the effect of CYP2D6 interacting drugs on tamoxifen treatment outcome

Tamoxifen indication	Outcome	Trial reference
Adjuvant breast cancer treatment	Coadministration of paroxetine decreased the plasma concentration of endoxifen	[12]
Adjuvant breast cancer treatment	The plasma endoxifen concentration was slightly reduced in women taking venlafaxine, a weak inhibitor of CYP2D6, while it was substantially reduced in patients taking paroxetine, a potent inhibitor of CYP2D6	[104]
Adjuvant breast cancer treatment	Patients displaying a decreased CYP2D6 metabolism after co-prescription of a potent or moderate CYP2D6 inhibitor had significantly shorter time to recurrence and worse relapse-free survival	[103]
Breast cancer—stage not known	Women with breast cancer who received paroxetine in combination with tamoxifen were at increased risk for death from breast cancer and death from any cause	[105]

and CYP2D6 status in breast cancer patients treated with tamoxifen, and it was found that the clinical benefit of tamoxifen was significantly decreased in patients with decreased CYP2D6 activity, either due to the poor metabolite genotype or to concurrent treatment with CYP2D6 inhibitors [103]. In addition, pharmacokinetic studies have demonstrated that women who are poor metabolizers of CYP2D6, either by genotype (PM/PM) or by a CYP2D6 inhibitor like some of the serotonin reuptake inhibitors (paroxetine or fluoxetine) which are often co-prescribed to alleviate hot flashes, have lower endoxifen plasma concentrations than patients with normal CYP2D6 metabolism [12].

Based on the above available evidence, mandatory use of CYP2D6 genetic test requires additional data from randomized clinical trials. The FDA has agreed that CYP2D6 is a predictor of tamoxifen efficacy. They have recommended relabeling tamoxifen to say that CYP2D6 poor metabolizers who take tamoxifen have a higher risk for breast cancer recurrence and that testing is available. The AmpliChip CYP450 Test is an FDA-approved test that uses the Affymetrix microarray technology, i.e., the DNA microarray technology that allows the simultaneous testing of thousands of DNA sequences, combining hybridization in precise locations on a glass microarray and a fluorescent labeling system. This test classifies individuals into three CYP2D6 phenotypes: (1) ultrarapid metabolizers, (2) intermediate metabolizers, and (3) poor metabolites by testing 27 alleles, including seven duplications. In addition, it classifies individuals into two CYP2C19 phenotypes—extensive metabolizers and poor metabolizers—by testing three alleles.

Concerning the use of CYP2D6 inhibitors, strong CYP2D6 inhibitors such as the selective serotonin reuptake inhibitors paroxetine and fluoxetine, which are used to treat hot flashes, should be avoided because they severely impair formation of the active metabolites [106].

Pharmacogenomics and Trastuzumab: Clinical Implications

The ErbB family of type I receptor tyrosine kinases includes ErbB1 (also known as the epidermal growth factor receptor (EGFR or HER1), ErbB2 (also known as Her2), ErbB3, and ErbB4. All HER receptors have a similar structure, including an extracellular ligand-binding domain, a short hydrophobic transmembrane region, and a cytoplasmic tyrosine kinase domain. These receptor tyrosine kinases are widely expressed in epithelial, mesenchymal, and neuronal tissues where they play a role in regulating cell proliferation, survival, and differentiation. Increased expression of ErbB2 or EGFR has been correlated with poor outcome in breast cancer patients.

Trastuzumab (Herceptin™) is a humanized anti-ErbB2 monoclonal antibody that has been approved for the treatment in the adjuvant and the metastatic setting of breast cancers that either overexpress ErbB2 or demonstrate ErbB2 gene

amplification [107]. Trastuzumab binds to the extracellular domain of the ErbB2 receptor and exerts its antitumor effects via several mechanisms:

1. Prevents cleavage of the extracellular domain of HER2 and thereby activation of the receptor
2. Blocks the dimerization of HER2
3. Mediates activation of antibody-dependent cell-mediated cytotoxicity leading to tumor cell lysis
4. Promotes HER2 internalization

Trastuzumab has been approved for the treatment in the adjuvant and the metastatic setting of breast cancer patients who either overexpress ErbB2 or demonstrate ErbB2 gene amplification. Trastuzumab is indicated in early breast cancer as adjuvant treatment and as neoadjuvant treatment. In the case of metastatic $HER2^+$ breast cancer, trastuzumab is indicated in combination with an aromatase inhibitor in postmenopausal women with endocrine-responsive breast cancer not previously treated with trastuzumab, or as monotherapy after at least one or more chemotherapy regimens, or in combination with paclitaxel or docetaxel. A serious adverse event of trastuzumab is reduction of left heart ejection fraction, resulting to congestive heart failure. A number of SNPs have been reported in the extracellular, transmembrane, and cytoplasmic regions of HER2. However, thus far, there is limited knowledge on SNPs that could affect the binding, efficacy, or tolerability of trastuzumab.

The most thoroughly investigated SNP at clinical level concerns Ile655Val (codon 655GTC/valine to ATC/isoleucine in the transmembrane domain of the HER2 protein) that is a potentially functional SNP. Predictions based on in silico models have suggested that this SNP increases protein kinase activity. In addition, it has been suggested that the Val allele may constitute a risk factor for trastuzumab-induced cardiotoxicity. A nonsynonymous coding SNP rs4252633 has been identified in the extracellular domain of HER2 that is targeted by trastuzumab. However, thus far, the functional consequences of this SNP are not known. As stated above, antibody-dependent cell-mediated cytotoxicity via interactions with Fcγ receptors (FcγR) on leukocytes may contribute to the antitumor toxicity of trastuzumab. Single-nucleotide polymorphisms (SNP) in FCGR3A and FCGR2A genes lead to amino acid substitutions at positions 158 and 131, respectively, and affect binding of antibodies to FcγR. A very recent study found no correlation between Fcγ receptors IIIa and IIa and clinical outcome in trastuzumab-treated patients with HER2 nonmetastatic breast cancer [108] in agreement with another recent trial that came to the same conclusion for patients treated with taxane plus trastuzumab chemotherapy for HER2-positive metastatic breast cancer [109].

SNPs coding for hepatic cytochromes or cell surface transporters are not expected to affect trastuzumab pharmacokinetics, as trastuzumab does not appear to be metabolized by CYP2C9, CYP2C19, and CYP2D6 or to be a substrate for P-glycoprotein.

Pharmacogenomics and Lapatinib

Lapatinib is an orally active dual tyrosine kinase inhibitor of the epidermal growth factor receptor 1 (ErbB1) and human epidermal receptor 2 (HER2). Lapatinib is used in combination with capecitabine or letrozole in patients with progressive HER2-overexpressing metastatic breast cancer previously treated with an anthracycline, a taxane, and trastuzumab. Lapatinib downregulates tyrosine phosphorylation by binding to the ATP-binding domain. No coding SNPs have been identified in the ATP-binding domain of HER2; thus, it is not expected that HER2 polymorphisms affect safety and efficacy of tyrosine kinase inhibitors [110].

Although lapatinib has an acceptable safety profile for the treatment of breast cancer, serious drug-induced liver injury (DILI) in cancer patients receiving lapatinib has been reported. Although this clinical liver injury could be attributed to some of the metabolites of lapatinib, the role of pharmacogenomics should not be discounted. However, knowledge on the pharmacogenomics of lapatinib is quite limited. CYP2C9 seems to have a minor contribution to the metabolism of lapatinib. In addition, in vitro studies have shown that the drug is a substrate for

P-glycoprotein. However, currently, it is not known if polymorphisms of CYP2C9 or ABCB1 affect the pharmacokinetics of lapatinib.

Pharmacogenomics and Bevacizumab

A network of blood vessels is needed for the survival and growth of tumor cells and thus for primary tumor development, invasiveness, and metastasis. Therefore, angiogenesis, i.e., the process of growth of new blood vessels, is regarded as a key target for the development of new therapeutic strategies for breast cancer. A number of agents have been developed to inhibit the vascular endothelial growth factor (VEGF) pathway, which plays a key role in both normal and tumor angiogenesis. The most successful strategies have been based on direct inhibition of the VEGF ligand with a specific monoclonal antibody or inhibition of the VEGF receptor using small-molecule tyrosine kinase inhibitors (TKIs). Bevacizumab, a humanized monoclonal antibody directed against VEGF, is the first antiangiogenic treatment approved by the FDA and European Agency for the European Medicines Agency (EMA). However, a significant number of patients do not respond to angiogenesis inhibitor therapy. The mechanisms underlying the nonresponsiveness are still unknown. In addition, angiogenesis inhibitor therapy is associated with a number of serious adverse events, including gastrointestinal perforation, thrombosis, bleeding, hypertension, and proteinuria. Indeed, no genetic and molecular markers to predict or monitor the efficacy of bevacizumab or the resistance to bevacizumab have been found yet. Thus, there is a need for identifying methods of determining which patients respond well to angiogenesis inhibitor therapy. Since the best known mechanism of action of the antibody is the inhibition of VEGF, personalized treatment with bevacizumab could be attempted through investigation of polymorphisms at the VEGF gene as well as at other genes involved at the VEGF pathway. These genes include the VEGF gene, also referred to as VEGFA; homologues to VEGF including placenta growth factor (PlGF); VEGFB; VEGFC; VEGFD; the VEGF receptors, including VEGFR-I and VEGFR-2 (also referred to as FLTl and FLK1/KDR, respectively); the VEGF inducers, including hypoxia-inducible factors HIFl and HIF2; and the oxygen sensors PHDl, PHD2, and PHD3. However, up to now, research efforts have not come to any outcome with clinical implications. VEGF-A SNPs seem to be important for determining the risk, prognosis, and survival of breast cancer patients, but their role is controversial as predictors of benefit from bevacizumab treatment.

In a relevant study, Schneider et al. [111] reported the association between different VEGF-A genotypes and median overall survival of patients treated with bevacizumab (with paclitaxel chemotherapy) in a Phase III clinical trial of metastatic breast cancer. In a retrospective review of cases, it was shown that VEGF-A -2578AA genotype was associated with a superior overall survival in the arm treated with paclitaxel and bevacizumab versus paclitaxel alone. In addition, the authors demonstrated that patients carrying the VEGF-A -1154A allele had superior overall survival in the combination arm [111]. On the other hand, in another study, the authors analyzed 137 women with locally recurrent or metastatic breast cancer receiving first-line bevacizumab-containing therapy for VEGF-A polymorphisms at position −2578 C/A, −1498 T/C, −1154 G/A, −634 G/C, and +936 C/T [112]. No association was demonstrated among any of the above polymorphisms and clinical outcome. However, the analysis of the +936C/T polymorphism revealed that the patients who were homozygous for the 936C allele exhibited a marked tendency for a shorter time to progression than the patients bearing the 936 T allele [65]. It should be emphasized that with the exception of renal carcinoma, bevacizumab is coadministered with chemotherapy. Thus, part of the observed antiangiogenetic effect might be attributed to the chemotherapeutic agent, making research of pharmacogenomics of bevacizumab even more demanding.

Finally, an alternative approach has been proposed for the prediction of outcome of treatment with bevacizumab: instead of investigating

VEGF-A polymorphisms, the investigation of the polymorphisms of other angiogenesis-related genes has been suggested. However, even with this approach, no fruitful outcomes have been achieved up to now. On the other hand, Kim et al., using a genome-wide SNP screening with a human SNP array and an in vitro chemosensitivity assay in 118 colorectal cancers, attempted to identify SNPs of a new gene associated with response to bevacizumab treatment. Angiogenesis-unrelated genes, such as ANXA11 and LINS1, were found to be associated with response to bevacizumab treatment. However, the clinical implications of this finding, if any, have not yet been verified [113].

Pharmacogenomics and Breast Cancer Chemotherapy

Although pharmacogenomics could partially explain variability in efficacy and safety of breast cancer chemotherapy, currently scientific data are quite limited and often controversial and clinical implications are not straightforward.

Antimetabolites are structurally similar to naturally occurring nucleotides and act by incorporating into DNA or RNA or by inhibiting proteins involved in nucleotide metabolism. All pyrimidine antagonists are prodrugs and they are converted intracellularly into cytotoxic nucleosides and nucleotides. The most commonly used pyrimidine antagonists are 5-fluorouracil (5-FU), gemcitabine (dFdC), and cytarabine (ara-C) and the newer oral variants of 5-FU, capecitabine and tegafur. Theoretically, polymorphisms in thymidylate synthase, methylenetetrahydrofolate reductase (MTHFR), and dihydropyrimidine dehydrogenase (DPD) enzymes might influence the pharmacodynamics of fluoropyrimidines; however, currently relative data are quite limited. Polymorphisms in thymidylate synthase gene have been associated with worse outcome of capecitabine or 5-FU treatment in breast cancer patients. DPD polymorphisms have been associated with more severe toxicity, especially neurotoxicity of 5-FU or capecitabine treatment.

Cyclophosphamide is an alkylating agent that is commonly used in combination therapy to treat breast cancer. Cyclophosphamide is a prodrug that is converted to its active metabolite by the CYP450 oxidative enzymes. Cyclophosphamide is a substrate for the metabolizing enzymes CYP2B6, CYP2C9, CYP2C19, and CYP3A5. In addition, cyclophosphamide is also a substrate for the enzymes glutathione S-transferase (GST) and aldehyde dehydrogenase. The pharmacogenomics of cyclophosphamide have not been studied extensively because it is commonly used in combination with other chemotherapeutic agents.

Anthracyclines, doxorubicin, and epirubicin exhibit extremely variant pharmacokinetics among patients. Carbonyl reductases and aldo-keto reductases convert doxorubicin and epirubicin via phase 2 reduction reactions to doxorubicinol and epirubicinol, respectively, which are then inactivated by CYP3A4 and CYP3A5. Doxorubicin is a substrate for ABCB1 and the solute transporter SLC22A16. Polymorphic variants of the genes coding these enzymes or transporters have been identified, which might influence the systemic pharmacology of the drug, i.e., GST polymorphisms have been associated with improved outcome to doxorubicin treatment due to lower enzyme activity leading to greater drug availability. However, up to now, the contribution of polymorphisms of the genes encoding the abovementioned enzymes remains controversial.

The taxanes paclitaxel, docetaxel, and nab-paclitaxel have been investigated as first-line therapy for MBC requiring chemotherapy in numerous trials. Taxanes are typically used in combination with other chemotherapeutic agents, namely, anthracyclines. Taxanes exert their antitumor effects by inducing apoptosis indirectly by stabilizing normally dynamic microtubules through binding to sites on tubulin dimers. Drug resistance has been attributed to polymorphisms of the gene encoding β-tubulin. Circulating taxanes are taken up by SLCO1B3 into hepatocytes. The CYP450 enzymes CYP3A4, CYP3A5, and CYP2C8 are responsible for the conversion of taxanes into their metabolites. ABCB1 and ABCC2 will dispose the metabolites into bile canaliculi. Polymorphisms of genes encoding for proteins involved in the transport and clearance of taxanes reduce excretion of the

drugs, leading to development of toxicity in patients. Polymorphisms of CYP2C8 have been associated with decreased metabolism of taxanes, and ABCB1 polymorphisms have been associated with treatment resistance.

Conclusion and Future Perspective

Existing evidence suggests that genetic variation affects the outcome of breast cancer therapy. However, further research is needed, taking into account all known sources of genetic variation, before appropriate clinical recommendations are given in practice.

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Breast Cancer Gene Therapy

26

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Abstract

Breast cancer, one of the most common female malignancy around the world, is a major public health problem. It is estimated that 1 woman in 9 will develop breast cancer during her lifetime. Conventional therapies, such as radiotherapy, chemotherapy, and hormonal therapy, have become more efficient in recent years. However, even if response is relatively good to treatments for cancers detected and treated early, the prognosis remains poor for advanced cancers due to the presence of metastases. As alternative to these conventional therapies, gene therapy is increasingly designed as a treatment solution to treat different types of cancers, such as breast cancer, ovarian cancer, lung cancer, cervix cancer, etc. Gene therapy is to repair a defective gene by introducing a healthy gene having a sequence of genetic information (DNA or RNA) into a cell to modify the expression of specific genetic program of that cell. It permits to target the causes of a disease that it is due to the mutation of a single gene or a more complex disorder. However, this repair usually requires the use of a kind of Trojan horse, which will introduce a healthy gene into the genome of the mutant cell responsible for cancer. Defined in the broad sense, gene therapy includes immunogene therapy, suicide gene therapy, correction of tumor suppressor genes, as well as oncogenes and antiangiogenic gene therapy. At this time, since gene therapy is experimental and far from clinical application running, the current data do not allow to use this approach as a alternative treatment to conventional therapy.

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Keywords

Breast cancer • Gene therapy • Genetic information • Mutation • Oncogenes • Tumor suppressors • DNA • RNA • Vectors

Introduction

Recent knowledge of the human genome and the application of molecular genetics to human biology have allowed new discoveries leading to a better understanding of how to diagnose and treat a variety of diseases. More recently, tremendous advances in molecular medicine, such as immunology, virology, genetics, and tumor biology, have generated considerable enthusiasm for gene therapy [1]. Cancers are genetic diseases that develop in a multistep process resulting from an accumulation of a series of genetic changes, such as mutations in DNA-repairing genes, in oncogenes, and tumor-suppressor genes which regulate normal cellular function in the genome of cells, causing alterations in the communication between cells [2, 3]. Breast cancer is one of the most important malignancies in women that accounts for 21 % of new cancer cases throughout the world [4, 5]. Five percent to 10 % of breast cancer has a genetic origin, i.e., hereditary, and 85–90 % of cases (known as sporadic or nonhereditary) have environmental origins that are yet to be understood [6, 7]. Conventional therapies, such as radiotherapy, chemotherapy, and hormonal therapy, have become more efficient for early detected cancers (Table 26.1). However, they are unable to cure the disease permanently and they still remain poor for advanced cancers. In parallel to these conventional therapies, gene therapy—a therapeutic strategy that embeds genes in cells or tissues of an individual to treat a disease—could be an alternative approach that can be used in the treatment of both genetic and acquired diseases [1, 9–11]. Even though the accessible and practicable vector systems for gene therapy can favorably transfer the genes into cells, the ideal delivery vehicle has not been found yet (Fig. 26.1). In this regard, many studies are ongoing to find more efficient viral and nonviral vector systems. Different therapeutic approaches can be considered when the molecular and pathophysiological basis of disease is disbanded. Pharmacological approach for which one can try to design a specific drug based on the functional properties of the deficient gene product or genetics for which manipulation based on the insertion of “transgenes” compensate the dysfunction of endogenous genes supporting the production of the deficient protein in cells where the anomaly is expressed. The prospects for this type of treatment can be applied to constitutional genetic diseases due to the alteration of a single gene or acquired diseases such as AIDS, cancer, thromboembolic disease, and cardiovascular disease or still some degenerative neurological diseases [12–14]. Insofar as the cancer is linked to the production of multiple mutations in a single cell that causes dysfunction of certain genes and the proliferation is out of control, a gene therapy permits to replace a defective gene responsible for disease by intact gene-encoding proteins. To this end, it can help prevent the action of proteins activating the uncontrolled proliferation of cells or restoring the functions of proteins that control cell division [10, 15]. Gene therapy is not a treatment, but it is one method of treatment. The principle is to “inject” a “normal” gene into a cell, in order either to replace a defective gene or to make a substance to destroy tumor cells, such as interleukin which is still experimental and cannot be prescribed outside clinical trials [16–18]. Cancer gene therapy is the transfer of nucleic acids which may be genes, portions of genes, oligonucleotides, or RNA into cancer or normal cells. Gene therapy for cancer includes different approaches, such as mutation correction, enhancement of the immune response against tumors, suicide gene, and antiangiogenic therapy to correct genetic errors or immunomodulation to reverse the malignant state [19].

Table 26.1 Conventional therapies for breast cancer

Surgery	Breast-conserving surgery, defined as lumpectomy or partial mastectomy, combined with axillary node dissection and local radiotherapy may be used for early stage (stages I and II) breast cancers
Radiotherapy	Postsurgical radiation therapy involves irradiation of the anterior chest wall, the ipsilateral internal mammary lymph nodes, the apex of the axilla, and the supraclavicular node
Cyclophosphamide	An alkylating agent, cyclophosphamide cross-links DNA, which causes a misreading of the DNA template or promotion of degradation by nucleases and cell death
Doxorubicin and related anthracyclines (daunorubicin, epirubicin, idarubicin)	These exert their cytotoxic effects by a combination of DNA intercalation, interference with topoisomerase II, formation of free radicals, chelation of metals, and damage to cell membranes
Methotrexate	Methotrexate is an inhibitor of dihydrofolate reductase, an enzyme required to maintain the supply of reduced folates for DNA and RNA synthesis
5-Fluorouracil (5-FU)	5-FU exerts its cytotoxic effects through incorporation into RNA as fluorouridine triphosphate (FUTP). This incorporation interferes with RNA synthesis and function. Moreover, as fluorodeoxyuridine monophosphate (FdUMP), it interferes with thymidylate synthase, an enzyme required for DNA synthesis
Vincristine	Vincristine exerts a cytotoxic effect by binding to tubulin and preventing its polymerization. This action interferes with the formation of the mitotic spindle and mitosis
Mitomycin C	Mitomycin C is metabolized in vivo to an alkylating agent that binds to DNA and inhibits DNA synthesis
Mitoxantrone	Mitoxantrone interferes with DNA synthesis by intercalation with DNA or by inhibiting topoisomerase II
Paclitaxel	Paclitaxel causes mitotic arrest in cells by stabilizing polymerized tubulin and the mitotic spindle
VP-16	VP-16 causes DNA strand breaks in cells by binding to topoisomerase II and preventing rejoining of DNA strands
LHRH analogs	Goserelin is a synthetic peptide analog of LHRH that is used as palliative treatment for premenopausal women with estrogen and/or progesterone receptor positive metastatic breast cancer
Tamoxifen	Tamoxifen is indicated primarily for the treatment of ER-positive breast cancer. Tamoxifen may decrease the production of TGF-a by ER-positive tumor cells
Letrozole	The aromatase inhibitors anastrozole and letrozole block estrogen synthesis by inhibiting cytochrome P450 aromatase
Trastuzumab	Trastuzumab is a humanized, monoclonal antibody that binds specifically to the HER2/neu growth factor receptor. The antibody acts by blocking the ligand activation of the HER2/neu-mediated signal-transduction pathway or by activating antibody-dependent cellular cytotoxicity (ADCC)

Adapted from Reilly [8]

Basically two types of gene therapy have been applied to humans, germinal and somatic:

- Germinal gene therapy introduces transgenic cells into the germ line as well as into the somatic cell population, not only to achieve a cure for the individual treated, but some gametes could also carry the corrected genotype.
- Somatic gene therapy focuses only on the body, or soma, attempting to effect a reversal of the disease phenotype by treating some somatic tissues in the affected individual [20].

For cancer gene therapy, broadly speaking there are three strategies which are used to achieve the goals of gene therapy: (1) in vitro/ex vivo, which initially involves removal of the patient's target cells in order to culture in vitro and are then incubated for genetic modification with the viral vector carrying the therapeutic gene. The genetically altered cells are then reintroduced into the patient's

Fig. 26.1 Features required for an ideal vector

body. This method is used particularly with blood cells that are easy to remove and reinsert; (2) in situ consists of placing directly the transfer vector into the target tissue. This technique is used particularly in cases of cystic fibrosis (transfer vectors in the trachea and bronchi), muscular dystrophy (injection into the muscle of a vector carrying the gene for dystrophin), or cancer (tumor injection of a vector carrying the gene for a toxin); (3) in vivo involves injecting the vector carrying the therapeutic gene directly into the diseased cells of the organism via vectors and the vector is then supposed to reach specific target cells. But, of course, gene therapy is more difficult to achieve in vivo [13, 21].

Successful gene therapy requires decisions concerning the choice of suitable biological, chemical, and physical methods in order to modify the numbers of target cells necessary to obtain the desired therapeutic effect (Table 26.2).

Table 26.2 Comparison among biological, chemical, and physical methods of gene delivery

Gene delivery method	Advantages	Disadvantages
Biological method		
Retroviral vectors	High transfection efficiency in dividing and nondividing cells	Not easy to manufacture and require cold storage
Adenoviruses	Possible targeted delivery	Cumbersome quality control requirement
Adeno-associated viruses	Possible systemic delivery	High cost, immunogenicity and oncogenicity risks
Lentiviruses	Stable expression	Limited size of gene to be inserted
Other types viral vectors		
Physical methods		
Direct intramuscular injection of plasmid DNA	Higher local tissue transfection efficiency	Need for a specific instrument
Direct intracellular microinjection	Direct intracellular microinjection	Need of parameter optimization for different types of cells
Electroporation	Transfection in all cell types, even in difficult to transfect cells, is achievable	Higher tissue damage observed
Jet injection	Easy process standardization	
Particle bombardment (biolistics)	Less limit on gene size	
	Mostly ex vivo applicable	
Chemical methods		
DEAE-dextran	High in vitro transfection achieved	Low in vivo transfection efficiency
Calcium phosphate co-precipitation	Possible in vivo organ targeting	Low efficiency in primary and nondividing cells
DNA-polylysine-cell receptor conjugates	Less costly	Limited clinical success
Polybrene-DMSO	Less limit on gene size	Consistent reproducible large-scale manufacturing
Liposome-mediated DNA transfer	Simple to manufacture in small batches and storage conditions are more flexible	
	High commercial interest	

Adapted from Jinturkar et al. [22]

Basis of Gene Therapy for Cancer

Progress in cancer gene therapy is largely depending on the development of novel vectors with maximum therapeutic efficacy at the target site and minimal toxicity to normal tissues [23].

Vector Characteristics for Gene Transfer

Once the gene is selected for its therapeutic potential against a disease, a crucial step in gene therapy is to enter the new genetic information into the targeted tissue or cells [21, 24]. Several hurdles can prevent the efficient uptake sequence of genetic information into cells. Such hurdles include the physical makeup of the proteins and carbohydrates matrix of extracellular milieu, which can physically hinder the movement of exogenous materials. Secondly, immunologically mediated factors, such as opsonins and phagocytes, can recognize and digest materials identified as "foreign." In addition, both intracellular and extracellular enzymes such as DNAse and RNAse can destroy new genetic material to be transferred. In order to achieve efficient cellular uptake, these "genetic information cargoes" need to be carried within a vehicle. For this purpose, delivery of therapeutic genes involves the use of carrier vehicles, called vectors. For the optimum therapeutic effect, certain conditions must be fulfilled by the vector and the gene(s) to be transferred: (1) the vector should have high transfection efficiency; (2) the vector must particularly target disease cells, such as the tumor cells including metastatic cells; (3) for optimal gene expression, there should be controllable genes which can be combined with appropriate promoter enhancer sequences; and (4) the vector must have low toxicity and low immunogenicity (see Fig. 26.1) [25, 26]. Currently, gene therapy vehicles or vectors can be broadly divided as viral, nonviral, and physical methods. Each has own advantages and disadvantages, as shown in Table 26.3 [26–29].

Viral Vectors

Both RNA and DNA viruses are utilized as viral vectors [30]. Having the advantage of high delivery efficiency toward a variety of cells, they were the first vectors employed in gene therapy clinical trials [31]. Viruses with a small number of genes are constructed to introduce new genetic material into the cells. They are surrounded by a protective protein coat which allows transport into the cell where the viral genetic information can be produced using the host cell's own translational machinery. Hence they are suitable gene delivery vehicles [32]. Even though these properties make viruses extremely attractive as vectors for gene therapy, they are also responsible for their pathogenicity. The viral vectors used for gene transfer are genetically modified viruses by deleting so-called secure. The principle is to eliminate the virus sequences that encode proteins, including those associated with potential pathogenic behavior of the virus, and keep only those that are used to build the viral particle and ensure the infection cycle. The virus genome is rebuilt to carry the therapeutic gene sequences [28, 32, 33].

Retrovirus

Most retroviral vectors are derived from retroviruses and they are the basic tool used to get a good transfer and stable expression of a therapeutic transgene. Retroviruses are the first viruses tested whose genome is composed of single-stranded diploid RNA molecules (ribonucleic acid). The retroviruses genome size is about approximately 8–11 kb base pairs, and they can accept up to 7–10 kb of exogenous gene sequences [34–36]. Currently, 60 % of clinical protocols are based on the use of retroviral vectors derived from murine leukemia retrovirus (MLV, Moloney virus in particular) which is the principal virus used in vectorology [37]. When a retrovirus infects a host cell, it will introduce its RNA together with some enzymes, namely, reverse transcriptase and integrase, into the cell [28, 30, 34]. This molecule RNA retrovirus must produce a DNA copy of the RNA molecule before the integration into the genetic material of the host cell's DNA. Retroviruses are naturally

Table 26.3 Main characteristics of the vectors currently authorized in clinical practice or study

Vectors	Benefits	Disadvantages
Retrovirus	Integration into the genome of the host	Only infects cells in cycle
	Prolonged expression in dividing cells	Generally low transfer efficiency
	Nontoxic in the absence of helper virus	Limited transfer of 8–9 kb DNA
	Variety of potential target cells	
Adenovirus	Transfer of DNA 15 kb	Transient expression due to the non-integration into the genome host
	Variety of potential target cells	Highly inflammatory and immunogenic, making it difficult to repeated administration
	Ability to produce large amounts of virus	Risk of recombination in the case of exposure to wild virus with possibility of restoring infectivity of the virus used for the transfer
	Authorizing the stability in vivo	
	High expression of the transgene	
	No integration into the host genome: no risk of insertional mutagenesis	
Adeno-associated virus (AAV)	Nontoxic (no known association with human disease)	Low efficiency of infection requiring the use of a large number of viral particles
	Specific integration into the host genome at a site known (chromosome 19)	Low frequency of integration into the genome of the host
	Authorizing the stability in vivo	Requires using molecules produced by adenovirus to be introduced into the target cell
	Variety of potential target cells	Being introduced into the target cell
		Preparation is very difficult for large amounts of virus
		Difficulty in obtaining preparations containing no helper virus (required for adenovirus manufacture of AAV)
		Limited size of the transgene
Lentivirus	Infection of quiescent cells	Theoretical risk of recombination with wild-type virus highly pathogenic
	Integration into the genome of the host	Specificity restricted to CD4 positive (in the absence of modification of the envelope)
	Targeting possible by modification of the envelope proteins	Expression of the transgene unstable
Herpesvirus	Large payload capacity (30 kb)	Lack of integration in the genome of the extended potence
	Easy handling	Significant cytopathic effect (1st generation)
	High viral titers	High prevalence of seropositive
	Prolonged latency (neurons)	Possible reactivation of latent wild

integrated into the genome of the host cell if it is in mitosis, such as tumor cells [23, 28, 34]. RNA viral vectors under development include oncoretroviruses encoding structural genes of *gag, pol,* and *env*, and lentiviruses and spumaviruses, which contain additional viral proteins [38, 39].

Lentiviruses (slowly replicating retrovirus) are another family of retroviruses that have the ability to infect cells that do not divide and have been the most studied retroviral vectors for gene delivery in recent years. This is the type of virus that causes immune suppression of HIV (human immunodeficiency virus) that causes acquired immunodeficiency syndrome (AIDS) [39, 40]. Lentiviral vectors are called "complex" because in addition to *gag, pol*, and *env,* genes, they have three to six additional viral proteins such as *tat, rev, nef, vif, vpr,* and *vpu* [38, 39]. Since these proteins facilitate an active transport of the pre-integration complex through the nucleopore, contrary to other lentiviral vectors, they do not require the breakdown of the nuclear membrane and so they are also able transduce nondividing quiescent cells [28, 41–43].

Adenovirus

Adenoviruses are double-stranded DNA viruses whose genome consists of 36–38 kb DNA in size [28]. The genetic material is contained in a protein structure called capsid. Fixation of this capsid to target cells is made possible by the presence of long protein fibers that interact with the receptor cell membrane. In order to use human adenovirus for gene transfer, E1 and E3 genes, which are essential for its replication, are deleted and replaced by the therapeutic gene sequence. This type of adenovirus is called defective [45–47]. This recombinant adenovirus is produced in vitro by infecting human cells in culture which have the E1 and E3 genes, thereby allowing the production of recombinant adenovirus. The advantage of adenovirus is its ability to infect many cell types with surface receptors [48, 49]. For this reason, adenovirus vectors are used most frequently (approximately 24 %) in gene therapy, such as for cancer. Adenoviral DNA is not integrated itself, but it is found in the nucleus of the host cell as extrachromosomal, an episome [46, 50, 51]. Although the level of expression of the therapeutic gene is generally higher in the case of adenoviral vectors, they could be associated with an immune reaction at a higher rate. This type of vector, widely used in the 1990s, is now much less considered in gene therapy [46].

Adeno-associated Virus (AAV)

AAV is a member of single-stranded nonpathogenic parvovirus. It requires the presence of helper virus, such as adenovirus or herpes simplex virus (HSV) for their propagation [52]. AAV DNA can integrate stably and efficiently into the genome of infected cells. They have the distinction of integrating specifically into the genome (19q13.3 region), which reduces the risk of activation of proto-oncogene. However, the preparation of AAV is cumbersome since it requires the presence of a helper adenovirus which must be subsequently removed by various purification steps. To this end, a very large amount of AAV is necessary to conduct in vivo tests [53, 54].

Other Types of Viral Vectors

Beyond viral vectors commonly used in clinical practice and described in the previous section, many other viruses are described as vectors, such as herpes simplex virus (HSV), pox virus, and foamy viral vectors, and identified as potential carriers for gene delivery [28].

Nonviral Vectors

Even though viral systems have a number of advantages, they are not currently safe. In recent years, different nonviral gene delivery techniques have been developed trying to combine the advantages of viral vectors while overcoming their shortcoming. Nonviral methods present some advantages over viral methods with low host immunogenicity and big DNA size. Unlike viral vectors, they are easier to produce, to handle, and to store. However, they have an associated lower transfection efficiency than that of viruses to transfer genetic information into a large population of cells, making them difficult to use in some cases such as in the case of modification of a majority of tumor cells [23, 55, 56].

Transfer of Naked DNA

This is the easiest method of nonviral transfection. In this system, the DNA is injected directly into the tissue to form plasmid. The injection is done using a gene gun, to bombard the epidermis with microparticles coated with DNA [57]. DNA reaches the nucleus, where it remains in episomal form, thus allowing a transient expression of the protein of interest. This technique does not seem sufficient to correct genetic abnormalities but may be effective as a vaccination procedure, since low expression transgene is sufficient to trigger an immune response [58]. Other physical approaches such as electroporation, ultrasound, and hydrodynamic delivery are used to transfer naked DNA [59].

Liposome-Based Nonviral Vectors

Since there are many existing barriers to administer DNA alone, in order to improve the delivery of new DNA into a cell, the DNA must be protected against damage and its entry into the cell must be facilitated. To this end, new molecules, lipoplexes and polyplexes, have been created to protect DNA from undesirable degradation during the transfection process. Liposomes were first described in 1965 as a model of cellular

membrane [60] and quickly earned their candidacy for gene therapy [28, 56].

Liposome-based gene transfer is based on the encapsulation of the DNA molecule within a vesicle composed of one or more phospholipid bilayers enclosing an aqueous compartment containing different molecules such as pharmacological agents, proteins, and nucleic acids [61, 62]. There are three types of lipids used to prepare liposomes: anionic (negatively charged), neutral, or cationic (positively charged). In gene delivery, liposomes composed of cationic lipids are used most actively [63, 64]. Cationic complexes DNA-liposomes fuse with the cell membrane lipid and follow an endocytosis path. DNA released by endosomes may, in some cases, cross the nuclear membrane and be transcribed in the nucleus of the host cell [65]. Theoretically they have no limit as to the size of plasmid as vector, and since they are poorly immunogenic, they could be administered repeatedly [66].

Polymer-Based Nonviral Vectors

Biodegradable cationic polymers have also been studied for nucleic acid delivery in cancer gene therapy. These positively charged polymers, such as cationic polymers, dendrimers, and chitosan, are able to combine with anionic nucleic acids which are called polyplex. Gene transfer mediated by cationic polymer/DNA complexes (polyplexes) has been accomplished efficiently both in vitro and in vivo. Usually, in vivo gene transfer is less efficient than in vitro, and it is not well predicted by in vitro results [67, 68].

Gene Therapy for Breast Cancer

Today we know that many genes are involved in the transformation of a normal cell into a cancer cell by controlling cell division. They are divided into two major families of opposing action: proto-oncogenes and tumor suppressors (antioncogenes).

Proto-oncogenes determine the synthesis of proteins to stimulate cell division. When they mutate, they become oncogenes causing excessive proliferation of cells, such as the ErbB2/HER2 gene associated with breast cancer; the c-myc gene, associated with leukemia, stomach, lung, and breast cancer; the Bcl-2 gene, associated with certain lymphomas; and cyclin D1, associated with breast cancer [15]. All these oncogenes are genetically dominant: it is sufficient that one of the two alleles of proto-oncogene is mutated oncogene to become deleterious.

The tumor suppressors (antioncogenes) encode the proteins that inhibit cell division. Inactivation of these genes by mutations resulted in a multiplication of cells leading to the tumorigenesis [15, 69, 70]. Both copies of the tumor-suppressor gene must be mutated in order to eliminate brake function in cell division [2, 3]. This category includes the p53 gene, whose mutations are associated with nearly half the total cancers [71–74]; the RB gene, whose mutations are associated with retinoblastoma, bone, and bladder cancer as well as breast cancer [69, 75]; and the BRCA-1/BRCA-2 genes, whose mutations are associated with breast cancer and ovarian cancer. The genetic events affecting these genes are recessive and both alleles are inactivated in tumors [15, 76]. To this end, at the present, several approaches can be applied for breast cancer gene therapy. Current approaches in breast cancer gene therapy are summarized in Fig. 26.2.

Restore the Activity of Mutated Tumor Suppressor Gene

The activation of tumor suppressor genes are lost during oncogenesis, contributing to tumor growth such as p53 whose mutations are the most frequently found among various malignancies, including breast cancer. Wild-type p53 suppresses the expression of genes, contributing to uncontrolled cell proliferation or activate genes controlling programmed cell death (apoptosis) [2, 71, 72, 74]. The absence or inactivation of the wild-type p53 leads to uncontrolled cell proliferation. Therefore, it seems logical that the restoration of wild-type p53 activity in some tumor types prevents anarchic cell growth or leads to apoptosis [71–74]. In different preclinical models, various strategies, such as adenovirus, retroviral, and nonviral vectors, have been used with some success to deliver the gene coding for wild-type p53. For example, adenoviral p53

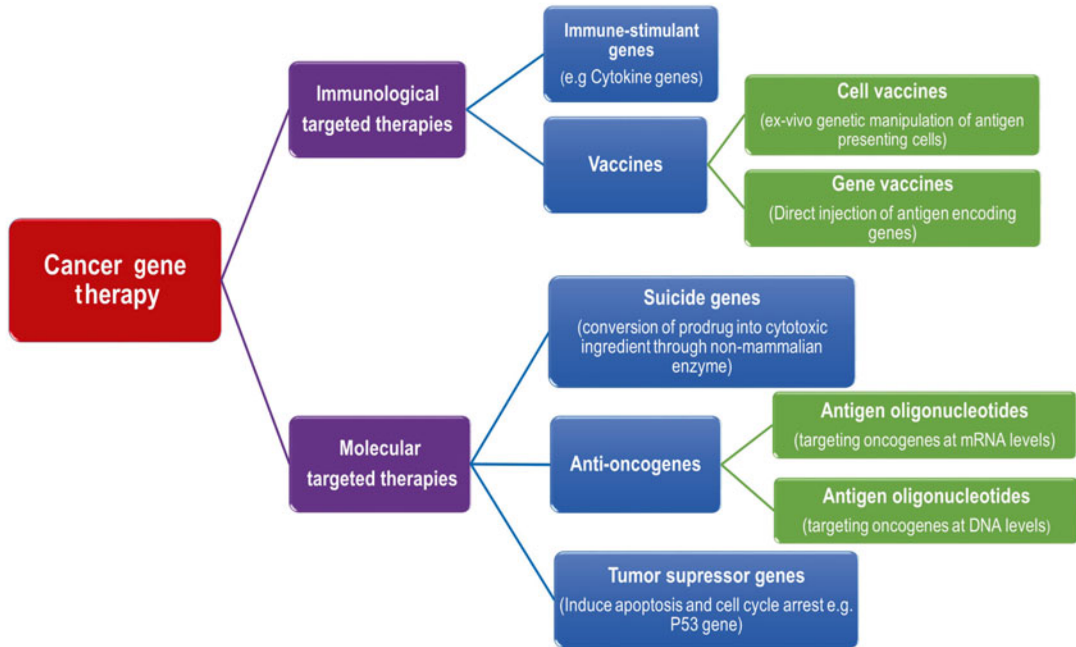

Fig. 26.2 Current approaches in breast cancer gene therapy (Adapted from El-Aneed [77])

gene therapy in combination with docetaxel has already been done and looks promising [78].

Modulation of Oncogene Function

Since the biological activity of oncogenes can be modulated and suppressed either on the RNA or the DNA level [77, 79, 80], these strategies involve the use of antisense oligonucleotides targeting oncogenes at mRNA levels through Watson-Crick base pairing, so inhibiting the translation step of protein synthesis as well as antigen nucleotides that target oncogenes at DNA level by blocking gene expression at the transcription level [77, 79–82].

Suppression of c-fos and c-myc Genes

c-fos and c-myc are cellular proto-oncogenes. When they are subjected to mutations, they behave as oncogenes and their overexpression stimulates cell proliferation [83–86]. It has been demonstrated that transfer of retroviral vector inserted c-myc and c-fos antisense mRNA into breast cancer cell line suppressed tumor formation in animal models [84].

Suppression of ErB2/HER2 Gene

Her2 (neu or c-erbB2) is encoded by proto-oncogene HER2/neu [87]. ErB2/HER2 overexpression in certain types of cancer, such as stomach, breast, and ovarian, is known to be an indicator of poor diagnosis and is strongly associated with increased disease recurrence [15]. For example, the ErB2/HER2 gene is amplified in approximately 20–30 % of breast cancer cases and is associated with a worse prognosis and resistance to hormone therapy [88, 89]. The development of monoclonal humanized murine antibody to ErbB2/HER2 protein (trastuzumab/Herceptin) has revolutionized the process of breast cancer treatment, especially in women who overexpress ErB2/HER2 [88]. Since ErB2/HER2 is overexpressed, it is important to downregulate its expression in tumor cells. Downregulation of ErB2/HER2 gene expression with adenoviral E1A gene encapsulated with liposomes injection into skin lesions or pleural/peritoneal effusion has been investigated as a form of antioncogenic gene therapy [90–92]. In mouse models, inhibition of HER-2/neu in vivo growth of breast cancer through adenovirus-mediated ribozyme method is also demonstrated [93].

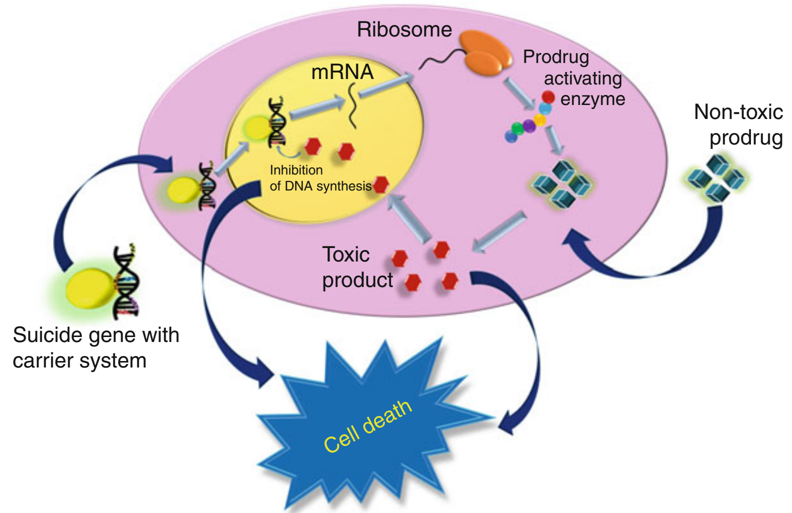

Fig. 26.3 Process in suicide gene therapy

Suicide Gene

Suicide gene therapy involves gene transfer that makes the target cells sensitive to a drug, such as chemotherapeutics suggested in the late 1980s using the enzymatic activity of thymidine kinase of herpes simplex virus type 1 (HSV1-TK) [94]. The specific objective is to transfer a gene-encoding drug-activating enzyme into tumor cells and treat with a prodrug form of chemotherapeutic agents which cause a high concentration of the activated drug in the tumor tissue [95, 96]. This approach permits the increase of therapeutic drugs in the tumor tissue while trying to preserve the surrounding healthy tissue. Suicide gene therapy has several advantages: First, suicide genes as well as their prodrugs are toxic to tumors that are resistant to chemotherapy. Second, only short-term gene expression is required. Third, only a fraction of the tumor cells within the tumor mass needs to express the suicide gene to kill the entire tumor [97, 98]. Additionally, during the application of suicide gene therapy, tumor cells elicit an immune response that can protect from subsequent relapse, as shown in Fig. 26.3 [97, 99, 100].

Antiangiogenic Gene Therapy

Angiogenesis is the process of growth of new blood vessels (neovascularization) from preexisting vessels, which plays an important role in the growth of malignant tumors and the development of metastases. The dependence of the tumor with respect to angiogenesis is a prime target for anticancer therapies [101]. To this end, knowledge of the complex mechanisms of angiogenesis, but also the identification of endogenous angiogenesis inhibitors, open the way for antiangiogenic gene therapy for cancer, such as breast cancer [102, 103], even though a growing number of endogenous angiogenesis inhibitors are discovered preclinical and clinical studies have shown that antiangiogenic therapy alone does not improve the survival of patients with breast or ovarian cancer. In this regard, combining antiangiogenic therapy with other strategies—both conventional and other gene therapy approaches—can produce better response [104].

Angiostatin and endostatin are the most important naturally occurring inhibitors of angiogenesis involved in gene therapy protocols. For example, in nude mice, inhibition of breast cancer is demonstrated through plasmid encoding angiostatin and endostatin combined with liposome [105, 106]. The association of angiostatin with tamoxifen showed better results in transgenic mouse breast cancer model [107].

Immunotherapy

Immunotherapy for cancer involves to induce or to amplify the host immune response against cancer

cells [108]. For immunotherapy, two approaches are possible: active in vivo immunotherapy and in vitro passive immunotherapy (immunotherapy adoptive). The former approach involves administering either genetically modified cells or tumor antigen-presenting (dendritic) cells, whose function is to recruit or stimulate antitumor immune effectors [109]. The majority of gene immunotherapy protocols engaged in the field of oncology is active immunotherapy [97]. Transfer of cytokine genes, such as encoding interleukins (IL-2, IL-4, IL-7, IL-12) as well as tumor necrosis factor (TNF-a) or interferon-g (IFN-g), into cellules tumorales aims to increase the expression of molecules of the major histocompatibility complex (MHC) class I on their surface. This allows an increase in the presentation of tumor antigens to cytotoxic T lymphocytes (LT) [97, 110]. As for passive immunotherapy, it involves increasing the immune response by providing antitumor effector cells (T lymphocytes) which are genetically modified in order to increase their cytotoxic efficacy [77, 109].

Increasing the Resistance of Healthy Tissues with Respect to Chemotherapy: MDR

Since tumor cells become progressively resistant to chemotherapy treatment, increasing the doses of chemotherapy is one way to overcome this chemoresistance. But the chemotherapy doses used are limited because of their toxicity in healthy tissues, such as hematopoietic tissue [111–113]. Multidrug resistance (MDR1) gene, which encodes the P-glycoprotein (Pgp), is a transmembrane protein involved in the efflux of toxic substances out of the cell [114], inducing protection of these cells toward chemotherapeutic agents [115]. Different groups have undertaken clinical studies of MDR1 gene therapy for advanced breast cancer or other neoplasms [116, 117]. Chemotherapeutic agents such as docetaxel and paclitaxel, which have good clinical activity in the treatment of breast cancer, are efficiently effluxed by P-gp and might be the best choice for this strategy [118, 119]. Recently, MDR1 mediated radio-protective gene therapy yielded a very promising method for reducing radiotherapy-related cytotoxicity of normal tissue cells leading to improve therapy success and the patient's quality of life [120].

EpCAM (Epithelial Cell Adhesion Molecule)-Mediated Gene Therapy

EpCAM is a cell surface molecule encoded by the EPCAM gene and is known to be highly expressed in almost all carcinomas leading to proliferation and neoplastic transformation [121]. Data suggests that EpCAM could be a potential novel target for breast cancer gene therapy [122].

Synthetic Lethal Approaches to Cancer Therapy

Synthetic lethal interaction is based on combination of two more gene mutations in the same cell leading to cell death. Each individual mutation is nonlethal by itself and is said to be viable [123]. Synthetic lethality was first described by the American geneticist Calvin Bridges in the early twentieth century [124], and 20 years later "synthetic lethality" name was coined by Theodore Dobzhansky [125]. In a synthetic lethal genetic screening, it is important to begin with a mutation that does not kill the cell and then systematically test other mutations at additional loci to determine which confer lethality [126–128]. Synthetic lethality approach may represent a novel way for the improvement and the maturation of the development for personalized targeted therapies concerning the tumor suppressor and DNA repair genes BRCA1 and BRCA2, mutations which can cause breast and ovarian cancer [129, 130]. About 5 % of breast cancers are hereditary and this can be explained by a germline mutation in BRCA1 or BRCA2 human tumor suppressors which also required double-strand breaks (DSBs) and are deficient in breast and ovarian cancers, [6, 7, 130]. Poly (ADP-ribose) polymerases (PARPs) that catalyze posttranslational modification (poly(ADP-ribosyl)ation) of proteins are enzymes involved in the repair of DNA damage, such as induced by chemotherapy and ionizing

radiation [130–132]. For the "synthetic lethality" concept of targeted cancer therapeutics, PARP inhibitors currently represent an exciting novel therapeutic class drug in BRCA1/2-deficient breast tumors with encouraging results leading significant clinical efficacy [124, 133, 134].

Conclusion and Future Perspective

When the concept of gene therapy was somatically proposed for humans more than 35 years ago, it was commonly accepted that the eventual clinical applications for the most part would concern the treatment of monogenic hereditary diseases [13, 21, 135, 136]. Current ongoing works show a significantly different outcome, and most of the protocols underway or planned at the international level have addressed cancer patients [5, 14, 29, 76]. These works are partly related to risk/benefit ratio in the favorable context, but also these technologies are likely to optimize conventional treatment or allow development of entirely original therapeutic options.

After an infatuation with initial phase results, which were probably premature, we are now witnessing a return of some opinions denouncing the ineffectiveness of transfer of genes for therapeutic purposes. Both of these positions are extreme. At present, available therapeutic strategies for breast cancer are very effective when the disease is diagnosed early. However, the more the stage is advanced, the more poor the prognosis is, causing the death of the patient due to the presence of metastases. In this regard, a better understanding of the genes and the development of gene therapy allow new hope in terms of recovery.

Even though gene therapy offers exciting and encouraging therapeutic prospects, the place of cancer gene therapy in the arsenal of currently available therapies is not yet asserted. One of the limiting factors for gene therapy is the choice of the vector which will convey the interest to the target cell. To this end, future focus needs to be on novel effective strategies concerning development of novel vectors for gene transfer as well as knowledge on signaling pathway in tumorigenesis. On the other hand, a reconsideration of the general interest of the public and institutions on the need for a rational basic scientific research is necessary for these efforts [23, 77, 137]. New strategies for gene therapy which are still ongoing offer promising and encouraging perspectives. It is hoped that some of them offer a real therapeutic benefit against various cancers in the near future.

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27 Clinical Trial Endpoints in Breast Cancer

Melvin George and Sandhiya Selvarajan

Abstract

Breast cancer is one of the leading causes of cancer among women worldwide. Although considerable progress has been made in the management of breast cancer, there is still a dearth of molecules which can change the bleak scenario of metastatic breast cancer. It is essential to use optimum endpoints which can pick the right drug candidate with favorable efficacy and toxicity. Overall survival is the gold standard endpoint in phase III clinical trials of breast cancer. However, the long time required to follow up patients makes it inconvenient as an endpoint, especially in the context of obtaining accelerated approval. Disease-free survival (DFS) as an endpoint can be an early indicator of improved survival. Several modifications of DFS such as invasive DFS and distant DFS have been introduced in recent years. Progression-free survival is currently the most preferred endpoint in breast cancer trials.

Objective response rate is a common endpoint in phase II clinical trials that gives a fair idea of the efficacy of the drug and helps the researchers to make a call on whether to continue drug development in larger phase III clinical trials. Pathologic complete response is an endpoint that has been gaining popularity in the setting of neoadjuvant chemotherapy of early-stage high-risk breast cancer. The FDA has released a draft guideline on the use of pCR in this setting, and a recent meta-analysis has demonstrated correlation with overall survival. As our knowledge of pathophysiology of breast cancer and experience with different endpoints increase, a surge in the number of new molecules that target breast cancer in both early and advanced stages may be anticipated.

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Endpoints • Breast cancer • Overall survival • Disease-free survival • Pathologic complete response • Quality of life • Adverse event assessment • Accelerated approval • Clinical trial

Introduction

One of the most crucial elements in the design of a clinical trial is the choice of an optimal and appropriate endpoint(s). As per the definition of the National Cancer Institute, an endpoint is an event or outcome that can be measured objectively to determine whether the intervention being studied is beneficial. An endpoint is always assessed using specified process as stated in the clinical trial protocol. There are several factors that are taken into consideration in the selection of the most appropriate endpoint for a clinical trial. In clinical trials of different molecules used in breast cancer, some of the most common endpoints that are used include overall survival, progression-free survival, adverse events, and quality of life. This chapter gives an overview of the different types of endpoints used in clinical trials of breast cancer.

Categorization of Endpoints

Although several endpoints can be used in the design of clinical trials, it is the *primary endpoint* which is the defining element of the clinical trial protocol, since it determines whether the study drug is effective in the patient population. The sample size of the study is dependent to a large extent on the primary endpoint. The endpoints that are used in breast cancer studies as the primary endpoints are usually commonly accepted across most groups. It is essential that the primary endpoint is well defined at the start of the study to avoid any ambiguity.

Secondary endpoints are additional variables that are studied to help the investigators obtain valuable information regarding the drug in question. In a clinical trial, it is likely that the sample size is not adequate enough to obtain a statistically significant result with the secondary endpoint. If there is no statistically significant difference with a primary endpoint, there is not much value in the statistical analysis of the secondary endpoints, though by convention all endpoints are measured by statistical analysis [1].

True endpoints are those endpoints that measure the direct clinical benefit to the patient, such as survival or improvement in the quality of life. Endpoints such as overall survival are considered true endpoints. *Surrogate endpoints* are those endpoints which are easier to measure and are meant to fairly represent a true endpoint. It is mandatory to validate a surrogate endpoint before it is routinely used to evaluate drug benefit [2–4]. Since inappropriate use of surrogate endpoints could lead to misleading conclusions, one should use them judiciously [5]. The list below gives the common criteria that are applied to validate a surrogate endpoint:

1. Endpoint must have a well-accepted standard definition.
2. Strong correlation of surrogate endpoint with clinical outcomes from several studies.
3. Well-powered prospective studies that prove the surrogate endpoint is predictive of clinical benefit.
4. Prospective studies to determine if the surrogate endpoint is generalizable to drugs with other mechanisms, other target organs, and other populations.

Overall Survival (OS)

The efficacy of new drugs in breast cancer treatment can be best assessed by the use of overall survival (OS). It is the most universally accepted endpoint because it directly measures the drug's benefit. As per the FDA, overall survival is defined as the time from randomization until death from any cause and is measured in the intent-to-treat population [6].

Most randomized trials are unlikely to find improvement in OS because the study is not adequately powered. Yet, it provides the most objective method of how efficacious the drug is. The sheer amount of time required to complete trials with OS as the primary endpoint makes it a daunting task. When one anticipates frequent change of regimen, OS may not be the best option [7].

OS, although considered to be the gold standard as an endpoint, has increasingly become less popular as a preferred endpoint in breast cancer [8]. For example, a study that analyzed RCT performed in advanced breast malignancy and published in eight leading journals revealed that only one of the 58 studies used OS as the endpoint [9]. Thus, using OS is likely to prolong the onset of using a novel molecule that is likely to be effective in breast cancer. In cases of clinical trials with a crossover design, the results of OS can get easily obscured as it becomes difficult to assess as to which arm of therapy the OS could be attributed to. The presence of second-line therapy introduces further confounding bias when only OS is used as the primary endpoint.

Although OS is less preferred as a primary endpoint, a search for phase III clinical trials performed in breast cancer that were registered in the year 2012 in the clinicaltrials.gov registry showed that 17 of the 22 trials used OS as a secondary outcome measure or endpoint [9, 10]. Since OS can be easily assessed and is the least ambiguous of outcome measures, it is used as a secondary outcome measure [11]. When a non-inferiority analysis is used in a clinical trial with OS as the primary endpoint, there is always the possibility of the new drug having the survival advantage of the standard drug.

Since OS has several drawbacks, many alternative endpoints have been proposed to circumvent the limitations associated with OS [7]. Some of the common surrogate endpoints used in clinical trials of breast cancer are disease-free survival, progression-free survival, and objective response rate. Figure 27.1 shows the advantages of surrogate endpoints over true endpoints such as OS. Although surrogate endpoints have the potential to reduce the size, duration, and cost of studies, these endpoints are not without their own limitations. Surrogate endpoints are generally more appropriate in a phase II clinical trial where the main goal of the study is to determine if the drug is worth further large-scale investment in the form of phase III clinical trials. However, in the context of phase III clinical trials, when one requires absolute certainty of drug's benefit, true endpoints may be almost indispensable [12].

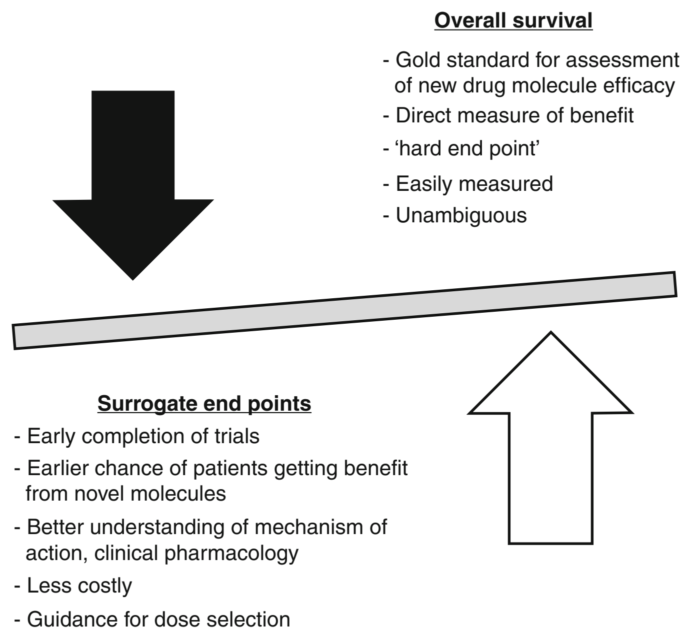

Fig. 27.1 Comparison of overall survival and surrogate endpoints

Disease-Free Survival (DFS)

Disease-free survival (DFS) is defined as the time from randomization until recurrence of tumor or death from any cause. DFS is a common surrogate endpoint used in breast cancer clinical trials, especially in the adjuvant setting following surgery or radiotherapy. In a situation where the survival is prolonged, it may not be practically feasible to measure the overall survival and the DFS may be a preferred endpoint in this scenario and can be an early indicator of improved survival. Patients without recurrent disease may be detected by DFS. Many of the hormonal therapies and cytotoxic chemotherapies that have been approved by the regulatory agencies have employed DFS as the primary endpoint in the exploratory and confirmatory clinical trials. It is essential that while designing a clinical trial with DFS as endpoint, the DFS definition should be clearly stated in the trial protocol. The schedule for follow-up must be stated without any ambiguity to avoid unscheduled visits, which can introduce bias into the study. Bias can be introduced into the study if the number of follow-up visits is greater in one arm of the study due to the development of toxicity in the other arm.

Although deaths that occur in breast cancer clinical trial can be due to disease recurrence, there is always a possibility of deaths being noted without any prior documentation of tumor progression. It is common practice to consider all deaths as recurrences to reduce the amount of bias. However, there is a possibility of overestimation of DFS if patients survive for a long period of time. DFS identifies the proportion of patients without disease recurrence [11, 13].

One of the major challenges in using DFS as the primary endpoint is the variable definition of DFS in different breast cancer trials. Some of the events that are included under breast cancer DFS endpoints by most trialists are contralateral breast cancer that includes invasive lobular or ductal carcinoma, such as ductal carcinoma in situ and in situ carcinoma and deaths from other causes. When there is variability in breast cancer DFS definitions, it becomes less appropriate to compare the different trial results and make meaningful conclusions. There is also a possibility of a treatment being deemed as improved by one particular definition of DFS but not by another [13].

A panel of experts comprised of medical oncologists, biostatisticians, and other scientific experts from several reputed institutions in the USA and Canada convened to standardize the guidelines for endpoint definitions in breast cancer trials. The panel suggested the replacement of DFS with a more specific endpoint termed invasive disease-free survival (IDFS) for early breast cancer adjuvant trials [13]. Listed below are the events included under IDFS:

- Ipsilateral invasive breast tumor recurrence (IIBTR): invasive breast cancer involving the same breast parenchyma as the original primary
- Regional invasive breast cancer recurrence: invasive breast cancer in the axilla, regional lymph nodes, chest wall, and skin of the ipsilateral breast
- Distant recurrence: metastatic disease breast cancer that has either been biopsy confirmed or clinically diagnosed as recurrent invasive breast cancer
- Death attributable to any cause, including breast cancer, non-breast cancer, or unknown cause
- Contralateral invasive breast cancer
- Second primary non-breast invasive cancer

Distant metastasis is the most critical parameter that influences the survival of the patient. Hudis et al. have proposed a novel endpoint termed *distant disease-free survival*, under which ipsilateral breast tumor recurrence, regional invasive recurrences, contralateral breast cancer, and all in situ carcinomas are avoided as events, since they have minimal potential to affect survival [13]. There is a strong correlation between distant disease recurrence and death. Distant disease-free survival includes only distant recurrence and death due to breast cancer and non-breast cancer deaths.

Objective Response Rate (ORR)

Objective response rate (ORR) is defined as the proportion of patients with tumor size reduction

of a predefined amount and for a minimum time period [12]. The ORR is the proportion of patients with a best overall response of confirmed complete (CR) or partial (PR) response. ORR is considered a direct measure of antitumor drug activity, but not a direct measure of clinical benefit [14]. It is important to state the definition of response without any ambiguity in the clinical trial protocol. It is not pertinent to include stable disease under objective response rate, as stable disease can reflect the natural history of the disease, thereby obfuscating the trial results. Since ORR can be achieved even in a short span of time in certain cases, it is one of the endpoints that is selected for accelerated approval. ORR was formerly considered an adequate endpoint for assessment and approval of an anticancer agent by the FDA. However, the subsequent realization that endpoints such as OS are more reflective of the drug's benefit made the regulatory authorities seek for OS in clinical trial protocols instead of ORR. Nevertheless, ORR is still considered a major endpoint in phase II clinical trials of breast cancer therapies [2].

Response rate can be most accurately determined only with the help of imaging technology such as X-ray, CT scan, or MRI using RECIST (Response Evaluation Criteria in Solid Tumors) criteria. The RECIST criteria are a standard set of guidelines that have been designed by the EORTC (European Organization for Research and Treatment of Cancer) and NCI (National Cancer Institute) of the USA and Canada to assess the progression of tumors [15]. The RECIST criteria were originally proposed in 2000 and were modified in 2009 to become known as RECIST 1.1 [16]. The FDA generally defines ORR as the sum of partial responses plus complete responses (CRs). According to RECIST 1.1 criteria, in a target lesion, CR is defined as the disappearance of all target lesions, while partial response refers to at least a 30 % decrease in target lesions. Progressive disease is a 20 % increase in the sum of target lesions, while stable disease is lack of sufficient shrinkage to qualify for partial response or progressive disease [16]. Since the treatment effect is directly attributable to drug activity, single-arm trial design may be applied when using ORR as an endpoint.

The RECIST criteria are useful in all situations when assessment of anatomical tumor burden is to be made and its response to therapy. The clinical significance of ORR must be determined by performing a risk–benefit analysis to ascertain the magnitude and duration of the effect [12]. Bruzzi et al. analyzed randomized trials which compared a standard FEC (fluorouracil, epirubicin, and cyclophosphamide) regimen with a dose-intensified FEC regimen and showed that tumor response is a highly significant predictor of survival [17]. However, in another analysis by Burzykowski et al., no endpoint could be identified as a good surrogate for overall survival [18].

Progression-Free Survival (PFS)

Progression-free survival (PFS) is defined as the time from randomization or treatment initiation until tumor progression or death. Since it usually requires a shorter follow-up period and smaller sample size than studies measuring overall survival (OS), and is not confounded by subsequent therapies, it is often used as a surrogate marker for accelerated approval of drug therapies. In situations where the deaths due to cancer are higher, as in advanced breast cancer, progression-free survival is a better indicator than time to tumor progression.

A study by Burzykowski et al. showed that tumor response is predictive of PFS in patients with advanced breast cancer and thus can be used as a surrogate marker of PFS. However, the authors rightly conclude that analyses regarding the validation of surrogate endpoints are "specific to well-defined disease, clinical outcome, and treatment" [18]. PFS is currently considered the most sensitive parameter for evaluation of the efficacy of a drug [19]. It is not surprising that a literature search across the clinical trial registry data of breast cancer phase III trials showed that PFS was the most common primary endpoint chosen, as illustrated in Fig. 27.2.

The advantage of PFS includes objective and quantitative assessment of the disease outcome.

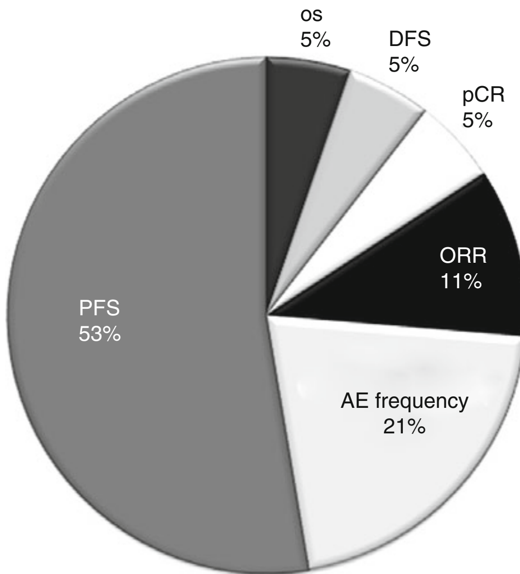

Fig. 27.2 Primary endpoints used in phase 3 clinical trials in breast cancer. Data is based on the list of studies registered in the clinicaltrials.gov registry in the year 2012 that are active. *PFS* progression-free survival, *OS* overall survival, *DFS* disease-free survival, *pCR* pathologic complete response, *ORR* objective response rate, *AE* adverse event

Moreover, PFS is not affected by crossover treatment. The limitations of PFS include frequent assessment of disease progression at reasonable intervals through various investigations including radiological evaluation in both the study arms. This may add to the cost of the trial. Moreover, the definition of PFS may differ across trials, as there are no standard regulatory criteria to define progression of disease. Hence it is not considered a statistically validated surrogate endpoint for survival. Further, missing data in the trial may obscure the analysis of PFS. This could be overcome by assigning follow-up visits in the earlier stage to assess progression, while censored visits may be assigned after radiological examination reveals no progression in the disease. In addition, assessment bias is a major disadvantage of PFS as progression cannot be precisely measured. This could be overcome by randomized and blinded study design [6].

In the present scenario, PFS is gaining preference over overall survival (OS) as the primary endpoint in randomized clinical trials conducted in solid tumors. This could be attributed to the availability of earlier outcome with PFS compared to OS. Moreover, PFS is not affected by second-line treatments for cancer [20]. PFS is being validated as a surrogate endpoint in various cancers including breast cancer and colorectal cancer. In advanced colorectal cancer, PFS has been found to be a valid surrogate as opposed to OS following the completion of first-line chemotherapy. In a literature search done on metastatic colorectal cancer after first-line chemotherapy, appraising PFS as a potential surrogate endpoint compared to OS, a promising correlation was shown with OS [21]. In contrast, PFS has not been validated as a surrogate endpoint in advanced breast cancer so far. Recent investigations have shown that tumor response is an acceptable surrogate for PFS in patients with advanced breast cancer since the correlation seems to be reasonably good [18]. However, the success of using PFS as a potential endpoint to interpret clinical outcome needs to be explored [9].

Time to Tumor Progression (TTP)

Apart from progression-free survival, time to tumor progression is one of the most common endpoints used in the field of breast cancer clinical trials. Time to tumor progression (TTP) is defined as the time from randomization to time of progressive disease and censors deaths that occur before progression of disease. Since PFS includes deaths, it is preferable to TTP as an endpoint in breast cancer clinical trials. While PFS assumes that patient deaths are related to tumor progression, TTP discounts deaths altogether. TTP is especially appropriate in a situation where the majority of deaths are not related to tumor progression [6].

It has been found that since 1975 there has been an increase in the number of clinical trials conducted with the use of PFS/TTP as primary endpoints, especially in breast, colorectal, and non-small cell lung cancers [22]. For drug approval, the FDA considers both PFS and TTP as the primary endpoints for evaluating efficacy. However, the choice among the two is based on the magnitude of the effect and the risk–benefit

profile of the drug product. During analysis of TTP deaths without documented progression are censored, which may lead to biased estimates. This can be overcome with PFS, which includes death in analysis and takes into consideration treatment effects not mediated through tumor burden. Hence PFS is preferred over TTP as a regulatory endpoint [23]. However, while assessing TTP or PFS, patients should be evaluated at regular intervals in all treatment arms including assessment of all disease sites. To reduce bias further, similar assessment techniques should be carried out during each follow-up visit. Nevertheless, a statistically significant difference in TTP or PFS between treatment arms need not inevitably mean clinical benefit [12]. Bowater et al. showed that "the time period between the start of treatment and disease progression (i.e., time to progression) has a strong tendency to extend, by roughly the same amount, the period between the start of treatment and death (i.e., overall survival)" [24].

Time to Treatment Failure (TTF)

Time to treatment failure (TTF) is defined as the time from randomization to discontinuation of treatment for any reason, including disease progression, treatment toxicity, and death. Sometimes TTF is mistakenly defined as the time from study entry to progression of disease or death. However, TTF being a composite endpoint comprises subjective assessment of symptoms in addition to reasons for discontinuation of therapy [25]. A prospective multicenter randomized study done to compare the safety and effectiveness of vinorelbine and melphalan in patients with anthracycline-refractory advanced breast cancer included TTF as one of the efficacy endpoints along with time to disease progression, survival, tumor response rates, and quality of life [26]. To be considered as a regulatory endpoint, the parameter assessed should differentiate between drug efficacy and adverse effect, which is lacking in TTF. Hence the FDA does not recommend TTF as a regulatory endpoint, and it is not considered for drug approval [6].

Pathologic Complete Response (pCR)

There has not been a uniform consensus as of yet on the definition of pathologic complete response (pCR). Some investigators define pCR as the absence of residual cancer in the breast and regional lymph nodes at the time of definitive surgery, whereas others have defined pCR as a complete response in the breast, irrespective of axillary nodal involvement [27–30]. As per the FDA draft guidance document, pCR is defined as the absence of any residual invasive cancer on hematoxylin and eosin evaluation of the resected breast specimen and all sampled ipsilateral lymph nodes following completion of neoadjuvant systemic therapy. pCR is especially useful in the setting of evaluating neoadjuvant systemic chemotherapy, and several trials have utilized pCR as an endpoint in these settings [31]. Since the pathologists are playing a crucial role in evaluating pCR, it is required that they be blinded to the treatment arms to avoid bias.

The Collaborative Trials in Neoadjuvant Breast Cancer (CTNeoBC) carried out a large-scale meta-analysis involving more than 13,000 patients to assess the correlation between pCR and DFS/OS and to also determine the types of breast cancer that pCR is most likely to predict clinical benefit [32]. The study showed that individual patients who attain a pCR, defined as either ypT0ypN0 or ypT0/isypN0, have a more favorable long-term outcome and the data show comparable OS. Thus pCR has an association with long-term outcomes such as OS. In the coming years as our understanding of pCR as an endpoint improves, it is more likely to be used as a primary endpoint in regulatory clinical trials.

Clinical Benefit Rate (CBR)

Clinical benefit rate (CBR) is the proportion of patients with a best overall response—of complete response (CR), partial response (PR), or stable disease (SD)—lasting more than 24 weeks as defined in RECIST 1.1. Patients are followed up for the duration of the study and for an expected average of every 8 weeks after

randomization. Disease control rate (DCR) and CBR are defined as the percentage of patients with advanced or metastatic cancer who have achieved complete response, partial response, and stable disease to a therapeutic intervention in clinical trials of anticancer agents [33].

Patient-Reported Outcomes for Global Health Status/QOL

Patient-reported outcomes, such as time to definitive deterioration in global health status/quality of life (QOL), offer additional valuable information about the nature of the study drug that complements the other conventional endpoints [34]. The major disadvantage with these endpoints is the absolute requirement for randomization and blinding to avoid bias in assessment of outcomes. One should be careful to distinguish the symptoms that arise due to the malignancy and those that arise due to drug toxicity [12]. Patient-reported outcomes such as QOL gain importance in the context of therapies which are not expected to offer any substantial benefit on patient survival. Although not considered important enough to warrant itself as a primary endpoint, QOL is not infrequently measured as one among the several secondary endpoints measured [1]. There is a remarkable heterogeneity in the types of QOL scales used by different groups. Some of the scales used to assess QOL in clinical trials with breast cancer include the European Organization for Research and Treatment of Cancer QOL questionnaires (EORTC QLQ-C30, EORTC QLQ-BR23), the Functional Assessment of Cancer Therapy questionnaires (FACT-G, FACT-B) and its subscales, the DBCG-89 questionnaire, the Medical Outcomes Study 36-Item Short-Form Health Survey (MOS-SF-36), the Hospital Anxiety and Depression Scale (HADS), and the International Breast Cancer Study Group (IBCSG) approach [35]. Due to the shortfall in the methodologic standards applied in evaluating QOL in clinical trials of breast cancer, assessment of QOL appears to have limited benefit in choosing the right therapy for the patient [36]. There is a definite need for more consistency among the different groups so as to make the results of different studies comparable with each other [37].

Adverse Event Assessment

In most clinical trials that evaluate new anticancer drugs, it becomes essential to document the safety of the new molecule in comparison to the standard molecules used in patient care. The *Common Terminology Criteria for Adverse Events* is published by the National Cancer Institute, NIH, USA. As per NCI, an "adverse event (AE) is any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medical treatment or procedure that may or may not be considered related to the medical treatment or procedure." For each AE described, its severity can be further graded on a scale of 1–5, with grade 1 referring to the mildest form of AE, grade 5 meaning death, and grades 2–4 in increasing order of severity. Each AE is clearly defined and thus leaves little room for ambiguity [38]. A search across the clinicaltrials.gov registry showed that AE frequency was one of the common primary endpoints among phase III clinical trials registered under breast cancer in 2012.

Less Commonly Used Endpoints in Breast Cancer

Relapse-free survival (RFS) is the time from randomization to the first relapse or death from any cause. Since RFS includes all deaths, it is considered a sensitive endpoint. Disease-free interval is a term used when one assesses only the recurrence of the tumor without including deaths [13]. It is essential that any modified endpoints that are used are well defined and known to all the investigators who are involved in capturing data and are presented with clarity at the time of scientific communication. Table 27.1 gives a comparison of the common endpoints used in breast cancer trials, and Table 27.2 gives a list of the different endpoints used for approval of breast cancer therapies.

Table 27.1 Comparison of endpoints commonly used in breast cancer clinical trials

Endpoints	Overall survival	Disease-free survival	Objective response rate	Progression-free survival	Time to tumor progression
Definition	Time from randomization to death from any cause	Time from randomization to recurrence of tumor or death from any cause	Proportion of patients with tumor size reduction of predefined size for minimum time period	Time from randomization to objective tumor progression or death	Time from randomization to objective tumor progression
Regulatory approval	Clinical benefit required for regular approval	Surrogate marker for regular or accelerated approval	Surrogate marker for regular or accelerated approval	Surrogate marker for regular or accelerated approval	Surrogate marker for regular or accelerated approval
Study design	Randomized trial	Randomized trial, blinding needed	Single-arm or randomized studies, blinding preferred	Randomized blinding studies preferred	Randomized blinding studies preferred
Benefits	Measures precisely direct benefit, universally accepted	Smaller sample size, shorter follow-up	Smaller studies, assessed earlier	Small sample, shorter follow-up; not affected by crossover or subsequent treatment	Small sample, shorter follow-up; not affected by crossover or subsequent treatment
Limitations	Longer duration of follow-up, includes death not due to cancer, results altered by crossover study or sequential therapy, requires larger studies	Subject to bias, does not measure the outcome precisely	Not a direct measure of benefit, only a subset of patients get benefited	Missing data will affect analysis, definitions may vary	Missing data will affect analysis, definitions may vary

Table 27.2 Examples of drugs approved for breast cancer supported by different endpoints

Serial no.	Endpoint used	Drugs approved
1.	Overall survival	Capecitabine Docetaxel
2.	Objective response rate	Anastrozole Letrozole Exemestane
3.	Disease-free survival	Trastuzumab Tamoxifen Letrozole Anastrozole
4.	Progression-free survival	Bevacizumab
5.	Time to progression	Fulvestrant Lapatinib Anastrozole Trastuzumab

Endpoints for Accelerated Approval

Accelerated approval is a regulatory pathway by which the FDA approves a drug on the basis of demonstrating efficacy via improvement in surrogate endpoints. However, for drugs approved by the accelerated approval pathway, it is mandatory on the drug manufacturers to carry out post-marketing studies to confirm the clinical benefit of the drug along with its safety profile [39]. Since this pathway can quicken the drug approval process, it is frequently employed in metastatic breast cancer, where there is a clear unmet medical need. Bevacizumab was granted accelerated approval for metastatic breast cancer by the FDA in 2008 on the basis of its improvement in progression-free survival and reduction in tumor

volumes as shown by objective response rate in the E2100 trial. However, further post-marketing studies carried out by Genentech revealed that bevacizumab did not improve overall survival nor provide any benefit in slowing disease progression. Further, the adverse effects of bevacizumab such as hemorrhage, increased risk of MI, heart failure, and intestinal perforation worsened the risk–benefit ratio further. The results of these post-marketing studies made the FDA revoke the license for approval of bevacizumab for the treatment of metastatic breast cancer. This series of events is a classic description of a scenario where accelerated approval with the help of surrogate endpoints need not necessarily translate into direct clinical benefits [40–42].

Conclusion and Future Perspective

Breast cancer continues to be the leading cause of cancer among women. Development of new molecules which improve survival with minimal adverse effects is an active area of ongoing research. It is important that the endpoints that are chosen in evaluating these drugs reflect the true potential of the drug in terms of its efficacy and safety without unduly affecting the time required for completion of the trial and the financial requirements. True endpoints such as overall survival, although considered universally as the gold standard endpoint, are fraught with limitations. Hence they are increasingly used as secondary endpoints rather than primary endpoints in phase III breast cancer trials. Progression-free survival is currently the most preferred primary endpoint for phase III clinical trials in breast cancer. New endpoints, such as pathologic complete response rate, are increasingly being used in breast cancer. However, their correlation with well-established endpoints such as overall survival and progression-free survival remains to be conclusively established. It is hoped that the ongoing evolution of endpoints that are being used in evaluating breast cancer therapies will lead to the discovery and development of superior drug molecules in the management of breast cancer.

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