Current Clinical Pathology
Series Editor: Antonio Giordano

Michael J. Murphy *Editor*

Molecular Diagnostics in Dermatology and Dermatopathology

Humana Press

CURRENT CLINICAL PATHOLOGY

ANTONIO GIORDANO, MD, PHD

SERIES EDITOR

For other titles published in this series, go to
www.springer.com/series/7632

Michael J. Murphy
Editor

Molecular Diagnostics in Dermatology and Dermatopathology

Humana Press

Editor
Michael J. Murphy, MD
Division of Dermatopathology
Department of Dermatology
University of Connecticut Health Center
21 South Rd., Farmington, Connecticut 06030
USA
drmichaelmurphy@netscape.net

ISBN 978-1-60761-170-7 e-ISBN 978-1-60761-171-4
DOI 10.1007/978-1-60761-171-4
Springer New York Dordrecht Heidelberg London

Library of Congress Control Number: 2011921259

Printed on acid-free paper

Humana Press is part of Springer Science+Business Media (www.springer.com)

To my parents, Michael and Jacinta, without whose love and sacrifice this undertaking would not have been possible.

To Aileen, for her seemingly endless love, patience and support.

Preface

The era of molecular diagnostics in dermatology and dermatopathology is upon us. Advances in genomics are continually redefining our understanding of the pathogenesis and classification of skin disorders. Molecular testing is transforming patient care, facilitating diagnosis, staging, and prognostication of a variety of skin diseases, in addition to guiding the selection of appropriate treatment, monitoring of therapy, and identification of novel therapeutic targets. An understanding of the principles and potential applications of molecular technologies is now essential for any physician practicing in this field. The contents of this book will be of particular interest to dermatologists and dermatopathologists, as well as anatomic pathologists and other physicians/scientists who have an interest in skin disorders, or those who would like to expand their knowledge in this area. The book covers a broad range of cutaneous conditions and specifically emphasizes molecular testing strategies with immediate clinical relevance.

I would like to thank all my collaborators in Australia, Austria, Brazil, Canada, China, Denmark, Finland, France, Germany, Greece, Hungary, India, Israel, Italy, Japan, Korea, Mexico, The Netherlands, Scotland, Singapore, Spain, Sweden, Switzerland, Taiwan, Thailand, Turkey, United States of America, and Venezuela for contributing chapters and figures to this endeavor. My colleague Dr. Zendee Elaba deserves special mention for her help with illustrations. In particular, I wish to extend my sincerest gratitude to my dear friend Dr. Diane M. Hoss for her advice, encouragement, and help throughout the entire process.

I have often reflected on the need for a book such as this, that one can turn to while in the clinic, at the microscope, or in the laboratory. It has been intellectually rewarding to bring this goal to fruition. I sincerely hope the readers enjoy this book and use it as a reference guide in their daily practice of medicine.

Michael J. Murphy

Contents

Contributors

Michael J. Murphy, MD
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, Farmington, CT, USA

Wenyong Zhang, MD, PhD
Department of Pathology, New York Medical College, Westchester Medical Center, Valhalla, NY, USA

Jian Zhuge, PhD
Department of Pathology, Mount Sinai School of Medicine, New York, NY, USA

Laila Mnayer, PhD
Department of Pathology and Laboratory Medicine/Molecular Pathology and Cytogenetics, Hartford Hospital, Hartford, CT, USA

Zendee Elaba, MD
Department of Pathology and Laboratory Medicine, Hartford Hospital, Hartford, CT, USA

Melanie A. Carless, PhD
Department of Genetics, Southwest Foundation for Biomedical Research, San Antonio, TX, USA

Lyn R. Griffiths, PhD
Genomics Research Centre, GIHMR, School of Medical Science, Griffith University-Gold Coast, Bundall, QLD, Australia

Pedram Gerami, MD
Department of Dermatology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

Bryan Gammon, MD
Department of Dermatology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

J. Andrew Carlson, MD
Division of Dermatology and Dermatopathology, Department of Pathology, Albany Medical Colloge, Albany, NY, USA

Hensin Tsao, MD, PhD
Wellman Center for Photomedicine, Department of Dermatology and MGH Cancer Center, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Zhenyu Ji, MD, PhD
Wellman Center for Photomedicine and Department of Dermatology,Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Victor Neel, MD, PhD
Department of Dermatology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

John D. Pfeifer, MD, PhD
Lauren V. Ackerman Laboratory of Surgical Pathology, Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, MO, USA

Omar Jassim, MD, PhD
Department of Medicine, Washington University School of Medicine, St. Louis, MO, USA

Shih-Sung Chuang, MD
Department of Pathology, Chi-Mei Medical Center, Tainan and Taipei Medical University, Taipei, Taiwan

Antonio Subtil, MD, MBA
Departments of Dermatology and Pathology, Yale University School of Medicine, New Haven, CT, USA

Amanda Phelps, BA
Central Connecticut State University, New Britain, CT, USA

Markus Braun-Falco, MD
Department of Dermatology, Ludwig-Maximilians-Universität München, Munich, Germany

Pranab K. Mukherjee, PhD
Center for Medical Mycology, Department of Dermatology, University Hospitals Case Medical Center, Case Western Reserve University, Cleveland, OH, USA

Nancy Isham, M(ASCP)
Center for Medical Mycology, Department of Dermatology, University Hospitals Case Medical Center, Case Western Reserve University, Cleveland, OH, USA

Mahmoud A. Ghannoum, PhD
Center for Medical Mycology, Department of Dermatology, University Hospitals Case Medical Center, Case Western Reserve University, Cleveland, OH, USA

Avery LaChance, BA
University of Connecticut Medical School, Farmington, CT, USA

Diane M. Hoss, MD
Caris Life Sciences, Dermatopathology Division, Farmington, CT, USA

Frances J. D. Smith, PhD
Division of Molecular Medicine, Colleges of Life Sciences and Medicine, Dentistry and Nursing, University of Dundee, Dundee, Scotland, UK

W. H. Irwin McLean, PhD, DSc
Division of Molecular Medicine, Colleges of Life Sciences and Medicine, Dentistry and Nursing, University of Dundee, Dundee, Scotland, UK

Carlo Pincelli, MD
Department of Medicine, Dermatology Division, University of Modena and Reggio Emilia, Modena, Italy

Riccardo G. Borroni, MD
Department of Medicine, Dermatology Division, University of Modena and Reggio Emilia, Modena, Italy

Neil H. Shear, MD
Division of Dermatology and Clinical Pharmacology, Sunnybrook Health Sciences Centre, University of Toronto Medical School, Toronto, ON, Canada

Sandra Knowles, BScPhm
Division of Pharmacy and Clinical Pharmacology, Sunnybrook Health Sciences Centre, University of Toronto Medical School, Toronto, ON, Canada

Stephane Dalle, MD, PhD
Service de Dermatologie, Centre Hospitalier Lyon-Sud, Université Claude Bernard Lyon I, Pierre-Bénite, France

Deborah A. Payne, PhD
American Pathology Partners-Unipath LLC, Denver, CO, USA

Chapter 1
Introduction to Molecular Diagnostic Testing in Dermatology and Dermatopathology

Michael J. Murphy

> *"O brave new world," he repeated. "O brave new world that has such people in it. Let's start at once."*
>
> –Aldous Huxley, *Brave New World* (1932)

The complete sequencing of the human genome has ushered in an era of medical advances that was previously unimaginable. Scientists are continually discovering novel genetic and epigenetic mechanisms that are associated with human disease states and therapeutic responses. The ability to determine the underlying defect(s) in single-gene (Mendelian) diseases, many of which are rare, has improved both diagnosis in symptomatic patients and risk prediction of future disease in asymptomatic individuals. Potential applications of genomic discoveries include: (1) development of carrier, screening and diagnostic tests for single-gene disorders; (2) evaluation of several genetic loci in an effort to construct disease susceptibility profiles for non-Mendelian diseases, based on multiple gene and/or gene–environment relationships; and (3) pharmacogenomic testing to predict drug–genome interactions [1]. It has been estimated that ~5% of the ~25,000 genes in the human genome are of diagnostic significance; therefore, the potential exists to develop ~1,500 gene-based tests [2]. With regard to dermatologic conditions, exciting research is emerging and new applications are now being incorporated into clinical practice. Molecular diagnostic tests are transforming laboratory medicine and patient care, and becoming indispensable for physicians involved in the management of skin diseases, including dermatologists and dermatopathologists. Nucleic acid-based testing is becoming a crucial diagnostic tool, not only in the setting of inherited disorders (i.e., genodermatoses), but also for a wide variety of cutaneous solid and hematopoietic tumors, inflammatory dermatoses, and infectious conditions. In view of the increasing numbers of molecular diagnostic articles published in the dermatology literature, and potential application of these methodologies in clinical practice, a basic knowledge of the principles of molecular diagnostics is now essential for the physician who specializes in the diagnosis and/or treatment of skin diseases. Figure 1.1 illustrates the integration of research and diagnostic strategies in the study of skin diseases.

Essentially, molecular diagnostic testing involves the analysis of nucleic acid (DNA and/or RNA), using a wide variety of technologies. Simplistically, this field can be divided into "disease diagnostics" (i.e., risk prediction, disease identification, and recurrence detection) and "companion diagnostics" (i.e., drug responders, titration of efficacy, and patient selection for clinical trials) [2].

M.J. Murphy (✉)
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, 21 South Rd., Farmington, CT 06030, USA
e-mail: drmichaelmurphy@netscape.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_1,

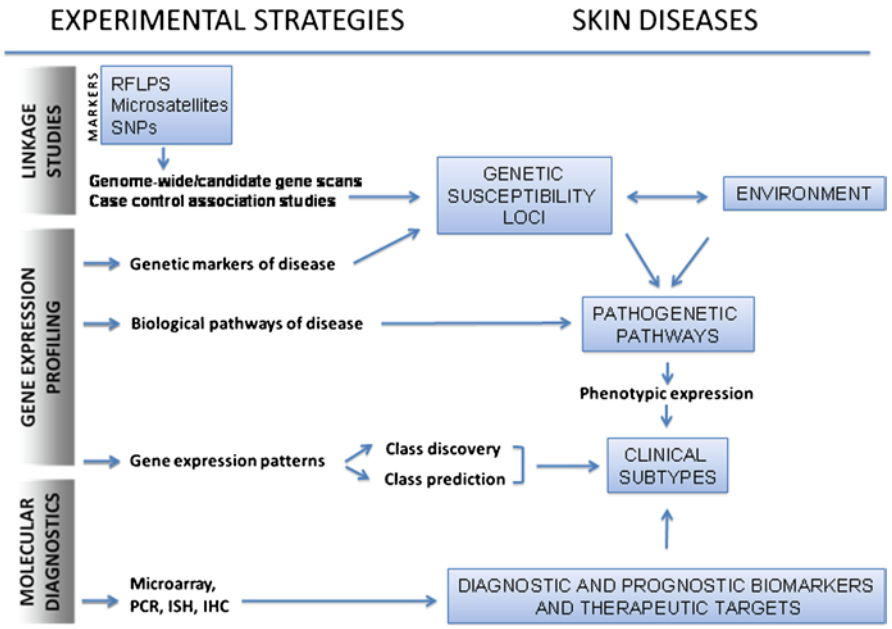

Fig. 1.1 Integration of research and diagnostic strategies in the study of skin diseases. Linkage studies facilitate an understanding of genetic markers and susceptibility loci. Microarray-based gene expression analysis enables a more thorough understanding of gene–gene and gene–environment interactions that are involved in disease induction and pathogenesis. Identification of disease-specific genetic signatures should provide a better classification of disease at the molecular level, redefining both known and unknown subtypes of disease. Data is incorporated into molecular diagnostic strategies for risk prediction, identification of diagnostic and prognostic biomarkers, and discovery of therapeutic targets of disease. *RFLPs* restriction fragment length polymorphisms, *SNPs* single nucleotide polymorphisms, *PCR* polymerase chain reaction, *ISH* in situ hybridization, *IHC* immunohistochemistry (Courtesy of Dr. Zendee Elaba, department of Pathology, Hartford Hospital, Hartford, CT, USA: Adapted from Dudda – Subramanya et al. [3])

To date, nucleic acid amplification methods have dominated this field; with the most readily recognizable of these being polymerase chain reaction (PCR). Newer technologies are now being incorporated and can facilitate parallel gene analyses (i.e., cDNA/oligonucleotide microarrays) and/or correlation of genomic changes with morphological features of disease [i.e., fluorescence in situ hybridization (FISH)]. A list of laboratories (including academic medical centers, community hospitals, and commercial reference facilities) offering clinical genetic testing services can be found on the GeneTests website [4]. Molecular diagnostic laboratories can either employ Food and Drug Administration (FDA)-approved kits or develop "home brew" assays, also known as laboratory-developed tests (LDTs). There is now a trend toward more widespread use of commercially available kits, and away from in-house assay development, largely due to concerns regarding intellectual property infringements and gene sequence patents. Of note, ~20% of human gene DNA sequences are currently patented; although, a recent law court decision has thrown the validity of some patents into question [5]. FDA approval is generally reflective of high-volume tests, and commercial kits are not currently available for all potential dermatologic applications. A list of FDA-approved molecular diagnostic tests, including genetic, microbial and solid tumor/hematopathology assays, is available through the Association for Molecular Pathology (AMP) website [6]. Broader assays that screen for genetic variations are being developed in parallel with more specific diagnostic tests for individual diseases (Fig. 1.1). As we move forward, an increasing emphasis will be placed on point-of-care (POC) testing, as well as the integration of genomics with other "omic" strategies, such as proteomics and metabolomics, and/or strategies that combine therapeutics with diagnostics ("theragnostics") [7]. Most molecular assays consist essentially of three steps: extraction and

purification of nucleic acid, amplification of the "target" sequence, and detection of the amplified product. The goal of POC testing is to streamline and miniaturize these processes, in order to develop hand-held devices for "bed-side" testing of those conditions (such as life-threatening infections) where rapid diagnosis is necessary for initiation of appropriate therapy.

The purpose of this book is to introduce the basics of molecular biology and molecular diagnostic methods most commonly used in the clinical laboratory, with an emphasis on the concepts and potential applications that are most relevant to dermatologic conditions. The contents of this book will be of particular interest to dermatologists and dermatopathologists, as well as anatomic pathologists and other physicians/scientists who have an interest in skin disorders. A broad range of cutaneous pathology is covered. In addition to chapters on infectious and inflammatory lesions, cutaneous lymphoproliferative disorders, melanocytic tumors, non-melanoma skin cancers and genodermatoses, the book also includes discussions of other dermatologic conditions, including cutaneous sarcomas and soft tissue proliferations, metastatic tumors, wound healing disorders, and alopecias. The application of pharmacogenetics and pharmacogenomics in patient management, and some of the regulatory, legal, coding, billing, reimbursement, and ethical considerations associated with molecular diagnostic testing in dermatology and dermatopathology are also outlined. The integration of newer diagnostic, staging, and prognostic molecular techniques with "traditional" clinical- and histopathological-based approaches is described, and a broad and comprehensive outline of current clinically relevant applications of these methodologies in dermatologic conditions is provided. Molecular studies that primarily investigate the pathogenesis of skin diseases (and can be found in other texts) have been largely excluded or have minimal discussion, unless they also have direct diagnostic and/or prognostic relevance or the possibility of such use in the near future.

Genetic Testing

There are a small percentage of individuals with common diseases who have rare, but inherited single gene mutations. These single gene mutations, which increase disease susceptibility, may reach frequencies as high as 1% in some population groups. More common genetic variants (DNA polymorphisms) are also associated with increased risks of developing common conditions. Interestingly, some polymorphisms can actually convey resistance to disease. Nonetheless, other genetic and/or environmental factors must play a role prior to the onset of disease in individuals with either these predisposing rare variants or polymorphisms. Acquired (somatic) mutations have also been found to play a significant role in many human malignancies, including skin tumors. In addition, the functions of genes involved in the development of chronic inflammatory disorders (such as psoriasis and atopic dermatitis) are being elucidated through comparative molecular genetic profiling of cells from diseased organs/tissues with corresponding normal cells. With such knowledge, interventions can be developed which either mitigate disease triggering events or facilitate early and effective therapy.

Despite remarkable progress, there are still many issues that impact the utility of genetic testing. Importantly, for many Mendelian genetic diseases, a "therapeutic gap" exists (i.e., it has proven easier to diagnose or predict a condition than to find means to prevent or effectively treat it). A positive test result does not necessarily indicate that the disease will inevitably develop or indeed, predict the age of onset, its severity and/or therapeutic response in those individuals in whom the condition does occur. In contrast, a negative test result may not definitively rule out future pathology. The disease could potentially occur even when tests fail to reveal predisposing polymorphisms or inherited susceptibility mutations. In addition, some tests may not detect all of the disease-causing mutations of a particular condition.

According to the National Institutes of Health-Department of Energy Task Force on Genetic Testing, "*the clinical use of a genetic test must be based on evidence that the gene being examined*

is associated with the occurrence of the disease in question, that the test itself has analytical and clinical validity, and that the test results will be useful to the people being tested (clinical utility)… Before a genetic test can be generally accepted in clinical practice, data must be collected to demonstrate the benefits and risks that accrue from both positive and negative results [8]." Analytical validity requires "*establishing the probability that a test will be positive when a particular sequence (analyte) is present (analytical sensitivity), and the probability that the test will be negative when the sequence is absent (analytical specificity). One key measure of analytical validity is accuracy, or the probability that the measured value will be within a predefined range of the true activity or concentration. Another measure of analytical validity is reliability, or the probability of repeatedly getting the same result. Analytical validation of a new genetic test includes comparing it to the most definitive or gold standard method* [8]." Clinical validation involves "*establishing several measures of clinical performance including: (1) the probability that the test will be positive in people with the disease (clinical sensitivity); (2) the probability that the test will be negative in people without the disease (clinical specificity); and (3) the probability that people with positive test results will get the disease [positive predictive value (PPV)] and that people with negative results will not get the disease [negative predictive value (NPV)]. Predictive value depends on the prevalence of the disease in the group or population being studied, as well as on the clinical sensitivity and specificity of the test… Two intrinsic features of genetic diseases, heterogeneity and penetrance, affect clinical validity* [8]." In its narrowest sense, clinical utility refers to the ability of screening or diagnostic tests to prevent or mitigate an adverse outcome, such as disability, morbidity, or mortality through medical intervention and/or the institution of healthier behavior, conditioned on the results of these tests [1, 8]. Of course, the clinical utility depends on a balance of benefits-to-risks of testing and access to appropriate interventions. More broadly, clinical utility encompasses the ability to inform clinical decision-making and/or impact outcomes (including psychological and economic) that are important to individuals, families, and society [1, 8]. It is necessary to understand the difference between screening and diagnostic tests. Diagnostic (confirmatory) tests are performed once clinical symptoms and/or signs of a disease are present. A potential benefit that comes with the identification of a disease-associated genetic aberration may be the ability to determine an individual's risk of developing that disease (i.e., a screening test), prior to the onset of symptoms and/or signs. Hence, early intervention, combined with increased vigilance, could reduce the risk of disease development or possibly prevent the disease from progressing to a more severe phenotype.

Molecular Diagnostics Market

Molecular diagnostic tests are rapidly becoming the standard of care in many medical specialties. This largely results from a combination of technological advances, bioinformatics, the explosion of molecular discoveries and biomarker publications in the scientific literature, FDA regulatory approval, evolving professional association and practice guidelines, the shift to evidence-based medicine, a more educated public, consumer-directed healthcare, litigation concerns among physicians, reimbursement decisions, the shifting of costs to consumers, and, in view of the unsustainable health care expenditures in the USA (17% GDP), the potential savings that can come as a result of preventative and/or more accurate testing [2]. As a result, both the growth and profitability of the USA molecular diagnostic testing market continue to increase exponentially. In 2001, only ~5% of all laboratory testing was based on DNA or RNA analysis [9]. Between 2005 and 2007, annual billable molecular tests were reported to have increased by 36%, and it is estimated that ~65 million molecular tests were performed in the USA in 2008 [2]. Based on the Washington G-2 Reports' 2008 Molecular Diagnostics Market Strategic Outlook Survey [2], ~65% of medical laboratories now offer molecular diagnostic testing, with a further ~23% planning to offer such assays. Current reports value

the molecular diagnostics market in the USA at $5.5 billion; a small, albeit significant portion of the ~$55 billion laboratory testing industry. In a 2005 survey, laboratories reported that molecular testing represented ~13% of total revenues [2]. By 2007, this had jumped to ~15.5% (an increase of 19.2%). Molecular testing now accounts for ~19% of the total revenue for laboratories which offer such assays, with an estimated growth of ~18% per year over the coming decade [2]. This percentage is expected to rise to 25% by 2014. Molecular diagnostics is predicted to represent 33% of laboratory revenues by 2018 [2]. In contrast, growth for most other areas of laboratory testing is predicted to be a modest ~5%. Currently, the molecular diagnostics market is dominated by infectious disease testing, which accounts for ~55% of all assays [2]. Some of the most commonly offered molecular-based tests are those for sexually transmitted diseases (i.e., chlamydia/gonorrhea, human papillomaviruses, and human immunodeficiency virus) and infections by other modes of transmission (i.e., hepatitis C virus). Other high-volume tests include those for coagulation screening (Factor II and Factor V Leiden), cystic fibrosis screening, and breast cancer prognostication/treatment selection (HER-2/neu) [2]. In the future, the areas that are expected to show the largest growth in this market are infectious disease assays and predictive testing (i.e., pharmacogenomics/pharmacogenetics) [2]. This explosion in molecular diagnostics likely reflects the fact that laboratories now recognize that molecular-based assays are increasingly necessary in day-to-day medical practice and/or represent a lucrative revenue stream. Two important factors that laboratories must consider when deciding to integrate molecular-based tests are: (1) potential clinical impact and (2) apparent demand, as assessed by send-out data [2]. The number one reason for performance of molecular testing is the effect of potentially more sensitive, more specific, and expedited test results on patient management decisions. Importantly, demand is now becoming more consumer-driven, coinciding with the advent of direct-to-consumer marketing and the development of less invasive sample procurement and testing procedures (i.e., "lab in a box"). Other issues include the time, personnel and resources required to develop such tests, FDA approval status, research applications, and cost data (i.e., cost per result performed in-house *vs.* send-out) [2]. For example, the average cost per molecular assay is ~$107 (median $57) compared with an average of ~$50 per non-molecular test (median $12) [2]. However, molecular-based assays generally have higher reimbursement rates than routine laboratory tests [2]. Coding, billing, and reimbursement issues are more fully explored in Chap. 23. Savings may also be realized through decreased test turnaround times and more accurate results, potentially impacting treatment selection and length of hospital stay. From a dermatological perspective, many surveyed laboratories currently offer molecular assays to detect herpes simplex virus and T- and B-cell surface receptor gene rearrangements (for work-up of cutaneous lymphoproliferative disorders), in addition to CYP450 genotyping (for drug response/adverse reaction prediction) [2]. Tests for methicillin-resistant *Staphylococcus aureus* (MRSA) and Epstein-Barr virus (EBV) are among those planned by many laboratories [2].

According to a recent publication by Washington G-2 Reports entitled "*Business Strategies for Molecular Diagnostics in the Lab 2009, including State of the Market Report*" [2], the field of molecular diagnostics can be viewed as a continuous and codependent cycle of innovations based on research and test development. First, molecular markers and clinical implications are uncovered by research-based strategies. Test development is promoted, and with clinical demand comes the need to improve test effectiveness and efficiency. This, in turn, continues to drive research and the cycle begins again [2].

Molecular Diagnostics in Dermatology and Dermatopathology

The uptake of molecular diagnostic testing in the specialties of dermatology and dermatopathology has been relatively low compared with other medical fields. A number of interesting factors could potentially account for this finding. Firstly, most dermatology offices are not hospital-based practices, where

the majority of molecular laboratories are located. Therefore, dermatologists may not be aware of the availability and clinical applications of nucleic acid-based assays offered. Secondly, decisions to order such assays could be based on perceptions (real or imagined) of how useful the results would be for diagnosis and/or management of skin disorders. Thirdly, providers may be concerned about the legal implications of testing or third-party coverage/reimbursement issues. With regard to molecular test development and usage, the practice of dermatology could potentially be viewed as a two-edged sword. In one respect, a distinct advantage of daily clinical practice is the accessibility of the skin, readily facilitating tumor screening strategies and early cancer detection. Nowhere is the necessity to correlate clinical and microscopic findings for diagnostic purposes more evident than in dermatology. However, the majority of skin disorders are easily diagnosed on the basis of morphological features, without the need for ancillary laboratory tests such as histochemical stains (i.e., PAS) or immunohistochemistry, let alone molecular studies. Physicians and insurance companies may be unwilling to stretch for that "unnecessary" test. Both the ability to remove relatively large pieces of tissue for histopathological review (compared with non-cutaneous sites) and, most importantly, the relative ease of repeating skin biopsies in instances of equivocal microscopic findings, have ensured that the burden of diagnosis and management in dermatology remain largely based on clinical features and/or microscopic analysis. Another factor possibly reducing the use of molecular diagnostics in clinical practice is the perception that such testing often produces erroneous results [10]. However, studies demonstrate that more errors originate during the pre-analytic and post-analytic phases of testing than during the analytic process itself [10]. The latter is tightly regulated by the Clinical Laboratory Improvement Amendments of 1988 (CLIA) and College of American Pathologists (CAP) guidelines (emphasizing control procedures and proficiency testing). Problems may arise with specimen handling and analysis, but are reported in only 0.06–0.12% of tests studied [10]. More importantly, inappropriate test selection (i.e., unwarranted testing) underlies many of the pre-analytic errors. Post-analytic errors commonly reflect not only problems in preparing reports, but also the accurate assessment of results. Studies have shown that a major contributor to these problems is poor understanding among healthcare providers of the limitations of molecular genetic assays and their proper interpretation. It is not enough for a physician to simply know that a test is positive or negative. Results must be interpreted in light of the patient's personal and family history, histopathological data, and results of other testing strategies. Many recommendations relate specifically to the molecular diagnosis of inherited diseases; largely a function of the dramatic increase in this form of testing. Of note, over the last 8 years, the number of screenings available has more than tripled from just over 400 to more than 1,300 [10]. However, guidelines are also applicable to molecular testing for other types of skin disorders. In the pre-analytic phase, laboratories should provide information about their tests to both healthcare professionals and patients, such as the intended use of the test, its limitations, appropriate collection of samples, and handling and transport of the specimens. Physicians should strive to provide important clinical data on the patient being tested. In some instances, relevant background information is also necessary. For example, in the case of inherited skin diseases, such as genodermatoses or familial melanoma, the patient's race or ethnicity, family history, and pedigree will be required. In the post-analytic testing phase, a laboratory must provide an interpretation of the results, as well as additional information where appropriate, in order to ensure that the referring physician can also make an accurate assessment of the data. This would include a description of the methodology, the nucleic acid targets of the test, and performance specifications and limitations. If required, the laboratory should make recommendations regarding repeat/additional testing. In the case of hereditary diseases, comments on follow-up genetic consultation or implications of test results for other family members may be appropriate.

The following sections outline some of the recent commercial advances vis-à-vis molecular testing in skin diseases. This discussion neither endorses nor recommends any particular company or its product(s). Instead, it is used to highlight the level of interest among commercial ventures for entry and capture of segments of the relatively untapped molecular diagnostics market in dermatology. Readers are directed to the respective websites in the reference list for additional information.

Melanoma

Between 5% and 10% of melanoma may be hereditary, with ~2% of all melanomas arising as a result of germline mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A), the most significant high risk melanoma susceptibility gene identified to date [11]. The likelihood of melanoma by age 80 in a patient with a germline mutation in CDKN2A is 58% in Europe, 76% in the USA, and 91% in Australia [11]. CDKN2A mutations also predispose to pancreatic cancer with a 25% risk of developing this tumor by age 80. Genetic testing is widely employed to identify those individuals with, or at risk of developing, hereditary breast/ovarian and colorectal cancers. However, genetic testing of CDKN2A with regard to hereditary melanoma is not currently part of routine clinical practice. A recent review outlined selection criteria for the genetic assessment of patients with familial melanoma [11]. Based on current guidelines, the higher rate of CDKN2A mutation in individuals with three or more primary melanomas, and/or families with at least one melanoma and two or more other diagnoses of melanoma and/or pancreatic cancer among first- or second-degree relatives on the same side of the family, warrant referral for genetic work-up [11]. Other melanoma-predisposing genes include cyclin-dependent kinase 4 (CDK4), cyclin-dependent kinase inhibitor 2A/p14 alternate reading frame (CDKN2A/ARF), and melanocortin 1 receptor (MC1R). Guidelines for molecular assessment do report that risk estimates associated with these genes have wide confidence intervals, but testing may be considered in some cohorts [11]. Testing might help identify individuals at significant risk for developing melanoma, who would require intensive skin cancer screening, education, and genetic counseling referral. Molecular testing for germline mutations in melanoma-predisposing genes is discussed in Chap. 5, and information on familial melanoma is available through the Melanoma Genetics Consortium (GenoMEL) website [12]. There are obviously important considerations regarding the clinical use and potential implications of genetic testing for melanoma risk. According to the GeneTests website [3], there are now five hospital-based and commercial laboratories in the USA offering testing for germline mutations in CDKN2A and/or CDK4. A Canadian-based company now offers a commercial test for MC1R gene variants [13]. According to the company website [13], "*the Genescreen™ consumer test is simple and noninvasive. The results of the test can assist consumers and help them to make decisions on their sun protection and sun lifestyle habits*." Such consumer-directed advertisement (in addition to the direct-to-consumer sales for some assays) can only enhance the profile of molecular testing.

In addition to germline aberrations, the study of somatic mutations in melanoma may reveal frequent cooperating oncogenic pathways responsible for the development and progression of this tumor. Subtypes of melanomas with common pathogenesis and clinical behavior may be identified, specific sites for targeted therapy may be revealed, and discovered genomic alterations may be used to develop diagnostic assays. In this regard, a California-based molecular diagnostics company describes its proprietary, patent-pending technology as unique for evaluating melanocytic neoplasms [14]. According to the company, this novel technology includes a number of highly validated genetic markers that allow for discrimination between benign melanocytic nevi and melanoma, in addition to better characterization of the risk of melanoma progression and/or metastasis [14]. The test evaluates the immunohistochemical staining patterns (i.e., intensity and distribution of expression) of five markers (ARPC2, FN1, RGS1, SPP1, and WNT2), selected on the basis of prior gene expression profiling studies using cDNA microarrays [15]. Significantly greater expression of each of these protein markers is seen in malignant compared with benign lesions. Based on comparison with the actual histopathological diagnoses, this multi-marker assay is reported to show 95% specificity and 97% sensitivity for diagnosing melanomas arising in melanocytic nevi, 95% accuracy in identifying both Spitz nevi and dysplastic nevi, and 75% accuracy in correctly diagnosing previously misinterpreted melanocytic lesions [15]. The company expects its multi-marker assay kits to capture a significant

share of the target market within the first 2 years; estimating sales of over $150 million (~10% market penetration) annually in the USA alone, and over $300 million worldwide [14]. Interestingly, a group of researchers from the same company have reported that multi-marker assays based on three proteins (NCOA3, SPP1, and RGS1; also derived from results of cDNA microarray analyses) add independent prognostic data, with regard to sentinel lymph node involvement and outcomes, in patients with melanoma [16]. Another California-based company is offering a patented combined tape-stripping and GeneChip assay (MelDTect™) for the detection of melanoma [17]. With this test, RNA is harvested from the surface layer of the skin by a patented noninvasive EGIR™ (Epidermal Genetic Information Retrieval) technique and analyzed using a 17-gene classifier. This assay is purported to demonstrate a sensitivity of 100% and a specificity of 88% in discriminating melanoma from melanocytic nevi (S. Chang, personal communication, 2010) [17]. Finally, a Florida-based company is now in the final stages of validating and commercializing a fluorescence in situ hybridization (FISH)-based test (MelanoSITE™) for the diagnosis of melanoma [18]. According to a recent press release, the company estimates that this FISH assay, which is based on Abbott Laboratories 4-probe set (6p25, 6 centromere, 6q23, and 11q13), will represent a $50–$100 million annual revenue opportunity over the next ~5 years [18]. The development of this test stems largely from the groundbreaking studies on melanocytic tumors undertaken at the University of California at San Francisco and Northwestern University in Chicago (see Chap. 5) [19–21].

Other Skin Disorders

Among common skin conditions, inflammatory diseases represent the group for which molecular diagnostics has had the least impact on to date. Many inflammatory disorders can be readily diagnosed on the basis of clinical–histopathological correlation, and current molecular studies of these entities are largely research-oriented. Yet, despite remarkable advances in our understanding of the pathogenesis of these diseases (particularly psoriasis and atopic dermatitis) and the availability of novel therapies, a significant number of patients defy accurate classification and/or do not respond to treatment. Of note, the EGIR™ platform, described above, has also been used to evaluate gene expression in a range of inflammatory dermatoses (psoriasis, allergic contact dermatitis, and irritant contact dermatitis), and could have a number of interesting future commercial applications in this setting, such as the selection and monitoring of therapy, as well as pharmaceutical/cosmeceutical research and development [17, 22–25]. In addition to both identifying specific genes associated with clinical response(s) in inflammatory skin diseases and promoting the development of novel therapeutic agents, molecular testing could be used to definitively categorize challenging cases and/or diagnose related conditions (see Chaps. 14 and 21). For example, it is estimated that between 20% and 40% of psoriasis patients will eventually develop psoriatic arthritis (PsA). Recently, a patent-pending commercial screening test for PsA was released, purportedly capable of identifying those at high risk for developing PsA before they experience arthritic symptoms, thus providing the opportunity to lessen joint damage through early medical intervention [26]. This assay determines the presence of a specific variation on the MICA (class I major histocompatibility complex chain-related gene A) immune response gene located on chromosome 6. According to the California-based company, an individual that tests positive for the MICA-A9 variant has ~60% chance of developing PsA, while an individual that tests negative for the MICA-A9 variant has ~70% chance of not developing PsA [26]. This company has also introduced a genetic screening test for male and female pattern baldness (androgenetic alopecia), that is based on the identification of specific variations in the androgen receptor (AR) gene, including (a) the presence or absence of a *Stu1* single nucleotide polymorphism (SNP) and (b) the number of CAG repeats [26]. According to the company website [26], "*a positive test result means that a man has the high risk genetic variation. Men who test positive have approximately a*

70% chance of going bald. Men who test negative have approximately a 70% chance of not going bald." The use of an additional genetic test to determine finasteride response would help "*doctors predetermine if patients will have a subtle, moderate, or great treatment response… allowing the physician to provide patients with the best treatment regimen to save their hair* [26]." Another test purportedly evaluates the risk of developing frequent outbreaks of genital herpes [due to herpes simplex virus-2 (HSV-2) infection], by analyzing for the presence or absence of specific mutations in the mannose-binding lectin (MBL2) gene [26]. A screening test that purportedly assesses a women's risk for developing moderate to severe cellulite (gynoid lipodystrophy), based on the presence of a specific variation of the angiotensin I-converting enzyme (ACE) gene located on chromosome 17, is also being marketed by the same company [26]. Finally, a Canadian-based company reports the development of genetic tests which can determine ultraviolet radiation (UVR) genetic susceptibility and the level of sun-related DNA damage, with results "*tailored into consumer friendly personalized recommendations for sun protection and treatment*" and offering "*new insight into diagnosis and new opportunities for selecting treatment regimes and monitoring strategies* [13]." Another test offered by this company is purported to assess the ability of skin care products to prevent or repair UVR-induced DNA damage [13].

Metastatic Tumors

The classification of cancers, based on their genomic signatures, has been advocated as an objective means to determine tumor behavior, identify therapeutic targets, define prognosis, and in some cases, assign lineage. With regard to the latter, there are up to 100,000 (metastatic) cancers of unknown primary (CUP) origin in the USA each year [2]. In addition, it is estimated that another 150,000 metastatic cancers require further differential work-up [2]. Global gene expression profiling-based molecular testing platforms, which employ quantitative reverse transcription (RT)-PCR and cDNA/oligonucleotide microarray technologies, are now being developed for this market by a number of USA and non-USA companies [27]. These assays are based on the premise that metastatic tumors retain the gene expression patterns of their respective primary lesions [27]. While a single test is expensive, in the range of $3,000 per assay, the overall accuracy of genomic cancer classification appears to be high (~80–90%) [27]. In contrast, a recent study reported that the average combined cost for non-molecular classification techniques (i.e., body imaging studies, tissue immunohistochemistry) for CUP is $18,000, with a success rate of only ~25% [2]. By extrapolation, a single successful identification with traditional approaches would cost in the region of $72,000 [2]. In dermatology, the ability to differentiate between a cutaneous metastasis and a primary skin tumor has obvious significance for prognosis, therapy, and survival outcome in individual patients, as well as implications for broader epidemiological studies. There are some anecdotal reports on the use of gene expression profiling to identify the organ/tissue origin for metastatic tumors in the skin. However, commercially available assays have not yet been validated for routine testing of cutaneous specimens. The use of molecular tests to differentiate between primary and metastatic tumors in the skin is discussed in Chaps. 6 and 9.

Pharmacogenetics and Pharmacogenomics

Pharmacogenetics and pharmacogenomics are now leading growth areas in molecular diagnostics. This is driven by the concept of "personalized medicine," and the potential tailoring of therapy to an individual patient, as genetic factors are known to be important determinants of both drug efficacy and drug toxicity. Although the terms are often used interchangeably, pharmacogenetics refers to

the study of variations in DNA sequence between individuals that result in differential drug responses; while, pharmacogenomics refers to the study of single or multiple genes using "omic" technologies, in an effort to guide drug selection and dosage, optimize efficacy, minimize adverse drug reactions (ADRs), and promote drug discovery and development [28]. The effects of treatment can be markedly heterogeneous among patients, and the average efficacy rate of many drug classes is just above 50% [28]. In addition, ~6% of hospitalizations occur as a result of ADRs [28]. The question that begs to be asked is: Can individualized drug dosing strategies be based on the results of gene profiling studies? Yes, is the simple answer. One example is the AmpliChip CYP450 Test (Roche Diagnostics Corporation, Indianapolis, IN); the first FDA-cleared pharmacogenetic test for analysis of an individual's metabolizing pathways, namely, the CYP2D6 and CYP2C19 genes. These two genes, which are components of the cytochrome P450 system, can greatly influence drug bioavailability. The AmpliChip CYP450 Test identifies the patient's genotype and provides a predicted phenotype (i.e., poor, intermediate, extensive or ultrarapid metabolizer) to the clinician for use in determining a therapeutic strategy. Furthermore, the molecular classification of a number of both inflammatory and malignant skin disorders is leading to the development of individualized therapies (i.e., PLX4032 for patients with metastatic melanoma harboring $BRAF^{V600E}$ mutations), in addition to providing insights into distinct clinical-histopathological features of particular diseases (i.e., age of onset, disease severity). Pharmacogenetic and pharmacogenomic data on a variety of major skin diseases, including discussions on susceptibility genes, personalized therapy, and genetic determinants of drug responses and ADRs, are provided in Chaps. 21 and 22.

Personal Genomic Services and Direct-to-Consumer Tests

In most instances, it is required that licensed physicians submit molecular diagnostic test requisitions and receive the results. Therefore, the vast majority of current molecular assays are marketed to physicians and laboratories rather than to consumers. However, the trend is reversing with the ongoing acquisition of genomic data, the development of newer user-friendly and less expensive technologies, and public awareness. Patients are now more actively involved in the decision-making process with regard to the delivery of their healthcare options, and a growing number of firms are advertising personal genomic services directly to consumers. According to a recent publication by Washington G-2 Reports [2], "*…personal genomics has come to the fore very rapidly with the help of the mainstream media… This area is directed to consumers. It has been very interesting to see the companies try to skate around exactly what they're offering, whether it's medical advice (i.e., predictive data) or recreational information about your own genome… It's a complex interaction of technology, knowledge, health care products, and ethical implications of the human genome project and science*." Companies involved in the personal genomics market include deCODE Genetics (Reykjavik, Iceland), 23andMe (Mountain View, CA), Navigenics (Redwood Shores, CA), SeqWright (Houston, TX), Knome (Cambridge, MA), and Personal Genome Project (Boston, MA). Most use microarray technology or full-genome sequencing to search for SNPs, with results often presented in terms of relative risk (i.e., chance of developing a particular disease compared to the average risk). SNPs are probably the most important category of genetic changes influencing common diseases; stemming from their ability to influence disease risk, in addition to drug efficacy and side effects. It is estimated that nine of the top ten leading causes of death have a genetic component, with one or more SNPs influencing disease risk [2]. Conceptually, the knowledge of any genetic risk will encourage individuals to reduce it by engaging in healthier behaviors and/or the adoption of efficacious therapy. However, genetic alterations are only one of a number of factors that influence an individual's risk of disease. Risk assessment models must also take into account family history, lifestyle, and environmental influences. The American College of Medical Genetics states that

problems with direct-to-consumer (DTC) testing can include "*lack of informed consent, inappropriate testing, misinterpretation of results, testing that is inaccurate or not clinically valid, lack of follow-up care, misinformation, and other adverse consequences* [29]." In the UK, the Human Genetics Commission has issued "*A Common Framework of Principles*" for DTC genetic testing services [30]. This wide-ranging set of principles, which companies must follow in order to protect consumers from potential harm, covers the purpose and scope of tests, advertising and marketing claims, regulatory data, information for consumers, including counseling, consent and test accuracy, provision and interpretation of results, sample handling and laboratory procedures, data protection and complaints [30]. According to the Human Genetics Commission, these principles promote the following: (1) purchasers of DTC tests must be made aware of possible outcomes and limitations of testing, such as information to be gained and how to act on it, in addition to other factors that might play a role in disease development, including lifestyle and environment; (2) tests for major hereditary diseases, such as cancer, should only be provided if counseling is available both pre- and post-testing; (3) test claims must be supported by scientific evidence; (4) the "consumer" must give consent for testing, and samples and genetic information must be keep secure; and (5) testing should only be performed by accredited laboratories [30]. Recent articles in the dermatology-focused literature have discussed the challenges of translating genetic tests for skin disorders into clinical and public health practice, in addition to highlighting concerns that the expanding market of DTC testing is beginning to blur the distinction between classic diagnostic assays and personal genomics services [29, 31]. It will take time for results and implications of genome-wide association studies and whole genome analyses to be fully appreciated, and therefore, it is necessary to educate both consumers and physicians on the utility and limitations of any information obtained from genetic testing. Point-of-care (POC) genetic education resources have been proposed [31]. For example, SNPedia is a Wikipedia, which shares information about the clinical effects of variations in DNA, citing peer-reviewed scientific publications [32]. Currently, it contains data on ~9,910 SNPs, with descriptions of genes, chromosomes, population diversity, and personal genomics companies offering tests [32].

The role of genetic and epigenetic changes in both benign and malignant skin disorders and their function in disease susceptibility, phenotype, severity, and treatment response continues to be investigated. This ever-growing knowledge is beginning to provide us with avenues for the development of more accurate and specific diagnostic and prognostic assays, cancer staging tests, and safer, more effective, and targeted therapies, in addition to strategies for disease prediction and prevention. It is truly a brave new world in the realm of dermatology and dermatopathology.

References

1. Grosse SD, Khoury MJ. What is the clinical utility of genetic testing? Genet Med. 2006;8:448–50.
2. Business Strategies for Molecular Diagnostics in the Lab. In: Murg S, Terry M, editors. Including state of the market report, 2nd ed. Newark: Washington G-2 Reports; 2009.
3. Dudda-Subramanya R, Alexis AF, Siu K, et al. Alopecia areata: genetic complexity underlies clinical heterogeneity. Eur J Dermatol. 2007;17:367–74.
4. GeneTests. http://www.genetests.org/ (2010). Accessed 2 Sept 2010.
5. Kesselheim AS, Mello MM. Gene patenting – is the pendulum swinging back? N Engl J Med. 2010;362:1855–8.
6. Association for Molecular Pathology. http://www.amp.org/ (2010). Accessed 2 Sept 2010.
7. Pene F, Courtine E, Cariou A, et al. Toward theragnostics. Crit Care Med. 2009;37(1 Suppl):S50–8.
8. Holtzman NA, Watson MS. Promoting safe and effective genetic testing in the United States. Final report of the task force on genetic testing. http://www.genome.gov/10001733. Accessed 2 Sept 2010.
9. Netto GJ, Saad RD. Diagnostic molecular pathology: an increasingly indispensable tool for the practicing pathologist. Arch Pathol Lab Med. 2006;130:1339–48.

10. Chen B, Gagnon M, Shahangian S, et al. Good laboratory practices for molecular genetic testing for heritable diseases and conditions. Morbidity Mortality Weekly Reports. 2009;58:1–29.
11. Leachman SA, Carucci J, Kohlmann W, et al. Selection criteria for genetic assessment of patients with familial melanoma. J Am Acad Dermatol. 2009;61:677.e1–14.
12. GenoMEL, the Melanoma Genetics Consortium. http://www.genomel.org/ (2010). Accessed 2 Sept 2010.
13. Genesis Genomics. http://www.genesisgenomics.com/ (2010). Accessed 2 Sept 2010.
14. Melanoma Diagnostics, Inc. http://www.melanomadiagnostics.com/ (2010). Accessed 2 Sept 2010.
15. Kashani-Sabet M, Rangel J, Torabian S, et al. A multi-marker assay to distinguish malignant melanomas from benign nevi. Proc Natl Acad Sci USA. 2009;106:6268–72.
16. Kashani-Sabet M, Venna S, Nosrati M, et al. A multimarker prognostic assay for primary cutaneous melanoma. Clin Cancer Res. 2009;15:6987–92.
17. DermTech International. http://dermtech.com/ (2010). Accessed 2 Sept 2010.
18. NeoGenomics Laboratories. http://www.neogenomics.org/ (2010). Accessed 2 Sept 2010.
19. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005;353:2135–47.
20. Bauer J, Bastian BC. Distinguishing melanocytic nevi from melanoma by DNA copy number changes: comparative genomic hybridization as a research and diagnostic tool. Dermatol Ther. 2006;19:40–9.
21. Gerami P, Jewell SS, Morrison LE, et al. Fluorescence in situ hybridization (FISH) as an ancillary diagnostic tool in the diagnosis of melanoma. Am J Surg Pathol. 2009;33:1146–56.
22. Wong R, Tran V, Talwalker S, et al. Analysis of RNA recovery and gene expression in the epidermis using non-invasive tape stripping. J Dermatol Sci. 2006;44:81–92.
23. Benson NR, Papenfuss J, Wong R, et al. An analysis of select pathogenic messages in lesional and non-lesional psoriatic skin using non-invasive tape harvesting. J Invest Dermatol. 2006;126:2234–41.
24. Morhenn VB, Chang EY, Rheins LA. A noninvasive method for quantifying and distinguishing inflammatory skin reactions. J Am Acad Dermatol. 1999;41:687–92.
25. Wong R, Tran V, Morhenn V, et al. Use of RT-PCR and DNA microarrays to characterize RNA recovered by non-invasive tape harvesting of normal and inflamed skin. J Invest Dermatol. 2004;123:159–67.
26. DermaGenoma. http://www.dermagenoma.com/ (2010). Accessed 2 Sept 2010.
27. Bender RA, Erlander MG. Molecular classification of unknown primary cancer. Semin Oncol. 2009;36:38–43.
28. Pincelli C, Pignatti M, Borroni RG. Pharmacogenomics in dermatology: from susceptibility genes to personalized therapy. Exp Dermatol. 2009;18:337–49.
29. Bowers J. DNA goes DTC. Genetic testing firms bring personal genomics to the consumer. Dermatol World. 2009;19:22.
30. Human Genetics Commission. http://www.hgc.gov.uk/Client/index.asp?ContentId=1 (2010). Accessed 2 Sept 2010.
31. Levy H. Genetic testing goes commercial. Skin & Allergy News. 2009;40:12.
32. SNPedia. http://www.snpedia.com/. Accessed 6 May 2010.

Chapter 2
Principles of Molecular Biology

Jian Zhuge and Wenyong Zhang

The rapid development of molecular biology in recent decades has dramatically changed the way we practice medicine. With the help of an impressive arsenal of new technologies, including high-throughput sequencing and microarrays, we are now well-equipped to probe into the molecular nature of diseases. Which set of genes are involved? What are the genetic and epigenetic alterations associated with these genes? In this chapter, we will describe the basic concepts of molecular biology, including genes, types of mutations, and gene expression.

DNA

DNA (deoxyribonucleic acid) is the genetic material of cells. It is composed of individual units called nucleotides. A nucleotide is composed of three subunits: a five-carbon sugar (deoxyribose), a phosphate group, and a base. There are four types of bases in DNA: adenine (A), guanine (G), thymine (T), and cytosine (C). Adenine and guanine are purines, and thymine and cytosine are pyrimidines. A polynucleotide chain is formed by linking the adjacent nucleotides via 5′→3′ phosphodiester bonds. In 1953, Watson and Crick solved the structure of DNA – demonstrating that a DNA molecule is composed of two complementary polynucleotide chains forming the double-helix structure [1]. The double-helix chains are stabilized by hydrogen bonds formed between the opposing A–T and C–G bases on the two complementary polynucleotide chains (Fig. 2.1).

RNA

In contrast to DNA, RNA (ribonucleic acid) contains the sugar ribose instead of deoxyribose, and uracil (U) instead of thymine (T). An RNA molecule is single-stranded and less stable than a DNA molecule. Cellular RNA serves diverse functions, carried out by different families of RNA molecules, including messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small interfering RNA (siRNA), and microRNA (miRNA). Ribozymes are RNA molecules with catalytic

W. Zhang (✉)
Department of Pathology, New York Medical College,
Westchester Medical Center, 100 Woods Road, Valhalla, NY 10505, USA
e-mail: zhangw@wcmc.com

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_2,

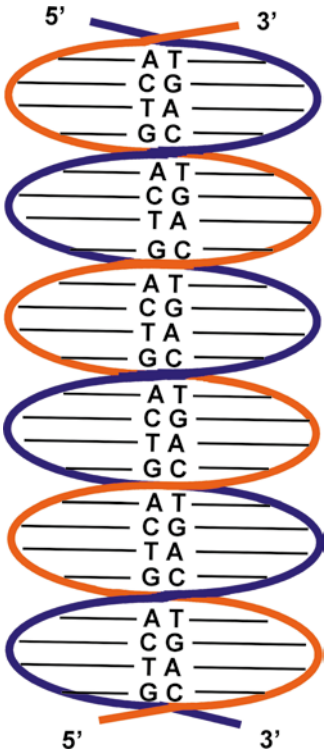

Fig. 2.1 Double-helix structure of DNA. The double-helix structure of DNA is stabilized by the hydrogen bonds formed between A–T and C–G bases on the two antiparallel complementary strands. *A* adenine, *T* thymine, *G* guanine, *C* cytosine

functions. Because RNA molecules have diverse functions, proponents of the "RNA world" theory suggest that RNA may have preceded DNA and protein in life's long evolutionary journey [2, 3].

The Human Genome

The human genome is composed of slightly more than three billion base pairs of DNA, organized into 46 chromosomes (22 pairs of autosomes and 2 sex chromosomes). The Human Genome Project (HGP) has taught us that there are approximately 20,000–25,000 protein-coding genes, representing only ~1.5% of the entire genome size [4]. The remainder of the human genome includes regulatory sequences, RNA genes, pseudogenes, repeat sequences, and other sequences for which no known functions are currently understood.

Repeat DNA Sequences in the Genome

Much of the so-called "junk DNA" (noncoding DNA) in the human genome contains repeat sequences. There are two types of repeat sequences: (a) tandem repeat and (b) interspersed repeat.

Tandem repeats are two or more nucleotides repeated as a unit one after another in the same orientation. Examples of tandem repeats include microsatellites (2–6 nucleotides long) and minisatellites (longer than microsatellites, but shorter than 60 nucleotides in length). Microsatellites are useful markers for identity testing, bone marrow chimerism study, microsatellite instability (MSI) testing, and gene dosage studies, such as loss of heterozygosity (LOH) or gene duplication analyses.

Interspersed repetitive DNA sequences, also known as retrotransposons, are repeat elements characterized by RNA intermediates [5]. In mammals, interspersed repetitive DNA constitutes approximately half of the genome. There are two types of interspersed repeat: (a) LTR (long terminal repeat) retrotransposons and (b) non-LTR retrotransposons. Non-LTR retrotransposons are further divided into SINEs (short interspersed nuclear elements) and LINEs (long interspersed nuclear elements). LINEs bear similarities to retroviruses, in that they encode the enzyme reverse-transcriptase which transcribes LINEs RNA into many DNA copies for integration into new genomic loci, thus providing a mechanism for genomic expansion. However, LINEs do not have the LTRs found in retroviruses (i.e., they are not functional retroviruses). LINEs account for approximately 20% of the human genome. SINEs are typically less than 500 bases in length, and do not encode reverse-transcriptase. They constitute about 14% of the human genome. The most common SINEs in the human genome are Alu sequences.

Eukaryotic Gene Structure and Function

A gene is the hereditary unit of a living organism. The classical concept of genes is centered around the notion that one gene encodes for one protein/enzyme. A classical eukaryotic gene is composed of exons, introns, and regulatory sequences. Exons are stretches of DNA sequences that are represented in the mature form of RNA, including mRNA and tRNA; while, introns are the intervening DNA sequences between exons that will be spliced from the maturing RNA molecule. An RNA transcript usually consists of multiple exons spliced together. A single gene may produce several different transcripts by alternative splicing. The regulatory sequences of a gene include promoters, enhancers, silencers, insulators, and locus control regions (LCRs). A promoter is a region of DNA that facilitates transcription by binding to transcription factors (TFs) and RNA polymerase II. A gene can have several promoters, usually located upstream of the transcription start site. The location of the promoter is designated by counting back from the transcription start site (i.e., −34 refers to 34 base pairs upstream). An enhancer is another type of gene regulatory element that is located either upstream or downstream of the gene, and which regulates gene expression from a greater distance.

Large-scale genomic studies have begun to challenge the classical concept of genes [6]. Data from the International Encyclopedia of DNA Elements (ENCODE) project revealed that genes are surprisingly flexible in the sense that "genes know no borders" (i.e., when a gene is transcribed, the transcript often contains not only the gene itself, but also a portion of the next gene). Such fusion transcripts are estimated to constitute 4–5% of the traditionally recognized gene sequences. In addition, a large number of novel transcription start sites, many of which are located hundreds of thousands of bases away from known start sites, as well as new promoters, have been identified. Surprisingly, nearly a quarter of the newly discovered promoters are located at the end of genes, rather than all at their beginning, as originally thought.

Only 1–2% of human DNA sequences code for proteins. However, genomic studies have shown that much of this noncoding "junk DNA" is transcribed [6]. Among thousands of RNA molecules that are transcribed from the noncoding DNA, the family of functional noncoding RNA (ncRNA) continues to expand. This family now includes tRNA, rRNA, miRNA, siRNA, small nuclear RNA (snRNA), piwi-interacting RNA (piRNA), and long ncRNA.

Telomere

A chromosome is a thread-like structure composed of a long strand of DNA and associated proteins. The chromosomal ends in eukaryotes are sealed and stabilized by special regions called telomeres. DNA at the telomeric regions is characterized by tandem repeat sequences. For example, human telomeres consist of 2–50 kilobases of TTAGGG tandem repeat sequences.

During DNA replication, the ends of chromosomes cannot be completely replicated, resulting in a shortened copy of DNA. Therefore, telomeric sequences can provide protection against the loss of vital DNA during this process. However, telomeres themselves are subject to shortening during DNA replication, unless they can be replenished by the action of telomerase, a modified RNA polymerase only active in the germ cells of most eukaryotes [7].

Somatic human cells lack telomerase, and therefore telomeres are shortened during each round of replication in these cells. In addition, oxidative stress can also result in telomere shortening. As telomeres are continuously reduced in length during replication, somatic cells will eventually reach the limit of their replicative capacity and enter senescence. Cellular senescence is thought to play an important role in the suppression of cancer development [8]. The link between telomere and cancer is well established. Cancer cells have found ways to circumvent the replicative limit imposed by shortened telomeres. In fact, most cancer cells possess telomerase activity that can replenish and maintain their telomeres [9]. In addition, some cancer cells may employ an alternative lengthening of telomeres (ALT) pathway [10], which involves the transfer of telomere tandem repeats between sister chromatids. Telomeres are not only important in cancer research, but also for aging studies [11]. Several premature aging syndromes, such as Werner syndrome, Bloom syndrome, and ataxia-telangiectasia, are characterized by an accelerated rate of telomere attrition [12]. Telomere shortening contributes to stem cell dysfunction and loss of tissue regeneration [13]. However, the use of telomere length or its attrition rate as aging biomarkers *in vivo* remains to be established.

Mitochondrial DNA

A mitochondrion contains 2–10 copies of mitochondrial DNA (mtDNA). There are 100–10,000 copies of mtDNA in a human somatic cell. The human mitochondrial genome is a circular DNA molecule with 15,000–17,000 bases, encoding 13 proteins, 22 tRNAs, and one each of the small and large subunits of rRNA. The 13 protein-coding genes of the mitochondrial genome are primarily involved in energy metabolism: subunits 1, 2, and 3 of the cytochrome c oxidase complex; cytochrome b; subunits 6 and 8 of the ATP synthase complex; and six subunits of NADH dehydrogenase. Because human sperms contain far fewer copies of mtDNA than ova, mtDNA typically follows a maternal line of inheritance.

Even though a mitochondrion contains its own DNA, it is nuclear DNA that encodes the majority of its approximate 1,500 proteins, which are transported into the mitochondrion following assembly in the cytoplasm. Therefore, genetic disorders affecting mitochondria can show Mendelian inheritance patterns. Pure mitochondrial genetic disorders show only a maternal pattern of inheritance. Because mitochondria are the “power plants” of the cell, mitochondrial diseases tend to affect organs with high energy requirements, such as muscle, heart, brain, and nerve. Some of the notable mitochondrial diseases include Leber hereditary optic neuropathy (LHON), mitochondrial encephalomyopathy, lactic-acidosis with stroke-like symptoms (MELAS), and myoclonic epilepsy and ragged red fibers (MERRF). Mitochondrial diseases are characterized by considerable heterogeneity,

due to variable distribution of defective mtDNA from organ to organ. Of note, frequent mutations in the mitochondrial genome have been reported in both melanoma and non-melanoma skin cancers [14, 15]. Mutant mtDNA in tumor cells might alter mitochondrial-mediated apoptotic pathways to prevent cell death and/or confer a selective growth advantage [14, 15].

MicroRNA

MicroRNAs (miRNAs) are a family of 19- to 22-nucleotide, noncoding small RNAs that primarily function as gene regulators. It is estimated that there may be 1,000 unique miRNAs in the human genome. Pri-miRNAs are transcribed from miRNA genes by RNA polymerase II or III. Transcription from these miRNA genes is most likely regulated by TFs that respond to multiple signals and/or are epigenetically controlled. The pri-miRNAs are processed by RNAase III enzyme Drosha complexed with DGCR8 (DiGeorge syndrome critical region gene-8) to form pre-miRNAs, which are ~70-nucleotide RNAs with an imperfect RNA duplex structure. A small number of pre-miRNAs are derived from introns via RNA splicing, and not processed by the Drosha-DGCR8 complex. These alternatively processed miRNAs are called "mirtrons" [16]. The pre-miRNAs are transported from the nucleus to the cytoplasm, and further processed by Dicer to generate an imperfect double-stranded RNA duplex called miRNA/miRNA*. The mature miRNAs contained in RISC (RNA interference silencing complex) bind to specific sites in the 3′-untranslated region of the target mRNA. If base-pairing between the miRNA and its target is perfect, the mRNA will be cleaved. Imperfect pairing between the miRNA and its target can elicit translational repression or mRNA destabilization by deadenylation (Fig. 2.2).

miRNAs can potentially regulate thousands of human genes, many of which are involved in transcriptional regulation or other basic cellular functions, such as cell differentiation, proliferation, and apoptosis. Deregulation of miRNA gene transcription may result from alterations in miRNA gene copy number, epigenetic mechanisms, or activity of the TFs that control transcription. A number of studies have shown that miRNAs may be useful biomarkers for cancer classification and prognostication, and represent potential therapeutic targets [17].

Replication, Transcription, and Translation

Replication

Replication of DNA is required to ensure that an exact copy of DNA will be passed down from the maternal cell to its progeny (Fig. 2.3). Watson and Crick first solved the double-helix structure of DNA, and suggested a copying mechanism for DNA replication. Each strand of DNA can serve as a template for the production of a new strand (semiconservative replication). DNA replication in eukaryotes is a parallel process, whereby many chromosomal sites are replicated simultaneously. A new strand of DNA is synthesized in the 5′→3′ direction, because nucleotides can only be added to the 3′ end of the growing nucleotide chain. Replication begins with helicase-mediated unwinding of the double-helix, producing the replication fork and allowing the two existing DNA strands to serve as templates for new strand formation. Only one of the two new strands can be synthesized continuously in the 5′→3′ direction as the replication fork opens. This is called the leading strand. The other strand, which is called the lagging strand, is formed by the joining of many discontinuous

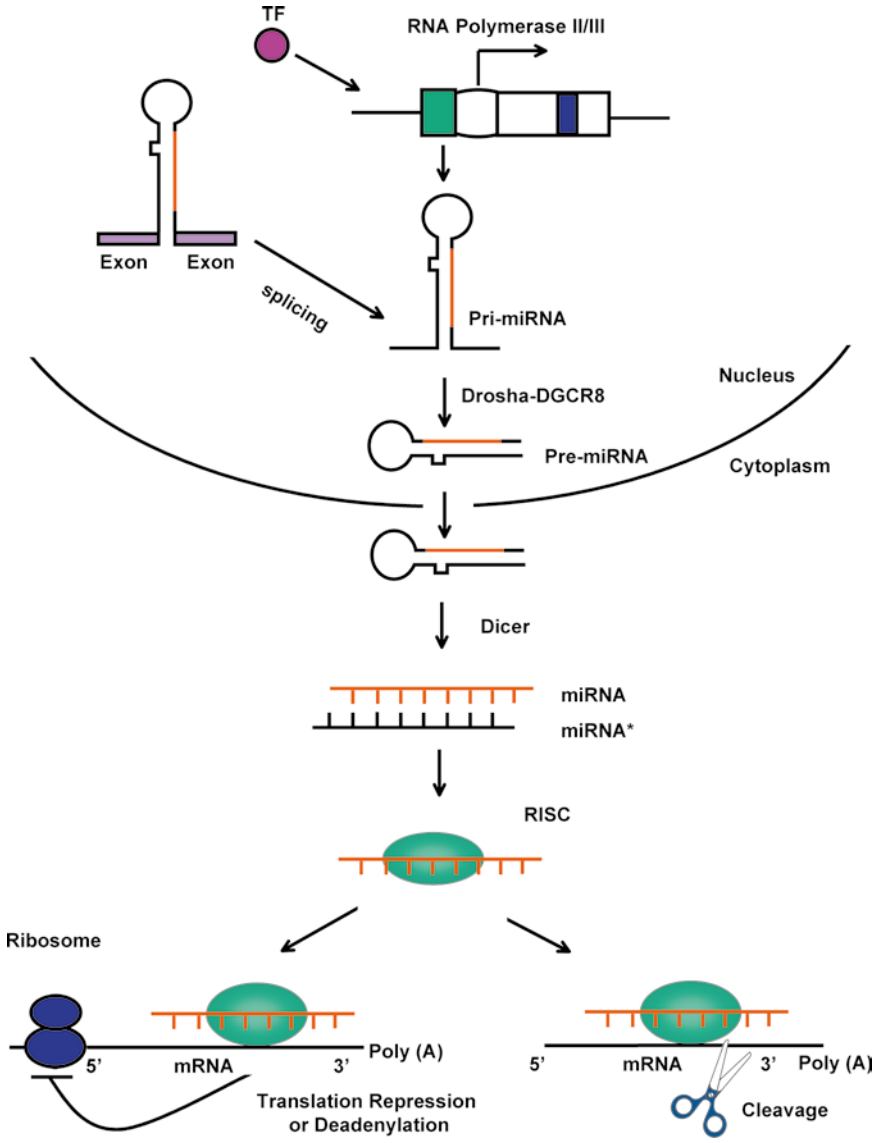

Fig. 2.2 Biogenesis of miRNA. Pri-miRNAs are transcribed from miRNA genes by RNA polymerase II or III, under the influence of transcription factors (TF). The pri-miRNAs are processed by Drosha-DGCR8 to pre-miRNAs. In an alternative pathway, miRNAs encoded in the intronic regions ("mirtrons") form pre-miRNAs directly via RNA splicing. The pre-miRNAs are transported from the nucleus to the cytoplasm, and further processed by Dicer to generate an imperfect double-stranded RNA duplex called miRNA/miRNA*. The mature miRNAs contained in RISC (RNA interference silencing complex) bind to specific sites in the 3′-untranslated region of the target mRNA. If base-pairing between the miRNA and its target is perfect, the mRNA will be cleaved. Imperfect pairing between the miRNA and its target can elicit translational repression or mRNA destabilization by deadenylation

small segments (Okazaki fragments) that are synthesized along the lagging strand template. The DNA polymerases involved in lagging and leading strand synthesis also have proof-reading 3′→5′ exonuclease activity.

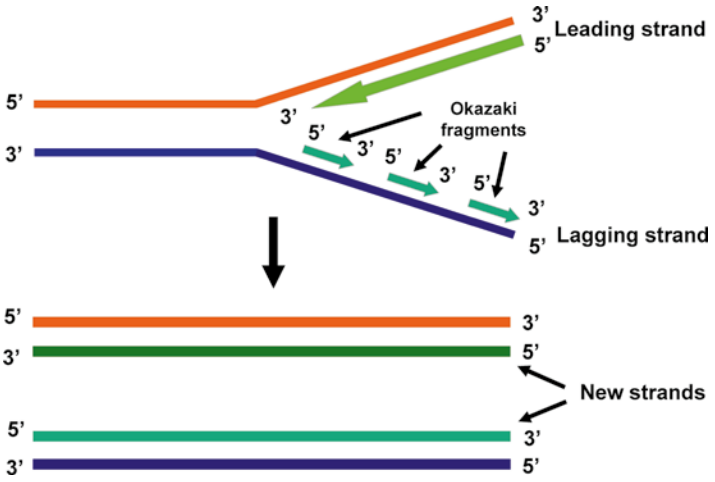

Fig. 2.3 DNA replication. DNA is unwound by helicase to form the replication fork. The leading strand is synthesized continuously in the 5′ to 3′ direction. The opposite strand (lagging strand) is formed by joining many discontinuous Okazaki fragments. DNA replication is semiconservative, in that each of the two newly formed DNA copies contains one old strand and one new strand

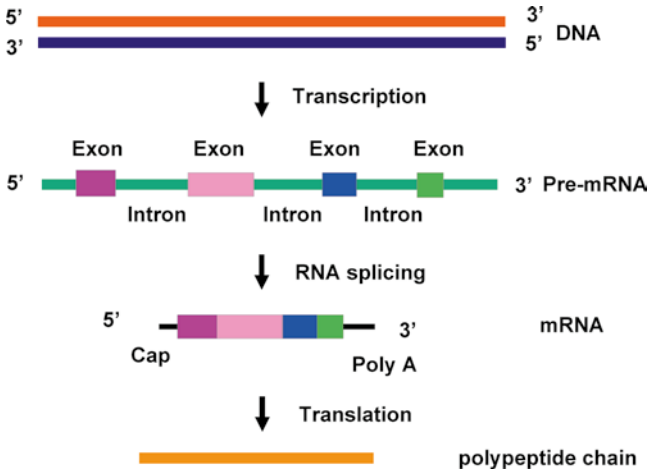

Fig. 2.4 Transcription and translation. These are two processes that decode genetic information carried by DNA. In transcription, an RNA molecule is synthesized based on its DNA template. The so-called pre-mRNA is processed by addition of a 5′ "cap," addition of a 3′ polyA tail, and RNA splicing which removes intronic sequences. The mature messenger RNA, which is called mRNA, is then transported to the cytoplasm. In the cytoplasm, the mRNA sequence dictates the synthesis of a polypeptide chain on ribosomes

Transcription

The information encoded in DNA dictates RNA synthesis and subsequent protein production. The directional information flow from DNA to RNA, and finally to protein has been called the "central dogma of molecular biology" (Fig. 2.4). The transcription of DNA into RNA is a highly regulated process, involving interactions between TFs, promoters and other regulatory elements. Transcription begins at the transcriptional "start site", which lies just upstream of the first coding sequence. From the

DNA template, the primary RNA transcript is synthesized in a 5′→3′ direction, catalyzed by RNA polymerase II. The primary RNA transcript contains both intron and exon sequences, and is processed in the nucleus by "capping" at the 5′ end and addition of a polyA tail to the 3′ end. The RNA transcript is then further processed by removal of its intronic sequences (RNA splicing). The fully processed RNA, now called mRNA, is transported into the cytoplasm where translation takes place.

Translation

Translation is the process by which a polypeptide chain is synthesized on the basis of the mRNA nucleotide sequence. Translation occurs on ribosomes in the cytoplasm. Eukaryotic ribosomes are composed of large (60S) and small (40S) subunits. The 60S subunit contains 5S, 5.8S, and 28S RNA, and associated proteins. The 40S subunit contains 18S RNA and associated proteins. Translation is mediated by tRNAs, adaptor molecules that have the dual functions of (a) carrying specific amino acids and (b) deciphering the correct codon sequences on mRNA though their anticodon regions. The first translated codon AUG corresponds to the amino acid methionine. The synthesis of a protein involves the successive addition of correct amino acids to the growing polypeptide chain, using mRNA as a template and based on the pairing of the anticodon region of tRNA to a specific codon of mRNA. Translation stops when a stop codon (UAG, UGA, UAA) is reached. A codon is a three-base combination that holds the instructions for translation, either indicating that a particular amino acid should be added or signaling translation initiation or termination. Since there are four different bases (A, T, C, G), the number of possible codons is 4^3, or 64. However, there are only 20 amino acids. Therefore, more than one codon may encode for one specific amino acid. In such cases, the codon is described as degenerate. Different degenerate codons have identical first two bases, varying only in the third base position.

Common Types of Mutations

Mutations are changes in DNA sequences (Table 2.1). At the nucleotide level, common mutations include point mutations, which can be further defined as silent mutations, nonsense mutations, missense mutations, deletions, and insertions. At the genomic level, mutations include amplifications (gene duplication), interstitial deletions, and chromosomal translocations, or inversions.

Copy Number Variation

The development and use of new genomic technologies, such as comparative genomic hybridization and microarrays, have resulted in increased recognition of copy number variation (CNV) as a common type of human genetic mutation. Studies of humans from different ethnic backgrounds have shown 1,447 CNV regions, covering ~12% of the human genome [18]. CNVs can involve a single gene or a contiguous set of genes. Variation in the copy number of dosage-sensitive genes may contribute to human phenotypic variability and disease susceptibility.

Table 2.1 Common types of mutations

Type of mutations	Characteristics
Nucleotide level	
Silent	No change in amino acid
Missense	Change in amino acid
Nonsense	Introduction of a stop codon causing premature termination of translation
Insertion, deletion	Insertion or deletion of nucleotides may result in frameshift
Genomic level	
Amplification	Multiple copies of a chromosomal region; cause increased gene dosage
Interstitial deletion	Intrachromosomal deletion; may cause gene fusion or loss of heterozygosity
Translocation	Interchange of genetic material from nonhomologous chromosomes
Inversion	Reversing the orientation of a chromosomal segment
Copy number variation (CNV)	Changes in the copy number of a chromosomal segment; can be caused by deletion or duplication

Single Nucleotide Polymorphism

Single nucleotide polymorphism (SNP) refers to a variation in the nucleotide sequence among different individuals of a species. SNP is the most common type of genetic variation, occurring every 100–300 bases in the human genome. The distinction between an SNP and a mutation is rather artificial. In general, if the allele frequency is at least 1%, it is called an SNP; otherwise, it is referred to as a mutation. However, the National Center for Biotechnology Information (NCBI) SNP database (dbSNP) contains SNPs that have allele frequency less than 1%. SNPs can occur in both coding and noncoding regions of the genome. If an SNP is located in the coding region, and it does not change the sequence of the polypeptide chain, it is called synonymous; otherwise, it is termed nonsynonymous.

The study of SNPs will lead to a better understanding of the genotype-phenotype relationship, and help determine an individual's predisposition to common diseases, and their response to the medications used to treat them. For example, studies in methotrexate-treated psoriasis patients suggest that functional SNPs in genes relevant to methotrexate metabolism may influence both the efficacy and toxicity of this drug (see Chaps. 21 and 22). In addition, geneticists can use detailed SNP maps and genome-wide association studies (GWAS) to identify disease-causing genes (genetic regions) [19].

DNA Methylation

DNA methylation is one form of epigenetic regulation, an inheritable influence on gene expression without changes in the DNA sequence. During this process, a methyl group is added to the C5 position of a cytosine pyrimidine ring. In human cells, DNA methylation typically occurs on a cytosine that is followed by a guanine (i.e., CpG dinucleotide). It is estimated that 70% of all CpG sites are methylated in mammals. The unmethylated CpG sites are concentrated in the 5′ upstream region of genes, including the promoter region, forming so-called "CpG islands." Methylation of CpG islands at the promoter region can negatively impact gene expression by blocking the access of TFs (Fig. 2.5). Promoter methylation may play an important role in carcinogenesis. More than half of all known human tumor suppressor genes, including retinoblastoma *(RB)* and *CDKN2A/p16*, are

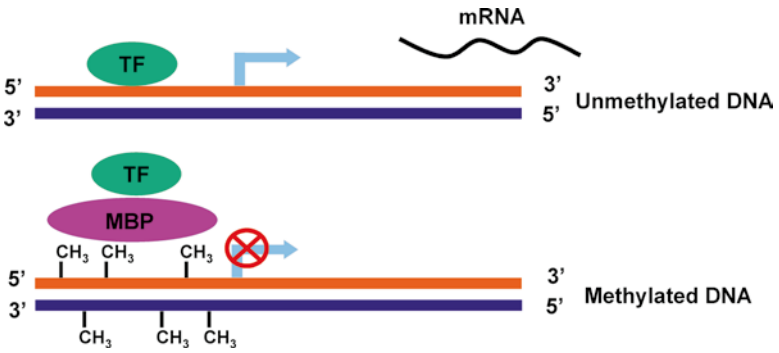

Fig. 2.5 Model of gene expression silencing by promoter methylation. In the unmethylated state, transcription factor (TF) can bind to the promoter region and activate transcription. If the promoter region is methylated, the methylated CpG site recruits methyl-binding proteins (MBPs), which further recruit other transcription repressors. TF access to the promoter is blocked and transcription is prevented

subject to promoter methylation in cancer [20]. Promoter methylation status of a select group of genes may serve as biomarkers for disease diagnosis, prognostication, and treatment response prediction. For example, methylation of the *MGMT* promoter is associated with a favorable response to temozolomide chemotherapy [21]. There is evidence to suggest that epigenetic dysregulation may be associated with not only skin cancers, but also other dermatologic disorders, such as psoriasis, atopic dermatitis, and cutaneous involvement by systemic lupus erythematosus [22].

Oncogenes and Tumor Suppressor Genes

An oncogene encodes a protein that is capable of transforming cells in culture or inducing cancer in animals. Oncogenes are derived from proto-oncogenes, normal cellular genes that provide pro-growth signals to cells. However, "gain-of-function" mutations or altered regulation of a proto-oncogene can transform it into an oncogene, resulting in excessive cellular growth. Oncogenes are classified according to their cellular functions. Common oncogenes that are associated with dermatologic neoplasms are listed in Table 2.2.

Tumor suppressor genes encode proteins that control cell cycle progression, repair damaged DNA, and regulate apoptosis. They function as "brakes," preventing uncontrolled cell growth and ensuring genomic integrity. Inactivation of tumor suppressor genes by mutations can tip the balance toward uncontrolled cell growth or unrepaired DNA damage, which may lead to cancer. An important difference between oncogenes and tumor suppressor genes is that typically both alleles of a tumor suppressor gene need to be inactivated in order to show a pro-cancer phenotype, whereas for oncogenes, an activating mutation residing on one of the two alleles is often sufficient to drive carcinogenesis. Another distinction is that oncogene mutations are often acquired, while mutations of tumor suppressor genes can be both acquired and inherited. A classic example of biallelic mutation in a tumor suppressor gene is provided by Knudson's study of the *RB* gene [23]. He proposed the "two-hit hypothesis" to explain that in familial retinoblastoma only one additional somatic mutation ("hit") is sufficient to cause disease, because one "hit" has been already inherited [23]. However, in the case of non-familial retinoblastoma, two separate somatic mutations are required to cause disease [23]. Examples of tumor suppressor genes associated with dermatologic neoplasms are provided in Table 2.3.

Table 2.2 Common oncogenes associated with dermatologic neoplasms

Functional groups	Mode of activation	Human skin tumors
Transcription factor		
MITF	Amplification	Melanoma [24]
STAT3	Activation	Mycosis fungoides [25]
Growth factor		
FGF2	Overexpression	Melanoma [26]
Growth factor receptors – tyrosine kinase		
KIT	Overexpression/point mutation	Melanoma [27]
c-MET	Overexpression/point mutation	Melanoma [28]
Cytoplasmic serine/threonine kinase		
mTOR	Activation	Melanoma [29]
G-protein		
K-RAS	Point mutation	AK [30], BCC [31], SCC [31]
N-RAS	Point mutation	Melanoma [32], congenital nevus [33]
H-RAS	Point mutation	Spitz nevus [34], SCC [35], BCC [31]
BRAF	*V600E* point mutation	Melanoma [36], benign nevus [36]
Cell cycle regulator		
CDK4	Amplification/point mutation	Melanoma [37], SCC [38]
CCND1	Amplification	Melanoma [39]
Apoptosis regulator		
BCL2	Translocation, amplification	Melanoma [40]
AKT3	Activation, amplification	Melanoma [41]
Wnt pathway		
β-catenin	Point mutation/activation	Melanoma [42], BCC [43]
Shh pathway		
SMO	Point mutation	BCC [44]

AK actinic keratosis, *SCC* squamous cell carcinoma, *BCC* basal cell carcinoma

Table 2.3 Common tumor suppressor genes associated with dermatologic neoplasms

Genes	Inherited tumor/syndrome	Non-inherited skin tumors
RB	Retinoblastoma	Many, including melanoma [45]
TP53	Li Fraumeni Syndrome	Many, including SCC, AK [46], BCC [44]
CDKN2A ($p16^{INK4a}$ and $p14^{ARF}$)	Familial melanoma	Many, including melanoma [37], MF [47], SCC [48]
XPA through XPG	Xeroderma pigmentosum	BCC, SCC [49]
NF1, NF2	Neurofibromatosis 1 and 2	Melanoma [50, 51]
PRKAR1A	Carney complex	Pigmented epithelioid melanocytoma [52]
PTEN	Cowden syndrome	Melanoma [37], MF [53]
PTCH1	Basal cell nevus syndrome	BCC [54]

SCC squamous cell carcinoma, *AK* actinic keratosis, *BCC* basal cell carcinoma, *MF* mycosis fungoides

References

1. Watson JD, Crick FH. Molecular structure of nucleic acids. A structure for deoxyribose nucleic acid. Nature 1953;171:737–8. JAMA. 1993;269:1966–7.
2. Woese C. The genetic code. New York: Harper & Row; 1968.

3. Gilbert W. Origin of life: the RNA world. Nature. 1986;319:618.
4. Claverie J-M. Gene number: what if there are only 30,000 human genes? Science. 2001;291:1255–7.
5. Belancio VP, Hedges DJ, Deininger P. Mammalian non-LTR retrotransposons: for better or worse, in sickness and in health. Genome Res. 2008;18:343–58.
6. Birney E, Stamatoyannopoulos JA, Dutta A, et al. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature. 2007;447:799–816.
7. Harrington L. Does the reservoir for self-renewal stem from the ends? Oncogene. 2004;23:7283–9.
8. Collado M, Blasco MA, Serrano M. Cellular senescence in cancer and aging. Cell. 2007;130:223–33.
9. Osterhage JL, Friedman KL. Chromosome end maintenance by telomerase. J Biol Chem. 2009;284:16061–5.
10. Muntoni A, Reddel RR. The first molecular details of ALT in human tumor cells. Hum Mol Genet. 2005;14 Suppl 2:R191–6.
11. Aubert G, Lansdorp PM. Telomeres and aging. Physiol Rev. 2008;88:557–79.
12. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. Nat Rev Genet. 2005;6:611–22.
13. Flores I, Benetti R, Blasco MA. Telomerase regulation and stem cell behaviour. Curr Opin Cell Biol. 2006;18:254–60.
14. Mithani SK, Smith IM, Topalian SL, et al. Nonsynonymous somatic mitochondrial mutations occur in the majority of cutaneous melanomas. Melanoma Res. 2008;18:214–9.
15. Dakubo GD, Jakupciak JP, Birch-Machin MA, et al. Clinical implications and utility of field cancerization. Cancer Cell Int. 2007;7:2.
16. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. Nature. 2007;448:83–6.
17. Mueller DW, Bosserhoff AK. Role of miRNAs in the progression of malignant melanoma. Br J Cancer. 2009;101:551–6.
18. Redon R, Ishikawa S, Fitch KR, et al. Global variation in copy number in the human genome. Nature. 2006;444:444–54.
19. Bosse Y, Bacot F, Montpetit A, et al. Identification of susceptibility genes for complex diseases using pooling-based genome-wide association scans. Hum Genet. 2009;125:305–18.
20. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003;3:253–66.
21. Hegi ME, Diserens AC, Gorlia T, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med. 2005;352:997–1003.
22. Millington GW. Epigenetics and dermatological disease. Pharmacogenomics. 2008;9:1835–50.
23. Knudson Jr AG. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA. 1971;68:820–3.
24. Garraway LA, Widlund HR, Rubin MA, et al. Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature. 2005;436:117–22.
25. Nielsen M, Kaltoft K, Nordahl M, et al. Constitutive activation of a slowly migrating isoform of Stat3 in mycosis fungoides: tyrphostin AG490 inhibits Stat3 activation and growth of mycosis fungoides tumor cell lines. Proc Natl Acad Sci USA. 1997;94:6764–9.
26. Halaban R. Growth factors and melanomas. Semin Oncol. 1996;23:673–81.
27. Beadling C, Jacobson-Dunlop E, Hodi FS, et al. KIT gene mutations and copy number in melanoma subtypes. Clin Cancer Res. 2008;14:6821–8.
28. Puri N, Ahmed S, Janamanchi V, et al. c-Met is a potentially new therapeutic target for treatment of human melanoma. Clin Cancer Res. 2007;13:2246–53.
29. Karbowniczek M, Spittle CS, Morrison T, et al. mTOR is activated in the majority of malignant melanomas. J Invest Dermatol. 2008;128:980–7.
30. Spencer JM, Kahn SM, Jiang W, et al. Activated ras genes occur in human actinic keratoses, premalignant precursors to squamous cell carcinomas. Arch Dermatol. 1995;131:796–800.
31. van der Schroeff JG, Evers LM, Boot AJ, et al. Ras oncogene mutations in basal cell carcinomas and squamous cell carcinomas of human skin. J Invest Dermatol. 1990;94:423–5.
32. Goldstein NB, Johannes WU, Gadeliya AV, et al. Active N-Ras and B-Raf inhibit anoikis by downregulating Bim expression in melanocytic cells. J Invest Dermatol. 2009;129:432–7.
33. Papp T, Pemsel H, Zimmermann R, et al. Mutational analysis of the N-ras, p53, p16INK4a, CDK4, and MC1R genes in human congenital melanocytic naevi. J Med Genet. 1999;36:610–4.
34. van Dijk MC, Bernsen MR, Ruiter DJ. Analysis of mutations in B-RAF, N-RAS, and H-RAS genes in the differential diagnosis of Spitz nevus and spitzoid melanoma. Am J Surg Pathol. 2005;29:1145–51.
35. Pierceall WE, Goldberg LH, Tainsky MA, et al. Ras gene mutation and amplification in human nonmelanoma skin cancers. Mol Carcinog. 1991;4:196–202.
36. Thomas NE. BRAF somatic mutations in malignant melanoma and melanocytic naevi. Melanoma Res. 2006;16:97–103.
37. Meyle KD, Guldberg P. Genetic risk factors for melanoma. Hum Genet. 2009;126:499–510.

38. Lazarov M, Kubo Y, Cai T, et al. CDK4 coexpression with Ras generates malignant human epidermal tumorigenesis. Nat Med. 2002;8:1105–14.
39. Sauter ER, Yeo UC, von Stemm A, et al. Cyclin D1 is a candidate oncogene in cutaneous melanoma. Cancer Res. 2002;62:3200–6.
40. Eberle J, Hossini AM. Expression and function of bcl-2 proteins in melanoma. Curr Genomics. 2008;9:409–19.
41. Robertson GP. Functional and therapeutic significance of Akt deregulation in malignant melanoma. Cancer Metastasis Rev. 2005;24:273–85.
42. Delmas V, Beermann F, Martinozzi S, et al. Beta-catenin induces immortalization of melanocytes by suppressing p16INK4a expression and cooperates with N-Ras in melanoma development. Genes Dev. 2007;21:2923–35.
43. Saldanha G, Ghura V, Potter L, et al. Nuclear beta-catenin in basal cell carcinoma correlates with increased proliferation. Br J Dermatol. 2004;151:157–64.
44. Reifenberger J, Wolter M, Knobbe CB, et al. Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. Br J Dermatol. 2005;152:43–51.
45. Nelson AA, Tsao H. Melanoma and genetics. Clin Dermatol. 2009;27:46–52.
46. Butani AK, Arbesfeld DM, Schwartz RA. Premalignant and early squamous cell carcinoma. Clin Plast Surg. 2005;32:223–35.
47. Navas IC, Algara P, Mateo M, et al. p16(INK4a) is selectively silenced in the tumoral progression of mycosis fungoides. Lab Invest. 2002;82:123–32.
48. Kanellou P, Zaravinos A, Zioga M, et al. Genomic instability, mutations and expression analysis of the tumour suppressor genes p14(ARF), p15(INK4b), p16(INK4a) and p53 in actinic keratosis. Cancer Lett. 2008;264:145–61.
49. de Boer J, Hoeijmakers JH. Nucleotide excision repair and human syndromes. Carcinogenesis. 2000;21:453–60.
50. Rubben A, Bausch B, Nikkels A. Somatic deletion of the NF1 gene in a neurofibromatosis type 1-associated malignant melanoma demonstrated by digital PCR. Mol Cancer. 2006;5:36.
51. Bianchi AB, Hara T, Ramesh V, et al. Mutations in transcript isoforms of the neurofibromatosis 2 gene in multiple human tumour types. Nat Genet. 1994;6:185–92.
52. Zembowicz A, Knoepp SM, Bei T, et al. Loss of expression of protein kinase A regulatory subunit 1[alpha] in pigmented epithelioid melanocytoma but not in melanoma or other melanocytic lesions. Am J Surg Pathol. 2007;31:1764–75.
53. Katona TM, O'Malley DP, Cheng L, et al. Loss of heterozygosity analysis identifies genetic abnormalities in mycosis fungoides and specific loci associated with disease progression. Am J Surg Pathol. 2007;31:1552–6.
54. Adolphe C, Hetherington R, Ellis T, et al. Patched1 functions as a gatekeeper by promoting cell cycle progression. Cancer Res. 2006;66:2081–8.

Chapter 3
Technologies in the Molecular Diagnostics Laboratory

Zendee Elaba, Michael J. Murphy, and Laila Mnayer

Molecular techniques are being increasingly employed in the field of dermatology, significantly enhancing the management of cutaneous disorders. These applications have become important diagnostic tools, not only in the setting of genodermatoses, but also in a wide range of cutaneous malignancies and infectious diseases. In addition, molecular testing has been used to select treatment, assess therapeutic response, and predict prognosis. This chapter provides an overview of the principles and applications of the molecular technologies most frequently used for the diagnosis and study of cutaneous diseases (Table 3.1).

Southern Blot Analysis

Developed in 1975, Southern blot analysis (SBA) is one of the oldest nucleic acid–based methods for quantitative detection of specific DNA sequences [1]. DNA to be analyzed is first digested by a restriction enzyme – a bacterial enzyme that recognizes specific DNA sequences (approximately 4–6 nucleotides long) and cuts the DNA at these restriction sites [2]. The digest is then run on a gel matrix, where the nucleic acid is denatured with NaOH in order to separate double-stranded DNA (dsDNA) into single-stranded DNA (ssDNA). The denatured DNA fragments, retaining their pattern of separation on the gel, are transferred to a nitrocellulose or nylon blotting membrane through capillary action, electrophoresis or vacuum transfer [3]. The blot is then incubated with a radiolabeled ssDNA probe that hybridizes with the complementary DNA sequence of interest, thereby forming a new dsDNA molecule. The position of the radioactively labeled target sequence is then visualized with the use of an x-ray film (autoradiography). Northern blot analysis is a variation of SBA that employs RNA, instead of DNA, as the test template.

SBA has long been considered the gold standard for detection of clonality in lymphoproliferative disorders, and can be used in the diagnosis and staging of patients with cutaneous T-cell lymphoma (CTCL) (see Chap. 10). However, while SBA readily detects monoclonal T-cell receptor gene rearrangements (TCR-GRs) in advanced stages of this disease, the lower sensitivity of this technology for detection of T-cell monoclonality is most evident in early patch and limited plaque skin disease. Monoclonal TCR-GRs can also be identified by SBA in the lymph nodes of patients with CTCL, predicting a poor clinical outcome and reduced probability of survival. Of note, SBA can detect identical TCR-GRs in the skin, lymph nodes, and peripheral blood of individual patients with

Z. Elaba (✉)
Department of Pathology and Laboratory Medicine, Hartford Hospital,
Hartford, CT, USA
e-mail: zendee.elaba@gmail.com

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_3,

Table 3.1 Molecular technologies and their applications in dermatology

Molecular technique	Method	Sample	Advantages	Disadvantages	Dermatologic applications
Southern blot analysis (SBA)	Nucleic acid hybridization and blotting	Fresh or frozen tissue	Specific; less probability of contamination	Requires fresh or frozen tissue; requires large amounts of DNA; longer turnaround time; low-throughput	Lymphoproliferative disorders, Merkel cell carcinoma
Polymerase chain reaction (PCR)	DNA fragment amplification	Fresh, frozen, or FFPE tissue	Sensitive and fast; requires minute amounts of DNA; can be used on FFPE tissue	Risk of product contamination; false-positive results	Lymphoproliferative disorders, genodermatoses, infectious diseases, melanoma, BCC, SCC, Kaposi's sarcoma
Reverse transcription-PCR (RT-PCR)	RNA amplification after reverse transcription into cDNA	Fresh, frozen, or FFPE tissue	Sensitive and fast; requires minute amounts of DNA; can be used on FFPE tissue	Risk of product contamination; requires pure RNA; problem of RNA degradation	Lymphoproliferative disorders, melanoma, BCC, SCC, Kaposi's sarcoma
Real-time PCR	Amplification and simultaneous quantification of DNA and RNA under real-time monitoring	Fresh, frozen, or FFPE tissue	Sensitive; quantitative measurement	Requires specialized instrumentation; higher cost	Lymphoproliferative disorders, melanoma, BCC, SCC, Kaposi's sarcoma, infectious diseases
Ligase chain reaction (LCR)	Amplification of probe, instead of target DNA	Fresh, frozen, or FFPE tissue	Can detect point mutations	Only able to detect known mutations; risk of product contamination	Infectious diseases
Transcription-mediated amplification (TMA); Nucleic acid sequence-based amplification (NASBA); Strand displacement amplification (SDA); Helicase-dependent amplification (HDA)	Isothermal nucleic acid amplification	Fresh, frozen, or FFPE tissue	Do not require thermocyclers	Inability to amplify long sequences; nonspecific background reactions due to low temperature test conditions	Infectious diseases
Multiplex ligation-dependent probe amplification (MLPA)	Multiplexed, PCR-based, ligation-dependent amplification	Fresh, frozen, or FFPE tissue	Detects multiple targets (up to 50) in a single assay; requires minute amounts of DNA	Requires specially designed probes	Melanoma, other melanocytic lesions, lymphoproliferative disorders

Fluorescence in situ hybridization (FISH)	Detection of DNA sequences in chromosomes by fluorophore-labeled probes	Fresh, frozen, or FFPE tissue; fresh or cultured cells	Applicable to interphase cells; in situ localization of signal	Requires an existent probe for a known target; risk of false-positive results	Melanoma, lymphoproliferative disorders, DFSP, HPV
Chromogenic in situ hybridization (CISH)	Same as FISH, but uses chromogen, instead of fluorophore	Fresh, frozen, or FFPE tissue	Does not require fluorescent microscope; stable signals; able to correlate results with morphology	Same as FISH; less intense detection signal	Melanoma, lymphoproliferative disorders
Hybrid capture	Chemiluminescent signal amplified hybridization	Mucosal swabs, skin biopsies	Easier, more convenient testing	Cross-reactivity; lower sensitivity than PCR	HPV, CMV
G-banding	Staining and identification of chromosomes by banding pattern	Cultured cells	Easy and inexpensive; whole genome analysis	Requires metaphase cells; does not detect intrachromosomal point mutations or balanced translocations	Lymphoproliferative disorders
Spectral karyotyping (SKY)	Painting the 24 chromosomes (22 autosomes, X chromosome, and Y chromosome) with differentially labeled probes	Cultured cells	All chromosomes simultaneously evaluated; whole genome analysis	Does not detect intrachromosomal point mutations or balanced translocations	Lymphoproliferative disorders, malignant fibrous histiocytoma, SCC
Loss of heterozygosity (LOH) analysis; Microsatellite instability (MSI) analysis	PCR-based microsatellite amplification	Fresh, frozen, or FFPE tissue; fresh or cultured cells	Fast; high-throughput	Variable threshold; need for non-tumor tissue/cells for comparison	Melanoma, BCC, SCC, Merkel cell carcinoma, lymphoproliferative disorders, keratoacanthoma, sebaceous tumors
Comparative genomic hybridization (CGH)	Detection of copy number changes through analysis of differentially labeled test and reference DNA	Fresh, frozen, or FFPE tissue	Whole genome analysis; no cell culture required for array-based CGH	Does not detect intrachromosomal point mutations or balanced translocations	Melanoma, Spitz nevi, genodermatoses
DNA microarray	Hybridization of DNA/RNA with DNA sequences spotted onto a microarray slide	Fresh, frozen, or FFPE tissue	Evaluation of thousands of molecules in a single experiment; whole genome analysis	Technically complex and expensive; requires high-quality mRNA extraction; massive data processing	Melanoma, BCC, SCC, atopic dermatitis, psoriasis, lupus erythematosus, systemic sclerosis
DNA sequencing	Determination of DNA nucleotide sequence	Fresh, frozen, or FFPE tissue	Highly specific; gold standard in mutation detection	Technically complex and expensive	Genodermatoses, infectious diseases

DNA deoxyribonucleic acid, *RNA* ribonucleic acid, *mRNA* messenger RNA, *FFPE* formalin-fixed paraffin embedded, *BCC* basal cell carcinoma, *SCC* squamous cell carcinoma, *cDNA* complementary DNA, *DFSP* dermatofibrosarcoma protuberans, *HPV* human papillomavirus, *CMV* cytomegalovirus

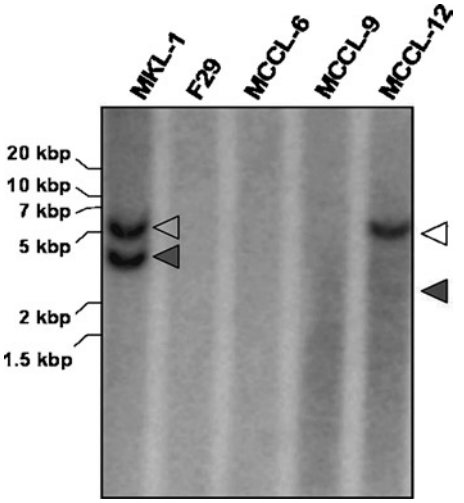

Fig. 3.1 *Monoclonal Merkel cell polyomavirus* (MCPyV) *integration in Merkel cell carcinoma* (MCC) *cell lines*. Southern blot for MCPyV genomic DNA in MCC cell lines: (1) MKL-1 and MCCL-12, both MCPyV-positive; and (2) MCCL-6 and MCCL-9, both MCPyV-negative. F29 represents feeder cells used to subculture MCCL-6, -9 and -12 cells. 30 μg of genomic DNA was digested with EcoRI, separated on a 1% agarose gel, transferred to a nitrocellulose membrane and probed with MCPyV-specific sequences. Similar to previously observed MCPyV integration patterns, the observed bands likely represent concatameric integrates digested to the unit size of viral genomes (*open arrowheads*), or virus/host junctions (*closed arrowheads*) (Courtesy of Drs. Nicole Fischer and Ingrid Moll, Institute of Medical Microbiology, Virology, and Hygiene; and Department of Dermatology and Venerology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany)

CTCL (see Chap. 10). SBA has also been used to determine *human T-cell lymphotropic virus type 1* (HTLV-1) integration patterns in cases of cutaneous adult T-cell leukemia/lymphoma (Fig. 11.6). In addition, SBA can identify the presence of a novel polyomavirus, known as *Merkel cell polyomavirus* (MCPyV), in Merkel cell carcinoma tumor samples (Fig. 3.1) [4].

A major limitation of SBA is its requirement for fresh or frozen tissue, in addition to the need for a large amount of high quality DNA (~10 μg in most standard protocols) [1]. Although SBA of DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue is feasible, degradation of DNA in such samples is a major problem that compromises testing [5]. As it is also both time-consuming and labor-intensive, SBA has now been largely relegated to research applications, and replaced by high-throughput target and signal amplification methods.

Target Amplification Methods

Nucleic acid target amplification is one of the most extensively used diagnostic molecular methods. It can be carried out by means of several different technologies, all of which aim to generate a detectable amount of amplicon from a small starting sequence (template) [6]. The polymerase chain reaction (PCR) is the most widely used DNA amplification technique. Other target amplification assays include: ligase chain reaction (LCR), transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), helicase-dependent amplification (HDA), and multiplex ligation-dependent probe amplification (MLPA).

Polymerase Chain Reaction

PCR is a rapid, sensitive, and specific methodology that permits the synthesis of multiple copies of a target nucleic acid sequence. Introduced in 1986 by Mullis et al. [7], it is one of the most universally utilized applications for detecting DNA (and RNA) from a wide variety of tissue sources. Target nucleic acid can be extracted from fresh/frozen or FFPE tissue, blood and other bodily fluids, mucosal scrapes, and fine needle aspirates [3].

The basic ingredients of a PCR mixture include the target DNA, DNA primers, free nucleotides, and the enzyme Taq polymerase. Each PCR cycle consists of three steps: denaturation, annealing, and elongation (Fig. 3.2) [8]. The reaction mixture is initially heated to separate the two strands of target DNA (denaturation). After strand separation, the temperature is lowered to allow the primers

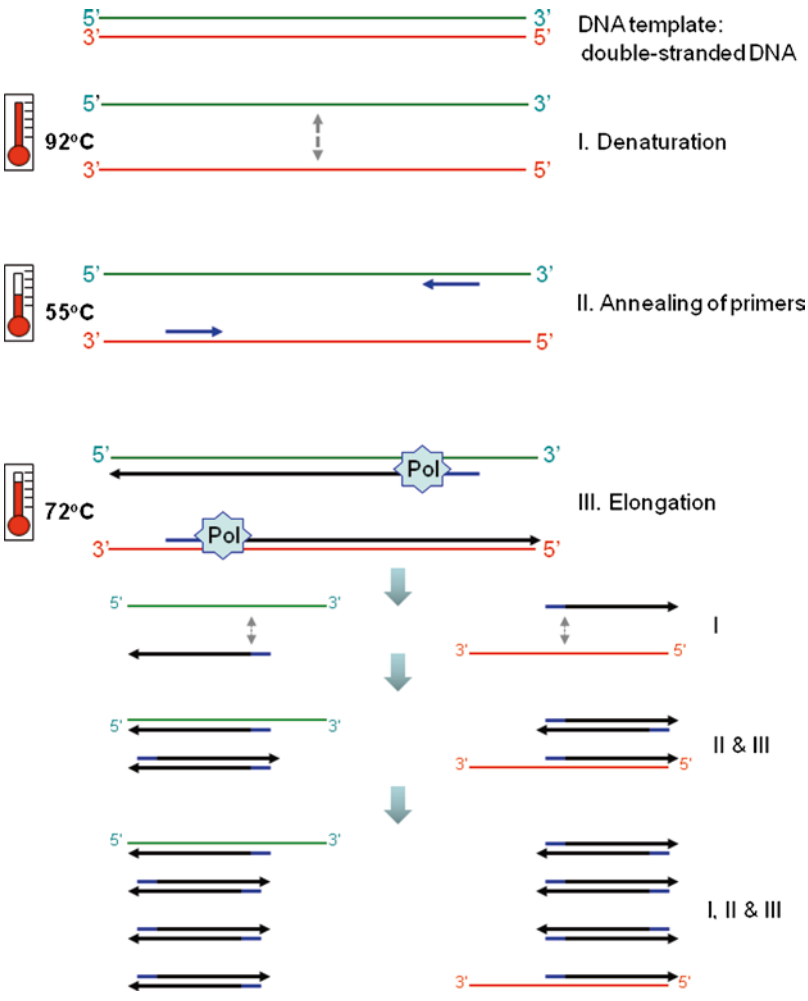

Fig. 3.2 *Polymerase Chain Reaction* (PCR). A regular PCR cycle usually commences with denaturation of the DNA template at high temperature (~92°C), after which the temperature is lowered (in this example to 55°C) in order to allow annealing of primers. This is then followed by a standard elongation step usually at 72°C. Pol, DNA polymerase enzyme

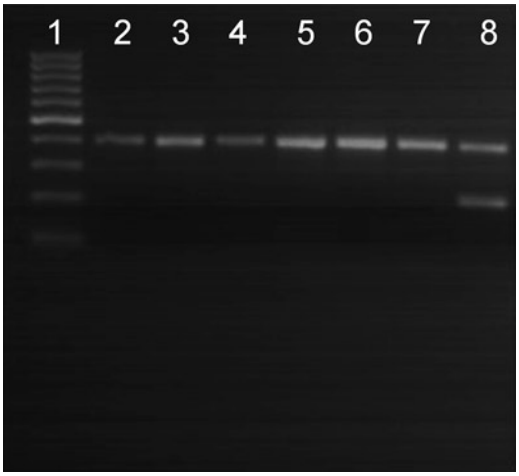

Fig. 3.3 *Pan-dermatophyte and Trichophyton rubrum multiplex PCR performed with DNA extracted from pure cultures of dermatophytes*. Lane 1: DNA marker, 2: *Microsporum canis*, 3: *Epidermophyton floccosum*, 4: *Trichophyton mentagrophytes*, 5: *Trichophyton interdigitale*, 6: *Trichophyton tonsurans,* 7: *Trichophyton violaceum*, 8: *Trichophyton rubrum.* Figure demonstrates (1) the pan-fungal band present for all dermatophytes and (2) the smaller band present for *T. rubrum* only (Courtesy of Drs. Anne Brillowska-Dabrowska and Maiken Cavling Arendrup, Statens Serum Institut, Copenhagen, Denmark)

to hybridize to complementary sequences (annealing) on template strands flanking the region of interest. Taq polymerase, which is a temperature-resistant DNA polymerase that is derived from the bacterium *Thermus aquaticus*, then initiates polymerization, adding nucleotides to the 3′ end of each growing DNA strand (elongation) [1]. At the end of each cycle, the PCR products are theoretically doubled, such that after n number of PCR cycles, the target sequence is logarithmically amplified to 2^n. The whole procedure takes place in an automated programmable thermocycler, in order to control: (1) the temperature at which each step occurs; (2) the length of time at each step; (3) the number of steps per cycle; and (4) the total number of cycles [9]. The resultant amplicons (double-stranded amplified target regions) are detected by means of either gel or capillary electrophoresis (Fig. 3.3) (see Section on "Analysis of PCR Products").

Because it is highly sensitive, fast, inexpensive, and applicable to DNA extracted from virtually any sample source, PCR has revolutionized the science of DNA detection. Its applications extend to most areas of molecular testing (i.e., detection of infectious agents, identification of gene mutations, translocations and amplifications, and forensic identity testing) [10]. In dermatology, PCR is widely employed, both as a research application and a clinically directed tool [11, 12]. PCR-based assays have been used for the evaluation of melanocytic and non-melanocytic skin tumors, lymphoid neoplasms, and infectious diseases [12].

Reverse Transcription-Polymerase Chain Reaction

The original PCR method has been extended to measure messenger RNA (mRNA), in a process referred to as reverse transcription-polymerase chain reaction (RT-PCR) (not to be confused with real-time PCR). This procedure can be broken down into two major steps: reverse transcription and PCR amplification. The mRNA isolated from cells or tissue is first reverse transcribed into complementary DNA (cDNA) using reverse transcriptase (RT) enzyme, a retroviral RNA-directed DNA

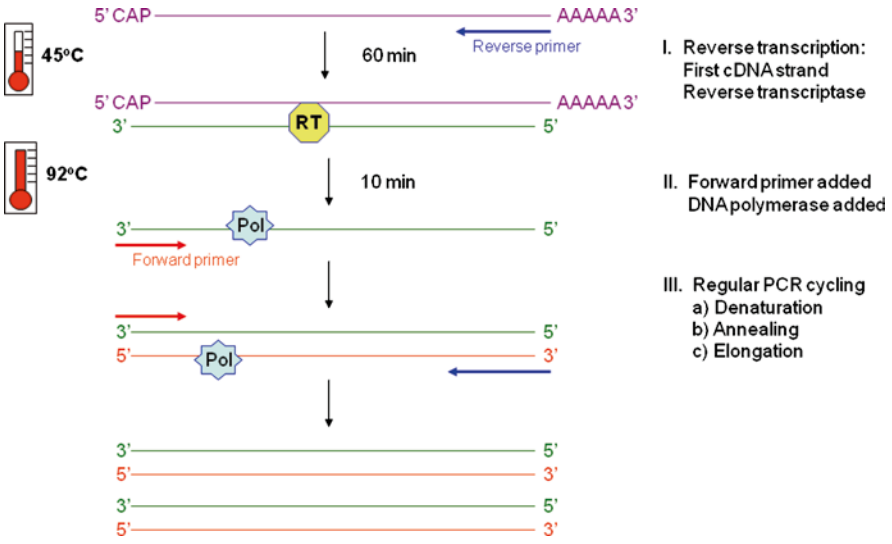

Fig. 3.4 *RT-PCR*. In the initial reverse transcription reaction, the RNA template, deoxyribonucleotide triphosphates (dNTPs), reverse primer, and reverse transcriptase (*RT*) enzyme are incubated at 45°C for 60 min in order to synthesize the first cDNA strand. The activity of RT is then terminated by heating to 92°C for 10 min. The forward primer, PCR buffer, dNTPs, and DNA polymerase enzyme (Pol) are then added, and the reaction is allowed to cycle in a regular PCR program that includes denaturation at 92°C, annealing of primer (55–60°C, depending on primer sequence), and elongation at 72°C

polymerase (Fig. 3.4) [1]. The resulting cDNA is then used as template for subsequent classic PCR amplification, with the remainder of the reaction proceeding as described previously for PCR.

Real-Time Polymerase Chain Reaction

Gel-based analyses of DNA and RNA, while effective, do not allow accurate quantification of PCR amplicons. The final (end-point) product may vary from sample to sample, and the ethidium bromide (EtBr) dye used for visualizing DNA in gel electrophoresis cannot resolve these subtle yield differences. This need for quantitation led to the development of real-time PCR in 1996 [13]. As its name suggests, real-time PCR quantifies the reaction products as the reaction proceeds. The instrumentation platform of real-time PCR includes a thermocycler, an optical system for fluorescence excitation and emission collection, and a computer equipped with data acquisition and analysis software.

There are two methods of molecule labeling in real-time PCR: (1) fluorescent dyes and (2) fluorescent probes. The simplest real-time PCR techniques utilize intercalating dyes (i.e., SYBR Green, EtBr), that insert into bases of DNA products, and fluoresce when bound [1]. After each cycle, the amount of fluorescence emitted from the dye is measured, with the degree of fluorescence corresponding to the number of copies of the target sequence [14]. The amount of fluorescence surges exponentially when a particular copy number is reached (Fig. 3.5). Sequence-specific probes are considered more specific than intercalating dyes. Examples of such fluorescent probes include the molecular beacon, TaqMan and fluorescence resonance energy transfer (FRET) probes [1]. In addition to delivering quantitative results, real-time PCR assays have the advantage of speed, since the analysis can be completed rapidly and no post-amplification manipulation is required. With the entire procedure performed in a single closed system, it is also less predisposed to contamination. Real-time PCR has been applied to the diagnosis of cutaneous infections (see Chaps. 15 and 16).

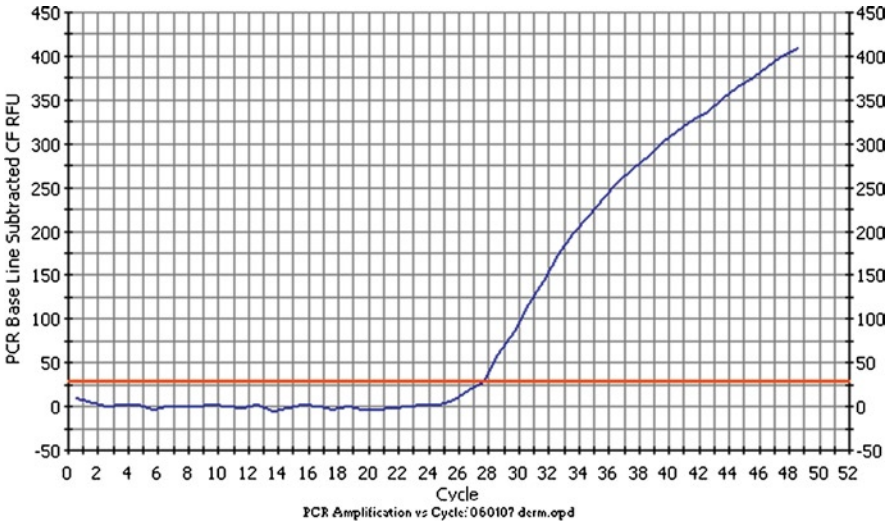

Fig. 3.5 *Amplification curve derived from real-time Trichophyton mentagrophytes-specific PCR assay of a skin scraping lesional specimen.* The *x*-axis shows the cycle number and the *y*-axis indicates the fluorescence units. An increase in fluorescence above the threshold (*horizontal orange line*) indicates detection of PCR products after 27–28 cycles (Courtesy of Dr. Michael Arabatzis, Medical School, University of Athens, Greece)

Ligase Chain Reaction

Similar to PCR, ligase chain reaction (LCR) accomplishes amplification of genetic material. However, it differs in that it amplifies the probe rather than the target DNA [15]. The procedure begins by heating the template DNA to separate the strands. For each DNA strand, a pair of oligonucleotide probes that are complementary to adjacent regions are used. When cooled to the annealing temperature, the two probes bind to each template strand and are subsequently joined together by a thermostable ligase. Insertions, deletions, or even single base pair (bp) alterations in the target sequence will prevent ligation, since the two probes must lie directly next to each other to be ligated [16]. In the following round of LCR, the original template strand and the newly ligated oligonucleotides are functionally equivalent to two new target strands that can each bind with complementary probes [17]. This leads to an exponential yield of products after repeated cycles. Detection of products is a multistep process involving: (1) a capture antibody that binds to the ligated product; (2) a second fluorescently labeled antibody that binds to this ligated probe-antibody conjugate; and (3) a substrate added to produce measurable fluorescence [15]. Like PCR, LCR can be used for RNA detection by incorporating reverse transcription prior to amplification. In dermatology, its utility lies mainly in the diagnosis of cutaneous infections (see Chap. 16).

Transcription-Mediated Amplification and Nucleic Acid Sequence-Based Amplification

Isothermal techniques involve reactions that proceed at a constant temperature, thereby eliminating the need for thermocyclers [18]. Transcription-mediated amplification (TMA) and nucleic acid sequence-based amplification (NASBA) are two isothermal nucleic acid amplification methods that operate on similar principles and are jointly described.

Both procedures begin with an RNA target. Heat denaturation results in the production of single-stranded RNA (ssRNA). This is followed by the addition of a ssDNA primer, which demonstrates a 3′ end complementary to the target RNA and a 5′ end that contains the promoter for T7 bacteriophage polymerase. A RT enzyme elongates the primer producing a cDNA copy of the RNA template and forming an RNA/DNA heteroduplex [19]. There is one major difference between TMA and NASBA methods. Following reverse transcription in TMA, the initial RNA template is hydrolyzed by the RT enzyme, while in NASBA, a separate enzyme, RNase H, degrades the RNA component of the heteroduplex, leaving the single-stranded cDNA [20, 21]. After RNA degradation, the single-stranded cDNA binds to a second primer at its 3′ end, and is extended by the RT to synthesize dsDNA molecules with an intact T7 promoter at one end. Recognizing this transcriptionally active promoter, T7 RNA polymerase produces numerous copies of RNA transcripts, with the dsDNA serving as template. The entire amplification reaction is performed at a constant temperature of 41°C [19], and with repeated cycles, exponential amplification up to 10^9-fold can be achieved within 90 min [21]. TMA and NASBA are most suited for RNA amplification, but can also be adapted to amplify DNA [17]. Both techniques have been successfully utilized for the detection of viral, bacterial, and mycobacterial pathogens in a variety of specimen types, including skin biopsies, blood, and amniotic fluid [19, 21].

Strand Displacement Amplification

Strand displacement amplification (SDA) is another isothermal amplification technique that is based on the combined action of a restriction enzyme and a DNA polymerase. The restriction enzyme nicks one strand of its corresponding double-stranded recognition site, and the DNA polymerase begins synthesis at the site of the nick, displacing the downstream DNA strand [22]. It is divided into two discrete phases: (1) a target generation phase that produces copies of the target sequences flanked by restriction sites, and (2) the exponential amplification phase brought about by serial nicking, strand displacement, and primer hybridization to displaced strands [23].

An advantage of SDA is its operation at a constant temperature (~40°C), which removes the need for thermocycler use [24]. However, a limitation of this method is its inability to amplify long target sequences. Another shortcoming of SDA is that by operating at a relatively low temperature, nonspecific background reactions through inconsistent primer hybridization can occur [24]. Clinical applications of SDA include the direct detection of infectious agents, such as *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* [25]. Recently, RT-SDA (modified with the addition of a reverse transcription step) has been used to measure human immunodeficiency virus (HIV) viral loads [26].

Helicase-Dependent Amplification

Helicase-dependent amplification (HDA) is another isothermal nucleic acid amplification technique that is unique in its use of a DNA helicase enzyme, instead of heat, to denature dsDNA [27]. The use of DNA helicases to unwind the double helix allows multiple cycles to be performed at a single incubation temperature, and eliminates the requirement for thermocycling [28, 29]. The remainder of the reaction proceeds essentially as for classic PCR, with DNA polymerases extending the primers annealed to the nucleic acid templates. The resultant dsDNA products are again used as substrates by DNA helicases, and following multiple reaction rounds, exponential amplification of the target sequence is achieved [30]. This platform is suited to both DNA and RNA amplification, and has been applied to the detection of *Clostridium difficile*, *Staphylococcus aureus*, and various RNA viral pathogens (i.e., HIV, West Nile virus, and enterovirus) [27, 31].

Multiplex Ligation-Dependent Probe Amplification

Multiplex ligation-dependent probe amplification (MLPA) is a PCR-based method that is used to determine copy number variations (CNV), or losses and/or gains of DNA sequence, in a test sample [32]. The MLPA probe is composed of two oligonucleotides (half probes), that are designed to recognize adjacent sites on the target sequence. Each half probe contains a universal PCR primer sequence and a sequence complementary to the target, known as the hybridization sequence [33]. When correctly hybridized to their respective target loci, they are ligated using a thermostable DNA ligase [34]. The ligated probes are subsequently amplified with universal primers (one of which is fluorescently labeled for detection) in a multiplex PCR. Multiplex PCR refers to a reaction that uses more than one pair of primers, with the goal of amplifying several segments of target DNA simultaneously. The PCR products are then quantified and resolved using electrophoresis [34]. The various probes are designed to differ in length/number of base pairs, by inserting stuffer sequences or extending the length of hybridizing sequences, such that each product has a distinct size, thereby facilitating separation [35]. The copy number of each target region is measured as a function of peak intensities of the MLPA products [33]. The relative quantity of each amplicon can be determined by comparing the peak pattern obtained on a given sample with known patterns from reference samples (Fig. 3.6).

Compared to other techniques, MLPA is high-throughput, requires only small amounts of starting DNA (unlike SBA), does not require cell culture for metaphase chromosome spread preparation (as in fluorescence in situ hybridization), and can be used to target any genomic sequences for copy number analysis, regardless of their size or proximity to each other [34]. MLPA has been used in

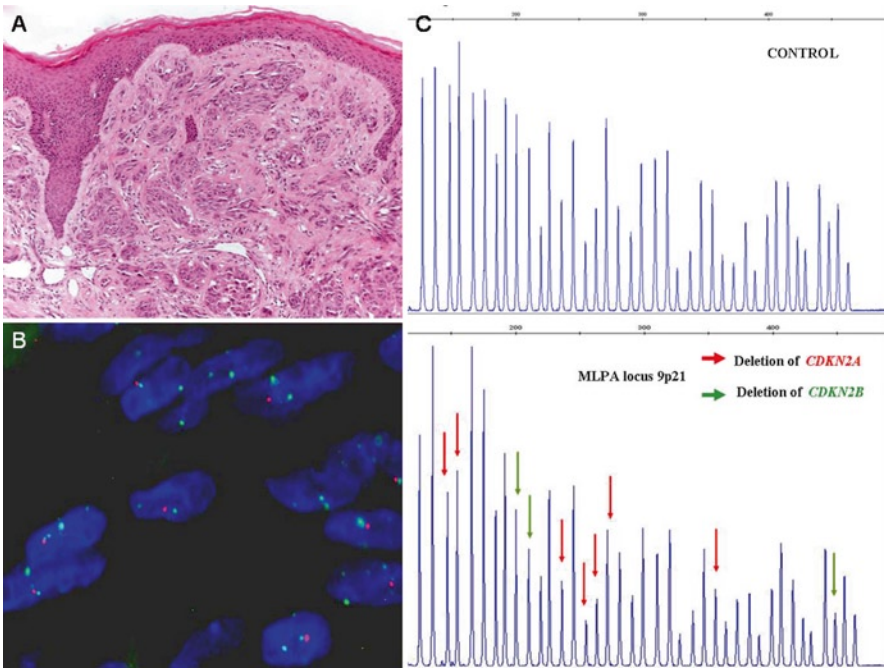

Fig. 3.6 *Molecular analysis of chromosome 9p21 in a Spitzoid melanocytic lesion*. (**a**) Spitzoid tumor from the ear lobe of a 23-year-old female. (**b**) Fluorescence in situ hybridization (FISH) analysis of the case showing deletion of 9p21; *red spot*-9p21 locus; *green spot*-centromere of chromosome 9. (**c**) Multiplex ligation-dependent probe amplification (MLPA) analysis of the same case exhibiting deletion of *CDKN2A* and *CDKN2B* at 9p21 locus (*bottom panel*) in comparison with the negative control (*top panel*) (Courtesy of Dr. Anna M. Cesinaro, University of Modena-Reggio Emilia, Modena, Italy)

dermatology as an adjunctive tool in the evaluation of melanocytic tumors. For example, copy number changes have been found to involve multiple genes in melanomas, but only one to two gene loci in Spitz nevi and common melanocytic nevi (Fig. 3.6) [36]. MLPA has also been used to demonstrate that loss of chromosomal region 9p21.3, and specifically inactivation of the CDKN2A gene that resides there, is associated with an unfavorable prognosis in primary cutaneous diffuse large B-cell lymphoma, leg type [37].

Analysis of PCR Products

The fundamental value of PCR in molecular testing is its ability to amplify targeted sequences from complex mixtures and provide adequate template for downstream applications. PCR products consist of DNA fragments, of defined length and sequence, which can be further mined for information ranging from basic genetic composition to unique mutations. The following discussion enumerates a number of methods employed for analysis of PCR products.

Electrophoresis

Analyses of PCR amplicons most commonly employ the process of electrophoresis, a molecular separation technique that uses an electric field to drive the migration of charged molecules through either a liquid- or gel-based medium. The movement of a molecule through the medium is dictated by the size and conformation of the molecule, the net charge of the molecule, the electric field strength or voltage, the pore size of the gel matrix, and the temperature [38]. By virtue of its phosphate groups, which carry negatively charged oxygen, the DNA molecule has an overall negative charge. Accordingly, it migrates to the positive pole (anode) when an electric field is applied. The two basic formats of electrophoresis are: (1) slab gel electrophoresis (i.e., electrophoresis through flat agarose or polyacrylamide gels) and (2) capillary electrophoresis (i.e., electrophoresis in tubular gels within capillary glass tubes). Electrophoretic methods are widely used in the evaluation of dermatologic disorders.

Agarose and Polyacrylamide Gel Electrophoresis

In both agarose gel electrophoresis (AGE) and polyacrylamide gel electrophoresis (PAGE), the gel matrix serves as a molecular sieve that separates DNA as a function of its size, with the smallest fragments migrating fastest and farthest (Fig. 3.3). A slab of gel is molded to contain wells, covered with buffer solution, and connected to positive and negative electrodes. Test PCR products, including positive and negative controls, and sizing standards are then pipetted into these wells. Size standards, also called DNA or RNA ladders, are nucleic acids of known fragment lengths that serve as controls for size determination [38]. A dye to visually track migration in the gel is also added [1]. Voltage is then applied to drive the movement of DNA molecules through the pores of the gel matrix.

Agarose gels, which are made from a seaweed-derived polysaccharide, have a large pore size (averaging 100–300 nm^3), with the degree of resolution dependent on the percentage of agarose in the gel [39]. For example, a 2% agarose gel can analyze products in the range of 50–2,000 bp [10]. On the other hand, polyacrylamide gels, which are made from a synthetic monomer, can be adjusted to provide smaller pores by manipulating both the acrylamide and cross-linking agent concentrations (i.e., a 12% polyacrylamide gel can analyze products in the range of 40–400 bp) [10]. Pore size

decreases with higher acrylamide concentration [19]. Accordingly, PAGE offers a higher resolving power than AGE, with the capacity for differentiation of smaller fragments down to a single base pair in length [39]. To visualize the bands separated by AGE and PAGE, the gel is soaked in EtBr solution (or EtBr is added to the gel prior to molding) and viewed with an ultraviolet (UV) transilluminator. Photographs are taken immediately as DNA molecules will diffuse throughout the gel over time.

Capillary Electrophoresis

Capillary electrophoresis (CE) operates on the same principle of molecular separation as gel electrophoresis (i.e., based on differences in charge, size, and hydrophobicity), but differs in that the separation occurs in a narrow-bore fused-silica capillary tube (~20–100 μm in internal diameter) [40, 41]. The tube is coated with polyimide, except for a section at the cathodic end of the tube that is left transparent as a window for photometric detection [42]. The ends of the capillary are placed in buffer reservoirs, and a low-viscosity polymer serves as the matrix that conducts current within the tube. The test sample is introduced into the tube either electrokinetically (with low voltage) or hydrodynamically (with pressure or suction) [43]. Application of high voltage (10–30 kV) causes the DNA fragments to migrate through the capillary tube [41]. Molecular transport through the matrix is primarily driven by two forces: (1) electrophoretic mobility of the molecule (i.e., rate of movement in solution when subjected to an electric field, generally determined by charge and mass), and (2) electro-osmotic flow of the buffer solution (i.e., motion of ions in a solvent) [43]. A variety of detection methods are available, based on UV absorbance, fluorescence, and conductivity. Newer high sensitivity detection systems include laser-induced fluorescence and electrochemical detection [44]. The data is processed and displayed as an electropherogram, which shows nucleic acids as peaks with different retention times (Figs. 10.2–10.5, 11.4, and 12.2) [45].

CE is widely used for the analysis of proteins, carbohydrates, chemicals, and pharmaceuticals. In the clinical molecular laboratory, the most common applications are (1) DNA fragment sizing or quantitation and (2) DNA sequencing. Compared to slab electrophoresis, which generally requires 3–4 hours procedure time, CE demonstrates significant time-saving, taking approximately 60 min to complete a run [1]. In addition, CE requires minute amounts of test sample, consumes limited quantities of reagents, and is easily automated for precise quantitative, high-resolution analysis. CE is now commonly employed for the evaluation of T-cell and B-cell monoclonality in cutaneous lymphoproliferative disorders (see Chaps. 10–12).

Restriction Fragment Length Polymorphism

Restriction analysis of PCR amplicons, initially described in 1985, is one of the oldest techniques used to analyze amplification products [46]. Restriction fragment length polymorphism (RFLP) refers to length variations in DNA sequences after digestion of the DNA samples with specific restriction endonucleases [47, 48]. There are multiple restriction sites throughout any region of DNA. Following digestion of the PCR product(s) with restriction enzyme(s), DNA fragments are separated by electrophoresis. The number of fragments and their relative sizes are reflected in (1) the banding patterns on PAGE or (2) fluorescence peak patterns on CE.

Single-Strand Conformation Polymorphism

Single-strand conformation polymorphism (SSCP) analysis is a PAGE-based technique, which operates on the premise that a single nucleotide substitution in a fragment of ssDNA is sufficient to cause a

mobility shift [49]. PCR products are heat-denatured to separate the strands and then electrophoresed through a non-denaturing polyacrylamide gel [50]. Each ssDNA assumes a folded configuration depending on its nucleotide sequence [51]. Without a complementary strand, the single strand is unstable and may undergo self-annealing (intra-strand base pairing) in order to achieve the most thermodynamically stable conformation. The resulting three-dimensional structure determines its migration rate through the gel matrix [52]. If the wild-type and mutant PCR products vary in sequence, even by as little as one nucleotide, they will be represented by two distinct bands corresponding to the two strands of the amplified molecule [39]. Developed in 1989 by Orita et al. [52], SSCP is a simple, inexpensive, and sensitive method for identification of unknown sequence variations, and has been used extensively to characterize polymorphisms in a variety of genes [52, 53].

Heteroduplex Analysis

Heteroduplex analysis is a gel electrophoresis-based mutation scanning method that distinguishes dsDNA. It is based on the differential mobility of DNA fragments containing one or more mismatched base pairs [54]. It requires (1) PCR-amplified DNA from a person with a presumed mutation in the gene of interest (test DNA) and (2) the same PCR-amplified region from a known normal control (reference DNA). The test and reference DNA are mixed, heated to denature the dsDNA, and then cooled to allow for reannealing of the single strands. This leads to the formation of a hybrid DNA molecule containing one strand from the test DNA and the other from the reference DNA. A DNA heteroduplex is formed if the two DNA strands have one or more mismatched base pairs, thereby making them focally unable to anneal and resulting in partially open DNA sequences [54]. By forming "bulges" and "bubbles" in the regions of base mismatch, these heteroduplexes lag in migration compared to their fully annealed homoduplex counterparts and are seen as separate bands on the gel (Fig. 10.1) [55].

Denaturing Gradient Gel Electrophoresis and Temperature Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are parallel methods of separating DNA molecules as a function of base composition and sequence-related properties, instead of according to size [56]. The principle behind these techniques is that different dsDNA molecules have unique melting properties, depending on the specific nucleotide composition of the DNA sequence [57]. Accordingly, they will have differences in mobility when subjected to a gradient of denaturing conditions. In the case of DGGE, the gradient is supplied by the inclusion of denaturing agents, usually urea and formamide [57]. For TGGE, conformational changes are induced by variations in temperature, brought about by water baths and cooling plates [58]. Denatured or partially unwound molecules will migrate more slowly, and differences in mobility can be visualized as distinct bands in the polyacrylamide gel [59].

Signal Amplification

Signal amplification techniques increase or amplify the signal generated by a probe molecule that has hybridized to the target sequence, instead of amplifying the actual target genetic material itself. In the process, these techniques minimize the possibility of contamination that is commonly associated with target amplification methods. Signal amplification technologies, relevant to the practice of dermatology, include in situ hybridization (ISH) and the hybrid capture assay.

Fluorescence In situ Hybridization

Fluorescence in situ hybridization (FISH) involves specific annealing of fluorescently labeled nucleic acid probes with complementary DNA or RNA sequences, and the subsequent detection of these labeled probes within fixed cells [60]. Slides can be prepared from fresh, frozen or FFPE tissue, as well as from cytologic preparations [61]. Nucleic acid probes are applied to the slide, and incubated at both high temperature and high humidity, in order to facilitate hybridization of the probe to its complementary sequence. Excess probe is washed away and the slide is read by fluorescence microscopy. A discrete fluorescent signal at the site of the bound probe is visualized. Results may be analyzed by a digital imaging system [62]. The number of spots per nucleus indicates the copy number of the chromosome locus analyzed. There are three primary types of FISH probes which outline: (1) the entire chromosome length (whole chromosome probes); (2) the centromeric region (alphoid or centromeric repeat probes); or (3) a specific region of a chromosome (locus-specific probes) [63].

With the probe hybridizing to the target sequence directly in the cells ("in situ"), FISH highlights the location of the sequence within the context of the cell or tissue of interest. This provides a link between cytogenetics and histology, allowing the observer to correlate genetic alterations with morphological features [64]. Its applicability to FFPE tissues is also an added benefit, since most skin biopsies are routinely processed in this way. In addition, the FISH technique can be performed on both metaphase and nondividing interphase cells, offering an advantage over G-banding, which is limited to analysis of metaphase spreads. FISH is useful in detecting gene amplifications, translocations [i.e., t(11;22)(q24;q12) in Ewing sarcoma/peripheral neuroectodermal tumor (Fig. 8.2)], microdeletions, and chromosomal duplications, in addition to viral infections [i.e., human papillomavirus (HPV)] [60, 65]. FISH protocols are employed in the diagnosis of cutaneous B-cell lymphomas and rarely T-cell lymphomas and leukemias (Figs. 3.7, 11.2, 12.4, 12.5, 13.1, 13.2, and 13.4). Recently, FISH has been reported to be a useful adjunct in the diagnosis and prognostication of melanoma and ambiguous melanocytic tumors (Figs. 3.6, 5.3, 5.5–5.8, 6.4, and 9.1) [66–68].

Variations of FISH

The two main strategies for detection of chromosomal translocations employ either (1) dual-color, dual-fusion probes or (2) dual-color, break-apart probes. Dual-fusion FISH (dFISH) utilizes two fusion probes with different fluorescence wavelengths in order to label structural chromosomal rearrangements. Each probe has a unique color by itself, and a third color is produced when the two probes are combined (fusion signal). This fusion signal can be used to identify reciprocal translocations that are known to involve two partner genes, for example, *IGH/BCL2* (Fig. 3.7a). Normal alleles will display their respective single-color signal, while nuclei containing the translocation will show a fused signal. Break-apart FISH detects translocations using differentially labeled DNA probes that are complementary to sequences proximal and distal to the breakpoint within a target gene (Fig. 3.7b). In normal nuclei, the signal of the selected gene appears fused. In nuclei containing a translocation, the gene in question will be represented by (a) one fusion signal for the normal allele and (b) two separated single-color signals due to disruption of the other allele.

Chromogenic In situ Hybridization

Some of the disadvantages of FISH include a requirement for fluorescence microscopy and the fading of fluorescence signals over time (often within a number of weeks) [69]. The method also poses

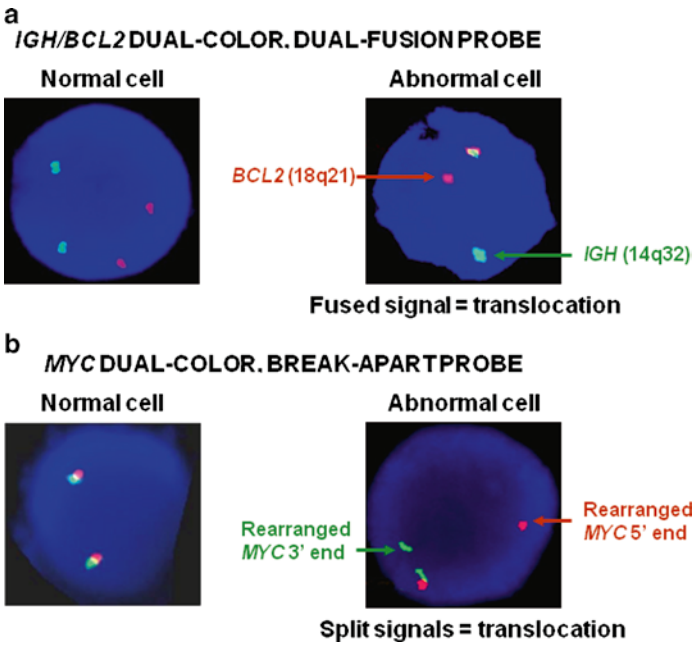

Fig. 3.7 *FISH dual-color, dual-fusion and dual-color, break-apart probes.* (**a**) In the abnormal cell, the *IGH/BCL2* fusion probe shows an extra set of fused red/green signals, in addition to the normal two signals, indicating the presence of the t(14;18) translocation in a follicular lymphoma. The normal cell contains two sets of separate red and green signals. (**b**) The MYC break-apart rearrangement probe for Burkitt lymphoma hybridizes to chromosomal band 8q24. The normal cell has two fused signals, while the rearranged cell shows the probe being split into its 5′ and 3′ portions that are labeled with Spectrum Orange and Spectrum Green, respectively (Courtesy of Lauren Wilson, Hartford Hospital, Hartford, CT, USA)

some difficulty in discerning tissue morphological features, because of the use of fluorescence and not light microscopy [70]. These practical limitations have been overcome through the introduction of chromogenic in situ hybridization (CISH), a modification of conventional FISH that uses probes labeled with different chromogenic substrates, hence eliminating the need for a fluorescent microscope. CISH detects DNA probes by employing peroxidase or alkaline phosphatase enzymatic reactions akin to simple immunohistochemistry (IHC) [71]. The use of hematoxylin counterstain makes histologic evaluation of a tissue section relatively straightforward. The hybridization signals of CISH appear as intracellular peroxidase-positive dots that are easily visualized with the 40× objective [72]. The signals and tissue morphology can be simultaneously evaluated by standard light microscopy, without having to consider storage conditions and time elapsed from test performance. CISH has been used to show loss of the melanocyte-specific gene melastatin (MLSN) in aggressive metastatic melanoma (Fig. 3.8) [73]. ISH can also be used to demonstrate immunoglobulin light chain restriction in cutaneous B-cell lymphomas (Fig. 12.3). In addition, ISH for detection of Epstein-Barr virus-encoded mRNA (EBER-ISH) is used in the work-up of subtypes of CTCL (Figs. 11.3 and 11.5).

Hybrid Capture Assay

The hybrid capture assay is a signal amplification technique that involves antibody capture and chemiluminescent signal detection [74]. This method uses an RNA probe to hybridize with denatured

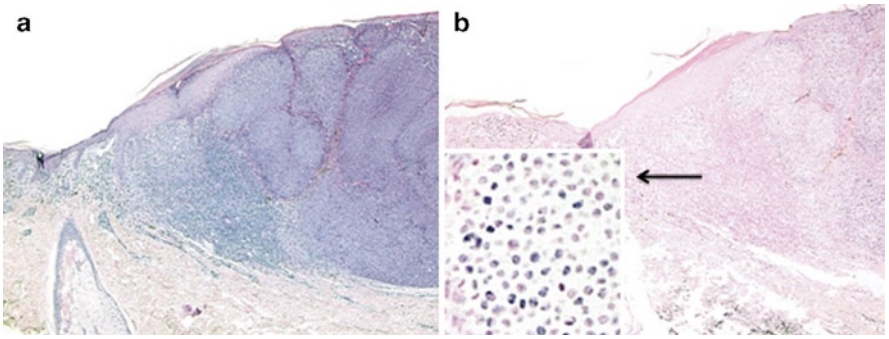

Fig. 3.8 *Chromogenic in situ hybridization* (CISH) *for melastatin* (MLSN) *expression, a marker of disease progression/aggressiveness in melanoma*. (**a**) Nodular melanoma of the back, 1.8 mm in Breslow thickness and ulcerated, that (**b**) demonstrated partial loss of MLSN mRNA (*inset*: half of the cells showed strong MLSN expression, whereas the remainder showed partial to almost complete loss of MLSN mRNA). The patient died of metastatic melanoma 2.5 years after diagnosis (Courtesy of Dr. J. Andrew Carlson, Albany Medical College, Albany, NY, USA)

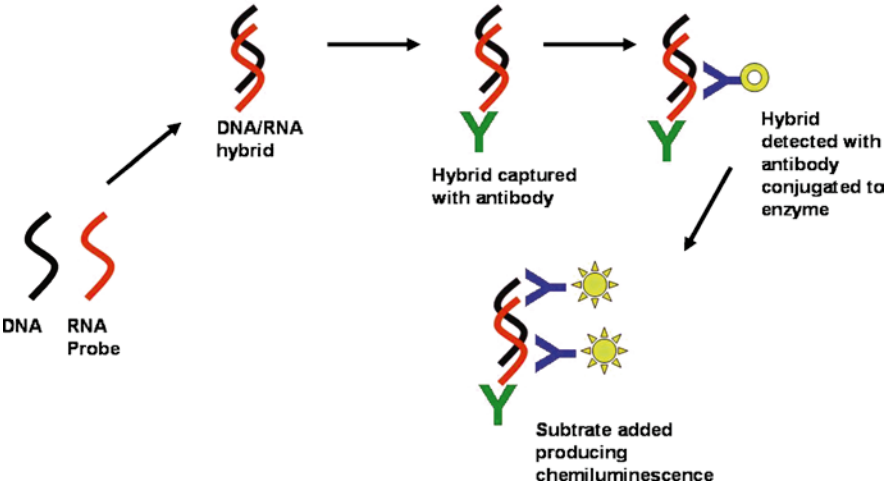

Fig. 3.9 *Hybrid capture assay*. RNA probe is hybridized to target DNA and the resultant hybrid is captured with an antibody. The hybrid is then detected by a second antibody conjugated to an enzyme. The addition of a chemiluminescent substrate generates light emission

target ssDNA. The generated DNA/RNA hybrid is captured through an antibody that is immobilized to the wall of a microtiter plate. This antibody specifically detects DNA/RNA duplexes, but not ssDNA or ssRNA. A second labeled antibody, conjugated to alkaline phosphatase and likewise designed to recognize DNA/RNA hybrids, is added in order to detect the bound primary antibody. This is followed by the addition of a chemiluminescent substrate which, after cleavage by the alkaline phosphatase, emits light that is measurable with a luminometer (Fig. 3.9) [75]. The light emitted is proportional to the number of copies of the target in the sample, and the concentration of DNA is determined from a standard curve [76]. Hybrid capture technology has been used to develop diagnostic tests for HPV, *Chlamydia trachomatis, Neisseria gonorrhoeae*, hepatitis B virus, and cytomegalovirus [77].

Other Molecular Techniques

G-Banding

G-banding is a cytogenetic staining technique that is designed to identify chromosomes on the basis of their unique banding patterns [78]. Prior to staining, the enzyme trypsin is applied to metaphase chromosomes in order to partially digest histones (i.e., the proteins that hold chromosomes together) and weaken DNA-protein interactions. A defined pattern of alternating light and dark bands is produced after application of Giemsa stain [79]. In addition to a distinct banding pattern, individual chromosomes are identified according to their size and centromere position. The chromosomes are then counted and characterized in a karyotype analysis, where any abnormality in chromosome number and/or structure would become apparent (Fig. 3.10). Karyotype–phenotype correlations have been documented for many hematopoietic and soft tissue neoplasms. In dermatology, G-banding studies have been used to identify chromosomal aberrations in CTCL and parapsoriasis [79–81]. G-banding is an easy and inexpensive technique which allows screening of the entire genome for chromosomal abnormalities. Its obvious limitation in dermatology is the requirement for dividing (metaphase) cells and hence, the need for cell cultures. However, skin biopsies are almost always received in formalin fixative. Even if fresh skin tissue is obtained, for example, in primary CTCL, it may be difficult to produce high-quality metaphase chromosomes because of the limited ability of skin lymphoma cells to proliferate *in vitro* [82]. For these reasons, other molecular cytogenetic methods, such as FISH and comparative genomic hybridization (CGH), are favored for dermatologic applications.

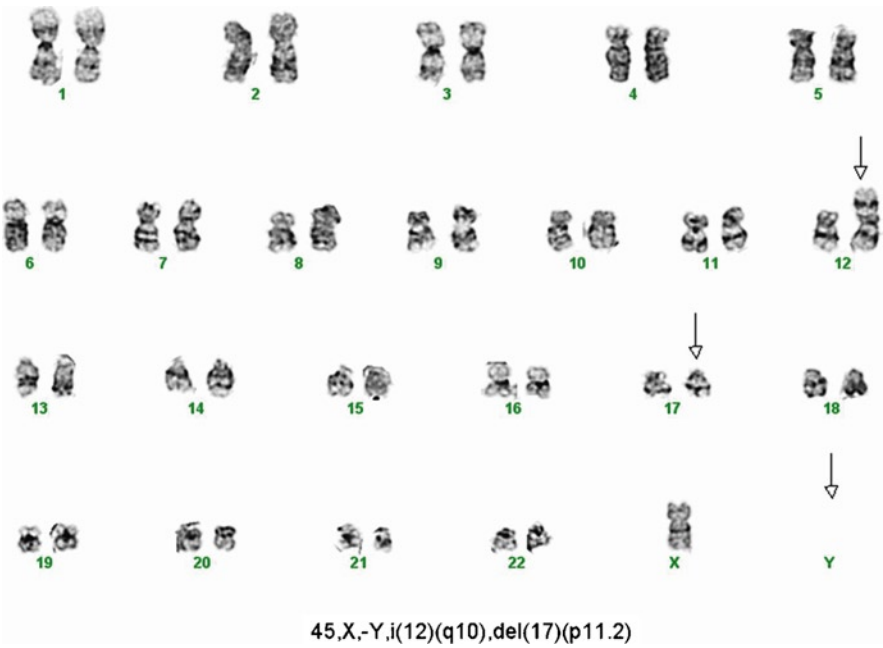

Fig. 3.10 *G-banding*. Follicular lymphoma demonstrating (1) loss of chromosome Y; (2) an unbalanced translocation involving chromosome 12; and (3) deletion of the short arm of chromosome 17 (Courtesy of Lauren Wilson, Hartford Hospital, Hartford, CT, USA)

Spectral Karyotyping

Spectral karyotyping (SKY) is a chromosome painting technique that labels all the chromosomes at the same time, providing a comprehensive screening test for cytogenetic changes [83]. Based on the technology of multicolor-FISH (M-FISH), 24 DNA probes (for the 22 autosomes, chromosome X, and chromosome Y) are prepared by flow-sorting human chromosomes and assigning each with a different fluorophore combination in order to arrive at a unique signature color [84]. The probes are then hybridized with a metaphase chromosome preparation, producing a multicolor karyotype, in which each homologous chromosome pair assumes its own distinct color (Fig. 3.11) [85]. This color-coding scheme efficiently highlights chromosomal translocations and rearrangements (i.e., in translocations, when a piece of a chromosome breaks off and attaches to another chromosome, SKY analysis would show a chromosome painted in one color with a smaller fragment of a different chromosome in another color attached to it). This technique expands on the diagnostic potential of FISH by facilitating the simultaneous visualization of all chromosomes and their abnormalities.

SKY has been used to demonstrate both numerical and structural aberrations in skin-homing T-lymphocytes in CTCL, and to illustrate the disappearance of these cells after treatment, raising a potential role for this technology in disease monitoring [86]. In analyses of mycosis fungoides (MF) and Sézary syndrome (SS) cell cultures, SKY has been used to identify several abnormalities (i.e., insertions and derivative chromosomes resulting from multiple rearrangements), which are not

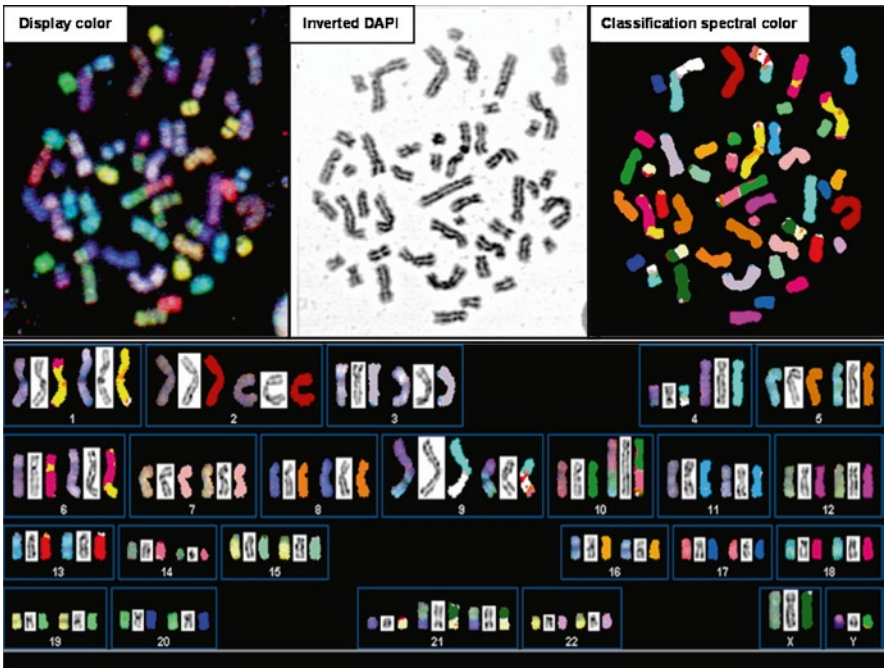

Fig. 3.11 *Spectral karyotyping (SKY) analysis of peripheral blood lymphocytes from a patient with Sézary syndrome.* The *top panels* show the same metaphase spread in (from left to right): display color, inverted DAPI, and classification spectral color. The *bottom panel* displays the karyotype with each chromosome shown in the same order (display color, inverted DAPI, and classification spectral color). The karyotype is as follows: 47,XY,t(1;6)(p32;q25),der(1)t(1;9)(q42;p22),t(4;9)(q12;p22),der(9)t(4;9)(q31;p22)t(9;14)(q31;q24),der(10)t(9;10)(?p24;q26)ins(10;14)(q26;q11.2q32),del(14)(q11.2q32),der(14),t(?7;14)(p21;q13),der(21)t(X;21)(p11.2;p11.2),+der(21)t(X;21)[20]/46,XY[14] (Courtesy of Dr. Denise Batista, Johns Hopkins University, Baltimore, MD, USA)

detectable by G-banding alone [87]. SKY has also been applied to the study of HPV-associated squamous cell carcinoma (SCC) [88] and malignant fibrous histiocytoma [89]. Similar to G-banding, limitations of the technique include its requirement for cell cultures and its inability to detect smaller intrachromosomal alterations, such as inversions and deletions [84].

Loss of Heterozygosity Analysis

Loss of heterozygosity (LOH) is a genetic alteration in which a heterozygous somatic cell (i.e., two different alleles at a single locus) becomes homozygous (i.e., two identical alleles at a single locus) or hemizygous (i.e., only one copy of the allele present, due to loss of the corresponding wild-type allele). This mechanism can lead to cancer through the loss of tumor suppressor genes, and is recognized as a key event in the evolution of tumors [90]. The classic pathway of tumor suppressor gene inactivation is described by the two-hit model, in which one allele is mutated and the other allele is lost, resulting in LOH at multiple loci [91]. This allelic imbalance (AI) causes disruption of normal cellular function and loss of regulatory mechanisms, leading to malignant neoplasia [92]. Mutational events that give rise to LOH include deletion, point mutation, mitotic recombination, gene conversion, and non-dysjunction [93]. Originally based on SBA and RFLP analysis, LOH studies are now largely carried out by microsatellite PCR-based methods, requiring significantly less DNA and permitting analysis of FFPE tissues [94, 95]. Among cutaneous malignancies, melanoma and various non-melanoma skin cancers [SCC, basal cell carcinoma (BCC), and Merkel cell carcinoma] have been shown to arise as a consequence of LOH [96]. However, AI analysis is not routinely employed in the study of melanocytic lesions, because of the need for DNA from peri-lesional non-tumor cells, which are often present in insufficient amounts in limited skin biopsy specimens [64].

Microsatellite Instability Analysis

Microsatellites [also called simple sequence repeats (SSR)] are repeated sequences of DNA, usually less than 10 bp in length and distributed over the entire genome [97]. The term microsatellite instability (MSI) refers to an abnormal increase or decrease in the number of tandem repeats at various microsatellite loci in DNA, due to defects in mismatch repair (MMR) proteins [98]. MMR proteins preserve genomic integrity. Their primary function is the elimination of mismatches of single nucleotide bases, that are caused by small insertions or deletions during DNA replication [99]. The MMR defect develops at the somatic level when the corresponding wild-type allele of a germline mutated DNA MMR gene undergoes an inactivating “second hit” [100]. This leads to the accumulation of replication errors and tumorigenesis [101].

MSI testing involves the comparison of allelic patterns within normal and tumor DNA. From unstained sections of FFPE tissue specimens, selected foci that represent the tumor to be tested and non-neoplastic/normal areas are microdissected for DNA extraction [98]. PCR amplification with sets of primers targeting the microsatellite regions is performed on the matched samples of tumor and normal reference DNA [102]. In 1998, the National Cancer Institute (NCI) recommended a panel of five microsatellite markers for the evaluation of MSI status in tumors. Using this panel, tumors can be classified as: (1) MSI-High (MSI-H), when MSI is detected in at least two of the five markers; (2) MSI-Low (MSI-L), if MSI is detected in only one marker; and (3) microsatellite stable (MSS), when none of the five markers shows MSI [103].

The prototypic skin-related disorder associated with MSI and MMR abnormalities is the Muir–Torre Syndrome (MTS). This is a rare autosomal dominant genodermatosis, characterized by sebaceous gland neoplasms and/or keratoacanthomas, associated with visceral malignancies [101]. Based on clinical and molecular evidence, it has been suggested that there are 2 types of MTS.

The most common subtype is considered a variant of hereditary non-polyposis colorectal cancer (HNPCC) and is characterized by early-onset tumors and germline mutation(s) in DNA MMR genes (i.e., MSH-2, MLH-1, MSH-6). A smaller subset of MTS, the pathogenesis of which remains undefined, presents with late-onset tumors and does not show MMR defects [104–106]. Skin lesions precede visceral malignancies in 22–60% of cases [107–109], highlighting the crucial role of dermatologists and dermatopathologists in the early identification of patients and their families, who stand to benefit from molecular testing and close clinical follow-up. Current evidence supports the use of IHC for loss of MMR protein expression as the initial screening test, followed by MSI testing [109, 110]. When IHC staining and MSI analyses are suggestive of MMR gene mutation, germline mutational analyses may be performed for confirmation [103].

Comparative Genomic Hybridization

Developed primarily as a tool for tumor cytogenetics, CGH is a FISH-based technology that facilitates the evaluation of genetic gains and/or losses over the whole genome [11].

Metaphase CGH

With this technique, equal amounts of tumor DNA (typically labeled with a green fluorochrome) and normal reference DNA (labeled with a red fluorochrome) are co-hybridized onto human metaphase chromosome spreads. During the hybridization process, the two DNA populations compete for their equivalent sequences on the substrate chromosomes [111]. The relative amount of tumor and reference DNA bound to a given chromosomal region reflects the relative abundance of these sequences in both samples. Therefore, differential fluorescence signals emitted by the metaphase spread indicate chromosomal gains or losses (copy number changes) in the tumor DNA relative to the reference DNA. The signals are read by fluorescence microscopy and quantified by image analysis to generate a green-to-red ratio profile [112, 113]. Increased green fluorescence intensity or high green-to-red ratio (>1.0) would represent DNA gain at a particular locus in the tumor, while increased red fluorescence intensity or low green-to-red ratio (<1.0) indicates DNA deletion at a particular locus in the tumor [114]. A chromosomal region with no copy number change (ratio of 1.0) will "stain" equally for both green and red, producing a yellow fluorescence signal.

Array CGH

In microarray-based CGH, the differentially labeled tumor and reference DNA samples are co-hybridized to an array containing genomic DNA targets or probes [115]. Replacing the metaphase spread, the target DNA fragments are spotted on glass slides as precise chromosome coordinates in order to provide a locus-by-locus measure of DNA copy number changes [116]. Chromosomal imbalances can be quantified and positionally defined. After hybridization and washes, microarray images are acquired on an array scanner. Test and reference fluorescence intensities are measured for each spot position, and intensity ratios are calculated (Fig. 3.12) [117]. Genomic gains in the tumor would be represented by green spots, while genomic losses are identified by red spots (if the tumor sample is again labeled with a green fluorochrome, as for metaphase CGH above).

Compared to conventional cytogenetic techniques, CGH does not require culture of target cells to obtain test DNA and can be performed on archival FFPE tissue. However, CGH only determines genomic aberrations that result in DNA copy number changes. Balanced translocations and point

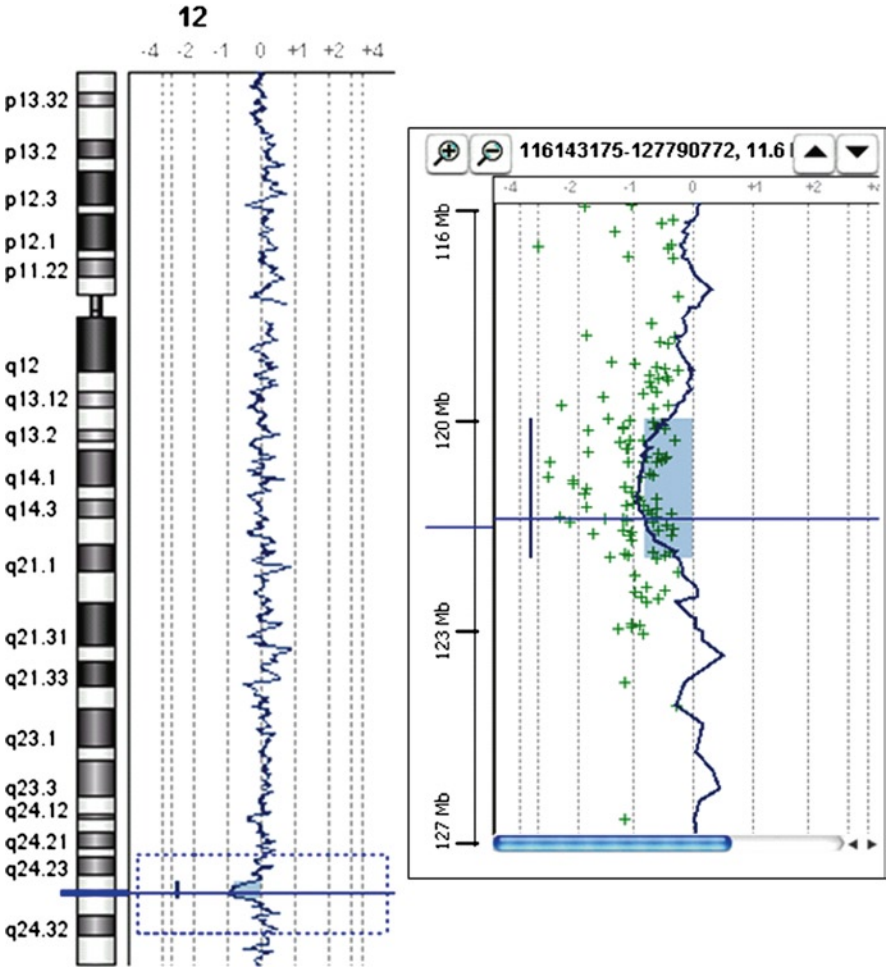

Fig. 3.12 *Array-based CGH of early-stage Mycosis Fungoides*. Chromosome 12 array-CGH profile (*left*) and detail of recurrent deletion at 12q24.31 region (*right*). *Blue vertical line* represents the deleted area as calculated by the ADM-2 algorithm (DNA Analytics software; Agilent Technologies) (Courtesy of Dr. Angelo Carbone, Catholic University of the Sacred Heart, Rome, Italy; and Dr. Laura Bernadini, San Giovanni Rotondo and CSS-Mendel Institute, Rome, Italy)

mutations are not detected [111]. CGH has been employed in the differentiation of melanoma from benign melanocytic lesions (Figs. 5.2–5.4) [118], as well as in the characterization of chromosomal changes in a number of genodermatoses [119, 120] and cutaneous lymphoma/leukemia (Fig. 10.11) [121–123].

DNA Microarrays

A DNA microarray is composed of thousands of DNA sequences, each representing a specific gene, spotted in a grid-like fashion on a glass microscope slide [124]. These DNA sequences, arranged in defined positions on the slide, function as probes and are complementary to genes of interest [125]. A genomic DNA or cDNA sample labeled with a fluorescent or chemiluminescent tag is hybridized to the array. The presence of bound DNA is exhibited by fluorescence or

Fig. 3.13 *cDNA Microarray analysis*. Total RNA was extracted from a cultured melanoma cell line, transcribed into cDNA, and hybridized with a customized cDNA microarray (GEArray™ Human Apoptosis and Cell Cycle Gene cDNA Microarray). Gene expression was measured by chemiluminescent detection and normalized against housekeeping genes on the microarray

chemiluminescence [126]. The array is laser scanned and the image analyzed for pattern and intensity of signal. Fluorescence or chemiluminescence intensity is displayed as one colored dot per gene location, with a single experiment capable of simultaneously profiling hundreds to thousands of genes (Fig. 3.13) [127]. Microarrays are being used to assess patterns of gene expression in order to derive disease-associated signatures and identify possible therapeutic targets [128].

Melanoma was one of the first skin tumors analyzed using DNA microarray technology (Fig. 6.2) [129, 130]. Non-melanocytic tumors, such as BCC and SCC [131, 132], in addition to CTCL (Fig. 10.10) [133], have also been subjected to gene expression profiling. DNA microarrays have been used to (1) illustrate differences in cytokine elaboration in a number of inflammatory skin diseases (i.e., psoriasis and atopic dermatitis), and (2) correlate these changes with disparities in the pathophysiology and propensity for superimposed microbial infection between these disorders [134, 135].

DNA Sequencing

The method developed by Sanger, Nicklen, and Coulson in 1977 forms the basis for most current DNA sequencing studies [136]. With Sanger sequencing, also known as the dideoxy sequencing reaction, an oligonucleotide primer is annealed to a ssDNA template. This is extended by DNA polymerase in the presence of four deoxyribonucleotide triphosphates (dNTPs), which serve as DNA building blocks. One of the dNTPs is fluorescently tagged so that the growing DNA strand is labeled. Also added to the reaction is one of four dideoxyribonucleotide triphosphates (ddNTPs), which serves as a base-specific chain terminator [137]. The ddNTPs are similar to the dNTPs, except that they lack a hydroxyl group at the 3′ end, which is required to form linkage with an incoming nucleotide [138]. In the original protocol for dideoxy sequencing, four separate primer extension reactions are performed, each carrying only one of the four possible ddNTP species (ddATP, ddGTP, ddCTP, or ddTTP, which would stop DNA synthesis specifically at either

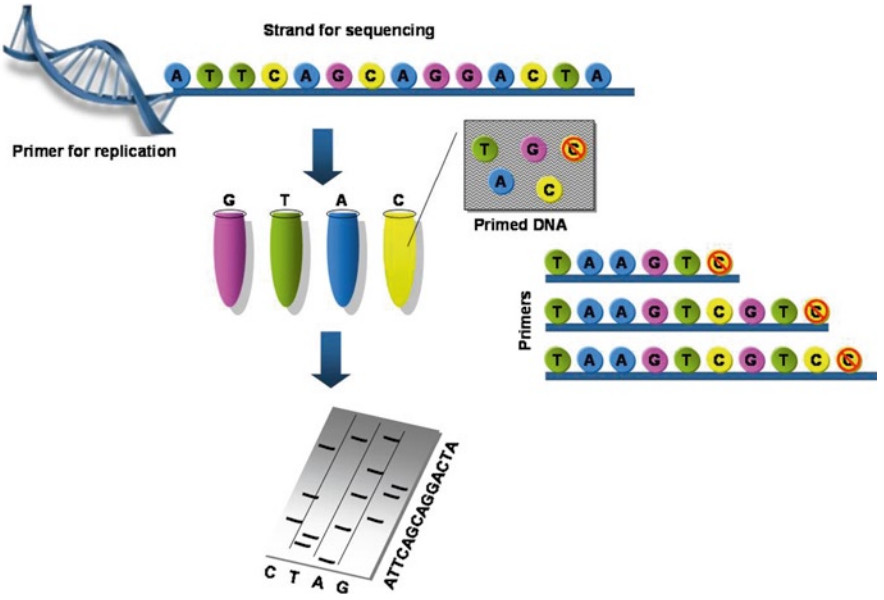

Fig. 3.14 *DNA sequencing: the Sanger method*. DNA denatured into a single strand is mixed with a primer and divided into four tubes that each contains DNA polymerase, four deoxyribonucleotide triphosphates (dNTPs), and a chain terminator (one of four dideoxyribonucleotide triphosphates (ddNTPs)). Each replication advances until a terminating nucleotide is added. The random addition of chain terminators yields DNA fragments of varying lengths, which are then separated by gel or capillary electrophoresis. The sequence of the original template strand can be derived from the results

A, G, C, or T, respectively). This results in terminated strands of various lengths from each reaction, corresponding to the positions of that nucleotide in the template sequence [137]. The products of the four reactions are subjected to gel electrophoresis for size-based separation, resulting in a ladder-like arrangement of bands which is "read" upward to give the nucleotide sequence of the template DNA (Fig. 3.14) [139]. In general, CE has replaced gel electrophoresis for DNA sequencing, dramatically increasing the speed of the procedure.

Newer technologies, collectively known as next-generation sequencing (NGS), are emerging as alternative methods for genomic analysis in genodermatoses (see Chap. 19). Briefly, in NGS, thousands to millions of ssDNA molecules are immobilized on glass slides or beads, PCR-amplified, and analyzed in a massively parallel way. For example, in the case of 454 sequencing (454 Genome Sequencer, Roche Applied Science), ssDNA is attached to beads and amplified by PCR in water-in-oil emulsion. The beads are then mixed with DNA polymerase and deposited in wells. Nucleotides are added to form cDNA strands. This incorporation process releases a pyrophosphate group which can be detected as emitted light. Across multiple cycles, the pattern of detected signals represents the sequence of templates in the individual beads [140]. NGS technologies are rapidly evolving. Given the current state of growth, it is anticipated that their use will become more widespread and routine as we continue to improve technical performance and develop better platforms.

Laser Capture Microdissection

Laser capture microdissection (LCM) is a valuable tool that facilitates molecular analysis of complex tissues. With this technique, specific populations of cells can be collected from a heterogeneous tissue section for extraction of DNA, RNA, and/or proteins [141]. Briefly, LCM entails placing a

transparent film or membrane over a tissue or cytology slide, visualizing the cells under microscopy, and selectively adhering the cells of interest to the film using an infrared laser [142]. Because of the short-duration, focused pulse of the laser, which is mostly absorbed by the membrane, the biologic molecules of the cells of interest largely remain intact [143]. The collected cells are then placed in buffer solutions for nucleic acid or protein extraction.

LCM is particularly useful in the study of dermatologic diseases. Because skin is an inherently heterogeneous tissue containing different cell populations (i.e., keratinocytes, melanocytes, infiltrating inflammatory cells), the precision of LCM is crucial in isolating targets for further analysis, such as lymphoid cells in cutaneous lymphoma [144, 145]. Material extracted using LCM is amenable to most molecular platforms. DNA obtained by LCM has been utilized for clonality studies in cutaneous B- and T-cell lymphomas [146, 147], fingerprinting mutational analysis in actinic keratosis and SCC [148], DNA sequencing in SCC [145], and LOH studies in melanoma [149, 150]. LCM has also been employed in the PCR-based detection of cutaneous infectious agents, including herpesviruses, mycobacterial species, and spirochetes [144].

References

1. Smith-Zagone M, Pulliam J, Farkas D. Molecular pathology methods. In: Leonard D, editor. Molecular pathology in clinical practice. New York: Springer; 2007. p. 15–40.
2. Amiss T, Presnell SC. Nucleic acids blotting techniques. In: Coleman WB, Tsongalis GJ, editors. Molecular diagnostics for the clinical laboratorian. 2nd ed. Totowa: Humana Press; 2006. p. 31–55.
3. Evans MR, Bertera AL, Harris DW. The Southern blot. An update. Mol Biotechnol. 1994;1:1–12.
4. Andres C, Belloni B, Puchta U, et al. Prevalence of MCPyV in Merkel cell carcinoma and non-MCC tumors. J Cutan Pathol. 2010;37:28–34.
5. Mies C, Houldsworth J, Chaganti RS. Extraction of DNA from paraffin blocks for Southern blot analysis. Am J Surg Pathol. 1991;15:169–74.
6. Payne DA, Vander Straten M, Carrasco D, et al. Molecular diagnosis of skin-associated infectious agents. Arch Dermatol. 2001;137:1497–502.
7. Mullis K, Faloona F, Scharf S, et al. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction 1986. Biotechnology. 1992;24:17–27.
8. Strachan T, Read AP. Amplifying DNA: PCR and cell based DNA cloning. In: Human molecular genetics. 3rd ed. New York: Taylor & Francis; 2003. p. 122–54.
9. Unger ER, Pincus MR. Introduction to molecular pathology. In: McPherson RA, Pincus MR, editors. Henry's clinical diagnosis and management by laboratory methods. 21st ed. Philadelphia: Saunders Elsevier; 2007. p. 1226–7.
10. Evans MF. The polymerase chain reaction and pathology practice. Diag Histopathol. 2009;15:344–56.
11. Braun-Falco M, Schempp W, Weyers W. Molecular diagnosis in dermatopathology: what makes sense, and what doesn't. Exp Dermatol. 2009;18:12–23.
12. Sra KK, Babb-Tarbox M, Aboutalebi S, et al. Molecular diagnosis of cutaneous diseases. Arch Dermatol. 2005;141:225–41.
13. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res. 1996;6:995–1001.
14. Netto GJ, Saad RD. Diagnostic molecular pathology: an increasingly indispensable tool for the practicing pathologist. Arch Pathol Lab Med. 2006;130:1339–48.
15. Sra KK, Torres G, Rady P, et al. Molecular diagnosis of infectious diseases in dermatology. J Am Acad Dermatol. 2005;53:749–65.
16. Nickerson DA, Ankener W, Delahunty C, et al. Genotyping by ligation assays. Curr Protoc Hum Genet. 2001; Chapter 2:Unit 2.6.
17. Killeen AA. Methods in molecular pathology. In: Principles of molecular pathology. Totowa: Humana Press; 2003. p. 89–139.
18. Payne DA, Sower L. Alternative methods for amplified nucleic acid testing. In: Coleman WB, Tsongalis GJ, editors. Molecular diagnostics for the clinical laboratorian. 2nd ed. Totowa: Humana Press; 2006. p. 75–84.
19. Deiman B, van Aarle P, Sillekens P. Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol Biotechnol. 2002;20:163–79.
20. Guatelli JC, Whitfield KM, Kwoh DY, et al. Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. Proc Natl Acad Sci USA. 1990;87:1874–8.

21. Loens K, Ursi d, Goossens H, Ieven M. Nucleic acid sequence-based amplification. In: Walker J, Rapley R, editors. Medical biomethods handbook. Totowa: Humana Press; 2005. p. 273–91.
22. Spargo CA, Fraiser MS, Van Cleve M, et al. Detection of M. tuberculosis DNA using thermophilic strand displacement amplification. Mol Cell Probes. 1996;10:247–56.
23. Walker GT, Little MC, Nadeau JG, et al. Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. Proc Natl Acad Sci USA. 1992;89:392–6.
24. Walker GT. Empirical aspects of strand displacement amplification. PCR Meth Appl. 1993;3:1–6.
25. Walker GT, Nadeau JG, Linn CP, et al. Strand displacement amplification (SDA) and transient-state fluorescence polarization detection of Mycobacterium tuberculosis DNA. Clin Chem. 1996;42:9–13.
26. Nycz CM, Dean CH, Haaland PD, et al. Quantitative reverse transcription strand displacement amplification: quantitation of nucleic acids using an isothermal amplification technique. Anal Biochem. 1998; 259:226–34.
27. Goldmeyer J, Kong H, Tang W. Development of a novel one-tube isothermal reverse transcription thermophilic helicase-dependent amplification platform for rapid RNA detection. J Mol Diagn. 2007;5:639–44.
28. An L, Tang W, Ranalli TA, et al. Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification. J Biol Chem. 2005;32:28952–8.
29. Gill P, Ghaemi A. Nucleic acid isothermal amplification technologies: a review. Nucleosides Nucleotides Nucleic Acids. 2008;27:224–43.
30. Vincent M, Xu Y, Kong H. Helicase-dependent isothermal DNA amplification. EMBO Rep. 2004;5:795–800.
31. Chow WH, McCloskey C, Tong Y, et al. Application of isothermal helicase-dependent amplification with a disposable detection device in a simple sensitive stool test for toxigenic Clostridium difficile. J Mol Diagn. 2008;10:452–8.
32. Kozlowski P, Jasinska AJ, Kwiatkowski DJ. New applications and developments in the use of multiplex ligation-dependent probe amplification. Electrophoresis. 2008;29:4627–36.
33. Shen Y, Wu BL. Designing a simple multiplex ligation-dependent probe amplification (MLPA) assay for rapid detection of copy number variants in the genome. J Genet Genomics. 2009;36:257–65.
34. Zhi J, Hatchwell E. Human MLPA Probe Design (H-MAPD): a probe design tool for both electrophoresis-based and bead-coupled human multiplex ligation-dependent probe amplification assays. BMC Genomics. 2008;10:407.
35. Takata M, Lin J, Takayanagi S, et al. Genetic and epigenetic alterations in the differential diagnosis of malignant melanoma and spitzoid lesion. Br J Dermatol. 2007;156:1287–94.
36. Takata M, Suzuki T, Ansai S, et al. Genome profiling of melanocytic tumors using multiplex ligation-dependent probe amplification (MLPA): its usefulness as an adjunctive diagnostic tool for melanocytic tumors. J Dermatol Sci. 2005;40:51–7.
37. Senff NJ, Zoutman WH, Vermeer MH, et al. Fine-mapping chromosomal loss at 9p21: correlation with prognosis in primary cutaneous diffuse large B-cell lymphoma, leg type. J Invest Dermatol. 2009;129:1149–55.
38. Stenau M, Piper MA, Unger ER. Molecular diagnostics: basic principles and techniques. In: McPherson RA, Pincus MR, editors. Henry's clinical diagnosis and management by laboratory methods. 21st ed. Philadelphia: Saunders Elsevier; 2007. p. 1228–49.
39. Highsmith Jr WE. Electrophoretic methods for mutation detection and DNA sequencing. In: Coleman WB, Tsongalis GJ, editors. Molecular diagnostics for the clinical laboratorian. 2nd ed. Totowa: Humana Press; 2006. p. 85–109.
40. Knight P. Capillary electrophoresis. Biotechnology. 1988;6:1226–7.
41. Tagliaro F, Turrina S, Smith FP. Capillary electrophoresis: principles and applications in illicit drug analysis. Forensic Sci Int. 1996;77:211–29.
42. Smith A, Nelson RJ. Capillary electrophoresis of DNA. Curr Protoc Mol Biol. 2004; Chapter 2:Unit 2.8.
43. Landers JP. Clinical capillary electrophoresis. Clin Chem. 1995;41:495–509.
44. Guttman A, Ettre LS. The evolution of capillary gel electrophoresis: from proteins to DNA sequencing. LCGC N Am. 2004;22:896–904.
45. Kleparník K, Bocek P. DNA diagnostics by capillary electrophoresis. Chem Rev. 2007;107:5279–317.
46. Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 1985;20:1350–4.
47. Green E. Restriction fragment length polymorphisms. In: Walker J, Rapley R, editors. Medical biomethods handbook. Totowa: Humana Press; 1998. p. 271–9.
48. Butler JM, Reeder DJ. Detection of DNA polymorphisms using PCR-RFLP and capillary electrophoresis. Meth Mol Biol. 2001;163:49–56.
49. Hayashi K. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Meth Appl. 1991;1:34–8.
50. Sood AK, Buller RE. SSCP and sequence analysis of p53 mutations in ovarian Tumors. In: Bartlett J, editor. Methods in molecular medicine: ovarian cancer: methods and protocols. Totowa: Humana Press; 2000. p. 323–8.

51. Sunnucks P, Wilson AC, Beheregaray LB, et al. SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. Mol Ecol. 2000;9:1699–710.
52. Orita M, Iwahana H, Kanazawa H, et al. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA. 1989;86:2766–70.
53. Han M, Robinson MA. PCR-SSCP analysis of polymorphism: a simple and sensitive method for detecting differences between short segments of DNA. Meth Mol Biol. 2003;226:327–34.
54. Wallace AJ. SSCP/heteroduplex analysis. Meth Mol Biol. 2002;187:151–63.
55. Bhattacharyya A, Lilley DM. The contrasting structures of mismatched DNA sequences containing looped-out bases (bulges) and multiple mismatches (bubbles). Nucleic Acids Res. 1989;17:6821–40.
56. Straten PT, Becker JC, Zeuthen J, et al. T-cell receptor clonotype mapping using denaturing gradient gel electrophoresis: analysis of clonal T-cell responses in melanoma. Meth Mol Med. 2001;61:339–51.
57. Fischer SG, Lerman LS. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. Proc Natl Acad Sci USA. 1983;80:1579–83.
58. Rosenbaum V, Riesner D. Temperature-gradient gel electrophoresis. Thermodynamic analysis of nucleic acids and proteins in purified form and in cellular extracts. Biophys Chem. 1987;9:235–46.
59. Knapp LA. Single nucleotide polymorphism screening with denaturing gradient gel electrophoresis. Meth Mol Biol. 2009;578:137–51.
60. Bartlett JM. Fluorescence in situ hybridization: technical overview. Meth Mol Med. 2004;97:77–87.
61. Yang X. In situ hybridization. Meth Mol Med. 2000;47:45–69.
62. Trask BJ. Fluorescence in situ hybridization: applications in cytogenetics and gene mapping. Trends Genet. 1991;7:149–54.
63. Wolff DJ, Schwartz S. Fluorescence in situ hybridization. In: Gersen SL, Keagle MB, editors. The principles of clinical cytogenetics. 2nd ed. Totowa: Humana Press; 2004. p. 455–90.
64. Blokx WA, van Dijk MC, Ruiter DJ. Molecular cytogenetics of cutaneous melanocytic lesions – diagnostic, prognostic and therapeutic aspects. Histopathology. 2010;56:121–32.
65. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors. Dermatofibrosarcoma protuberans and giant cell fibroblastoma. Cancer Genet Cytogenet. 2003;140:1–12.
66. Gerami P, Jewell SS, Morrison LE, et al. Fluorescence in situ hybridization (FISH) as an ancillary diagnostic tool in the diagnosis of melanoma. Am J Surg Pathol. 2009;33:1146–56.
67. Gerami P, Wass A, Mafee M, et al. Fluorescence in situ hybridization for distinguishing nevoid melanomas from mitotically active nevi. Am J Surg Pathol. 2009;33:1783–8.
68. Gerami P, Guitart J, Martini M, et al. Cyclin D1 homogeneous staining regions by fluorescent in situ hybridization: a possible indicator of aggressive behavior in melanomas. Arch Dermatol. 2008;144:1235–6.
69. Tanner M, Gancberg D, Di Leo A, et al. Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. Am J Pathol. 2000;157:1467–72.
70. Shipley J. Putting the colours into chromogenic in situ hybridization (CISH). J Pathol. 2006;210:1–2.
71. Pedersen M, Rasmussen BB. The correlation between dual-color chromogenic in situ hybridization and fluorescence in situ hybridization in assessing HER2 gene amplification in breast cancer. Diagn Mol Pathol. 2009;18:96–102.
72. Isola J, Tanner M. Chromogenic in situ hybridization in tumor pathology. Meth Mol Med. 2004;97:133–44.
73. Hammock L, Cohen C, Carlson G, et al. Chromogenic in situ hybridization analysis of melastatin mRNA expression in melanomas from American Joint Committee on Cancer stage I and II patients with recurrent melanoma. J Cutan Pathol. 2006;33:599–607.
74. Wang YF. Signal amplification techniques: bDNA, hybrid capture. In: Tang YW, Stratton CW, editors. Advanced techniques in diagnostic microbiology. New York: Springer; 2006. p. 228–42.
75. Knoepp SM, Kuebler DL, Wilbur DC. Resolution of equivocal results with the Hybrid Capture II high-risk HPV DNA test: acytologic/histologic review of 191 cases. Diagn Mol Pathol. 2007;16:125–9.
76. Butterworth LA, Prior SL, Buda PJ, et al. Comparison of four methods for quantitative measurement of hepatitis B viral DNA. J Hepatol. 1996;24:686–91.
77. Obiso R, Lorincz A. Digene Corporation. Pharmacogenomics. 2004;5:129–32.
78. Holmquist GP, Comings DE. Histones and G banding of chromosomes. Science. 1976;193:599–602.
79. Karenko L, Sarna S, Kähkönen M, et al. Chromosomal abnormalities in relation to clinical disease in patients with cutaneous T-cell lymphoma: a 5-year follow-up study. Br J Dermatol. 2003;148:55–64.
80. Karenko L, Hyytinen E, Sarna S, et al. Chromosomal abnormalities in cutaneous T-cell lymphoma and in its premalignant conditions as detected by G-banding and interphase cytogenetic methods. J Invest Dermatol. 1997;108:22–9.
81. Nowell PC, Finan JB, Vonderheid EC. Clonal characteristics of cutaneous T cell lymphomas: cytogenetic evidence from blood, lymph nodes, and skin. J Invest Dermatol. 1982;78:69–75.

82. Chevret E, Prochazkova M, Beylot-Barry M, et al. A suggested protocol for obtaining high-quality skin metaphases from primary cutaneous T-cell lymphoma. Cancer Genet Cytogenet. 2006;167:89–91.
83. Schrock E, du Manoir S, Veldman T, et al. Multicolor spectral karyotyping of human chromosomes. Science. 1996;273:494–7.
84. Jalal SM, Law ME. Utility of multicolor fluorescent in situ hybridization in clinical cytogenetics. Genet Med. 1999;1:181–6.
85. Macville M, Veldman T, Padilla-Nash H, et al. Spectral karyotyping, a 24-colour FISH technique for the identification of chromosomal rearrangements. Histochem Cell Biol. 1997;108:299–305.
86. Padilla-Nash HM, Wu K, Just H, et al. Spectral karyotyping demonstrates genetically unstable skin-homing T lymphocytes in cutaneous T-cell lymphoma. Exp Dermatol. 2007;16:98–103.
87. Batista DA, Vonderheid EC, Hawkins A, et al. Multicolor fluorescence in situ hybridization (SKY) in mycosis fungoides and Sézary syndrome: search for recurrent chromosome abnormalities. Genes Chromosom Cancer. 2006;45:383–91.
88. Ragin CC, Reshmi SC, Gollin SM. Mapping and analysis of HPV16 integration sites in a head and neck cancer cell line. Int J Cancer. 2004;110:701–9.
89. Weng WH, Wejde J, Ahlén J, et al. Characterization of large chromosome markers in a malignant fibrous histiocytoma by spectral karyotyping, comparative genomic hybridization (CGH), and array CGH. Cancer Genet Cytogenet. 2004;150:27–32.
90. Nakamura T, Sunami E, Nguyen T, et al. Analysis of loss of heterozygosity in circulating DNA. Meth Mol Biol. 2009;520:221–9.
91. Knudson Jr AG. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA. 1971;68:820–3.
92. Zhu X, Dunn JM, Goddard AD, Squire JA, et al. Mechanisms of loss of heterozygosity in retinoblastoma. Cytogenet Cell Genet. 1992;59:248–52.
93. Zheng HT, Peng ZH, Li S, et al. Loss of heterozygosity analyzed by single nucleotide polymorphism array in cancer. World J Gastroenterol. 2005;11:6740–4.
94. Farrand K, Jovanovic L, Delahunt B, et al. Loss of heterozygosity studies revisited: prior quantification of the amplifiable DNA content of archival samples improves efficiency and reliability. J Mol Diagn. 2002;4:150–8.
95. Ibbotson RE, Corcoran MM. Detection of chromosomal deletions by microsatellite analysis. Meth Mol Med. 2002;68:59–65.
96. Happle R. Loss of heterozygosity in human skin. J Am Acad Dermatol. 1999;41(2 Pt 1):143–64.
97. Strachan T, Read AP. Organization of the human genome. In: Human molecular genetics. 3rd ed. New York: Taylor & Francis; 2003. p. 240–74.
98. Thornland EC, Thibodeau SN. Hereditary nonpolyposis colorectal cancer. In: Leonard D, editor. Molecular pathology in clinical practice. New York: Springer; 2007. p. 223–32.
99. Peltomäki P. Deficient DNA mismatch repair: a common etiologic factor for colon cancer. Hum Mol Genet. 2001;10:735–40.
100. Kruse R, Rütten A, Hosseiny-Malayeri HR, et al. "Second hit" in sebaceous tumors from Muir-Torre patients with germline mutations in MSH2: allele loss is not the preferred mode of inactivation. J Invest Dermatol. 2001;116:463–5.
101. Ponti G, Ponz de Leon M. Muir-Torre syndrome. Lancet Oncol. 2005;6:980–7.
102. Gologan A, Sepulveda AR. Microsatellite instability and DNA mismatch repair deficiency testing in hereditary and sporadic gastrointestinal cancers. Clin Lab Med. 2005;25:179–96.
103. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998;58:5248–57.
104. Mangold E, Pagenstecher C, Leister M, et al. A genotype-phenotype correlation in HNPCC: strong predominance of msh2 mutations in 41 patients with Muir-Torre syndrome. J Med Genet. 2004;41:567–72.
105. Mathiak M, Rutten A, Mangold E, et al. Loss of DNA mismatch repair proteins in skin tumors from patients with Muir-Torre syndrome and MSH2 or MLH1 germline mutations: establishment of immunohistochemical analysis as a screening test. Am J Surg Pathol. 2002;26:338–43.
106. Mangold E, Rahner N, Friedrichs N, et al. MSH6 mutation in Muir-Torre syndrome: could this be a rare finding? Br J Dermatol. 2007;156:158–62.
107. Ponti G, Losi L, Pedroni M, et al. Value of MLH1 and MSH2 mutations in the appearance of Muir-Torre syndrome phenotype in HNPCC patients presenting sebaceous gland tumors or keratoacanthomas. J Invest Dermatol. 2006;126:2302–7.
108. Schwartz RA, Torre DP. The Muir-Torre syndrome: a 25-year retrospect. J Am Acad Dermatol. 1995;33:90–104.
109. Ponti G, Losi L, Di Gregorio C, et al. Identification of Muir-Torre syndrome among patients with sebaceous tumors and keratoacanthomas: role of clinical features, microsatellite instability, and immunohistochemistry. Cancer. 2005;103:1018–25.

110. Abbas O, Mahalingam M. Cutaneous sebaceous neoplasms as markers of Muir-Torre syndrome: a diagnostic algorithm. J Cutan Pathol. 2009;36:613–9.
111. Bauer J, Bastian BC. Distinguishing melanocytic nevi from melanoma by DNA copy number changes: comparative genomic hybridization as a research and diagnostic tool. Dermatol Ther. 2006;19:40–9.
112. Morrison C. Fluorescent in situ hybridization and array comparative genomic hybridization: complementary techniques for genomic evaluation. Arch Pathol Lab Med. 2006;130:967–74.
113. DeVries S, Gray JW, Pinkel D, et al. Comparative genomic hybridization. Curr Protoc Hum Genet. 2001; Chapter 4:Unit 4.6.
114. Kallioniemi A, Kallioniemi OP, Sudar D, et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science. 1992;258:818–21.
115. Beheshti B, Park PC, Braude I, et al. Microarray CGH. Meth Mol Biol. 2002;204:191–207.
116. Pollack JR, Perou CM, Alizadeh AA, et al. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet. 1999;23:41–6.
117. Redon R, Carter NP. Comparative genomic hybridization: microarray design and data interpretation. Meth Mol Biol. 2009;529:37–49.
118. Harvell JD, Kohler S, Zhu S, et al. High-resolution array-based comparative genomic hybridization for distinguishing paraffin-embedded Spitz nevi and melanomas. Diagn Mol Pathol. 2004;13:22–5.
119. Miozzo M, Castorina P, Riva P, et al. Chromosomal instability in fibroblasts and mesenchymal tumors from 2 sibs with Rothmund-Thomson syndrome. Int J Cancer. 1998;77:504–10.
120. Van Esch H, Hollanders K, Badisco L, et al. Deletion of VCX-A due to NAHR plays a major role in the occurrence of mental retardation in patients with X-linked ichthyosis. Hum Mol Genet. 2005;14:1795–803.
121. Hahtola S, Burghart E, Jeskanen L, et al. Clinicopathological characterization and genomic aberrations in subcutaneous panniculitis-like T-cell lymphoma. J Invest Dermatol. 2008;128:2304–9.
122. van Doorn R, van Kester MS, Dijkman R, et al. Oncogenomic analysis of mycosis fungoides reveals major differences with Sezary syndrome. Blood. 2009;113:127–36.
123. Dijkman R, van Doorn R, Szuhai K, et al. Gene-expression profiling and array-based CGH classify CD4+CD56+ hematodermic neoplasm and cutaneous myelomonocytic leukemia as distinct disease entities. Blood. 2007;109:1720–7.
124. Sellheyer K, Belbin TJ. DNA microarrays: from structural genomics to functional genomics. The applications of gene chips in dermatology and dermatopathology. J Am Acad Dermatol. 2004;51:681–92.
125. Friend SH, Stoughton RB. The magic of microarrays. Sci Am. 2002;286:44–49, 53.
126. Cheung VG, Morley M, Aguilar F, et al. Making and reading microarrays. Nat Genet. 1999;21(1 Suppl):15–9.
127. Wessagowit V, South AP. Dermatological applications of DNA array technology. Clin Exp Dermatol. 2002;27:485–92.
128. DeRisi J, Penland L, Brown PO, et al. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat Genet. 1996;14:457–60.
129. Kunz M, Ibrahim SM, Koczan D, et al. DNA microarray technology and its applications in dermatology. Exp Dermatol. 2004;13:593–606.
130. Clark EA, Golub TR, Lander ES, et al. Genomic analysis of metastasis reveals an essential role for RhoC. Nature. 2000;406:532–5.
131. Welss T, Papoutsaki M, Michel G, et al. Molecular basis of basal cell carcinoma: analysis of differential gene expression by differential display PCR and expression array. Int J Cancer. 2003;104:66–72.
132. Nindl I, Dang C, Forschner T, et al. Identification of differentially expressed genes in cutaneous squamous cell carcinoma by microarray expression profiling. Mol Cancer. 2006;5:30.
133. Li S, Ross DT, Kadin ME, et al. Comparative genome-scale analysis of gene expression profiles in T cell lymphoma cells during malignant progression using a complementary DNA microarray. Am J Pathol. 2001;158:1231–7.
134. Nomura I, Goleva E, Howell MD, et al. Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. J Immunol. 2003;171:3262–9.
135. Nomura I, Gao B, Boguniewicz M, et al. Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: a gene microarray analysis. J Allergy Clin Immunol. 2003;112:1195–202.
136. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA. 1977;74:5463–7.
137. Slatko BE, Albright LM, Tabor S, et al. DNA sequencing by the dideoxy method. Curr Protoc Mol Biol. 2001; Chapter 7:Unit 7.4A.
138. Atkinson MR, Deutscher MP, Kornberg A, et al. Enzymatic synthesis of deoxyribonucleic acid. XXXIV. Termination of chain growth by a 2',3'-dideoxyribonucleotide. Biochemistry. 1969;8:4897–904.
139. Shendure JA, Porreca GJ, Church GM. Overview of DNA sequencing strategies. Curr Protoc Mol Biol. 2008; Chapter 7: Unit 7.1.
140. Ansorge WJ. Next-generation DNA sequencing techniques. N Biotechnol. 2009;25:195–203.

141. Espina V, Wulfkuhle JD, Calvert VS, et al. Laser-capture microdissection. Nat Protoc. 2006;1:586–603.
142. Emmert-Buck MR, Bonner RF, Smith PD, et al. Laser capture microdissection. Science. 1996;274:998–1001.
143. Rekhter MD, Chen J. Molecular analysis of complex tissues is facilitated by laser capture microdissection: critical role of upstream tissue processing. Cell Biochem Biophys. 2001;35:103–13.
144. Yazdi AS, Puchta U, Flaig MJ, et al. Laser-capture microdissection: applications in routine molecular dermatopathology. J Cutan Pathol. 2004;31:465–70.
145. Agar NS, Halliday GM, Barnetson RS, et al. A novel technique for the examination of skin biopsies by laser capture microdissection. J Cutan Pathol. 2003;30:265–70.
146. Gallardo F, Pujol RM, Bellosillo B, et al. Primary cutaneous B-cell lymphoma (marginal zone) with prominent T-cell component and aberrant dual (T and B) genotype; diagnostic usefulness of laser-capture microdissection. Br J Dermatol. 2006;154:162–6.
147. Ke MS, Kamath NV, Nihal M, et al. Folliculotropic mycosis fungoides with central nervous system involvement: demonstration of tumor clonality in intrafollicular T cells using laser capture microdissection. J Am Acad Dermatol. 2003;48:238–43.
148. Agar NS, Halliday GM, Barnetson RS, et al. The basal layer in human squamous tumors harbors more UVA than UVB fingerprint mutations: a role for UVA in human skin carcinogenesis. Proc Natl Acad Sci USA. 2004;101:4954–9.
149. Bahrami S, Cheng L, Wang M, et al. Clonal relationships between epidermotropic metastatic melanomas and their primary lesions: a loss of heterozygosity and X-chromosome inactivation-based analysis. Mod Pathol. 2007;20:821–7.
150. Maitra A, Gazdar AF, Moore TO, et al. Loss of heterozygosity analysis of cutaneous melanoma and benign melanocytic nevi: laser capture microdissection demonstrates clonal genetic changes in acquired nevocellular nevi. Hum Pathol. 2002;33:191–7.

Chapter 4
Cytogenetics of Primary Skin Tumors

Melanie A. Carless and Lyn R. Griffiths

Skin tumors can arise as a result of cumulative genetic abnormalities, including chromosomal aberrations that can be described as either morphological (structural rearrangements) or molecular (copy number variations). Cytogenetic techniques have been used to examine both large and small chromosomal aberrations, and include karyotyping, comparative genomic hybridization, and fluorescence in situ hybridization. This chapter describes the recurrent aberrations associated with skin tumors, such as benign melanocytic nevi, melanoma, basal cell carcinoma, squamous cell carcinoma, actinic (solar) keratosis, Bowen's disease, keratoacanthoma, Merkel cell carcinoma, dermatofibrosarcoma protuberans, and cutaneous lymphomas, as detected by cytogenetic methodologies. A significant number of genomic aberrations are shared across different subtypes of skin tumors, including structural and numerical alterations of chromosome 1, −3p, +3q, +6, +7, +8q, −9p, +9q, −10, −17p, +17q and +20. Aberrations specific to certain skin cancers have also been detected, and include: loss of 18q in squamous cell carcinoma, but not its precursor, actinic keratosis; loss of 9q22 in sporadic basal cell carcinoma; and translocation involving 17q22 and 22q13 in dermatofibrosarcoma protuberans. These regions contain a number of potential candidate genes that are involved in aspects of cell signaling, proliferation, differentiation, and apoptosis. Cytogenetic methodologies continue to evolve with the advent of array-based comparative genomic hybridization, copy number variation microarrays, and next-generation sequencing. It is envisioned that cytogenetic analysis will continue to be employed for identification and further exploration of novel chromosomal regions and associated genes that drive skin tumorigenesis.

Introduction

Since the late 1950s, cytogenetic techniques have been employed to investigate chromosomal aberrations contributing to human disease, including those associated with solid and hematological tumors [1]. Historically, it has been considered the gold standard for prenatal diagnosis of chromosomal abnormalities, as well as for the diagnosis and prognostication of a number of human cancers, including chronic myelogenous leukemia (CML) [2], myelodysplastic syndrome [3], and Ewing sarcoma [4]. Cytogenetic analyses of premalignant and malignant skin lesions have identified a number of aberrant regions that appear to contribute to disease development and progression.

L.R. Griffiths (✉)
Genomics Research Centre, GIHMR, School of Medical Science, Griffith University-Gold Coast, PMB 50 Gold Coast Mail Centre, Bundall, QLD 9726, Australia
e-mail: L.Griffiths@griffith.edu.au

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_4,

Technologies

Some of the earliest cytogenetic studies investigated large chromosomal rearrangements that were easily detectable by microscopy using chromosomal banding patterns (i.e., G-banding). The identification of the Philadelphia chromosome in 1960 [5], later defined as a translocation between chromosomes 9 and 22 [t(9;22)] [6], was the first chromosomal abnormality found to be consistently associated with a specific malignancy (CML). Since then, many translocations, duplications, and inversions have been identified in various malignancies utilizing banding techniques. While karyotypic testing of hematological malignancies is extremely successful in determining chromosomal abnormalities, the analysis of solid tumors has been much more problematic, due to the necessity for short- or long-term cell culture in the latter, including skin tumors. Specifically, use of melanoma and non-melanoma skin cancer (NMSC) cell cultures has identified preferential growth of contaminating stromal fibroblasts in NMSC [7] and subclone selection in melanoma [8]. Caution is therefore warranted when interpreting cytogenetic abnormalities that are detected in samples derived from cultured skin tumors.

Comparative genomic hybridization (CGH) is a fluorescent-based technique that alleviates some of the problems associated with cell culturing, as DNA derived directly from a tumor sample can be used as a template to globally screen for gross (>20 Mb) copy number aberrations [9]. Whole-genome amplified and differentially labeled tumor and reference DNA are co-hybridized to normal metaphase spreads, such that imbalances are detected by changes in fluorescence values of the tumor DNA relative to the reference DNA [9, 10]. The main drawback of CGH analysis is that it detects only relatively large numerical or unbalanced alterations. However, it does not require the interpretation of complex tumor karyotypes or prior knowledge of aberrations for probe design, and can therefore easily detect previously unknown DNA copy number variation (CNV) [9, 10].

Fluorescence in situ hybridization (FISH) detects copy number changes, translocations, and inversions, that might otherwise be difficult to detect using standard karyotypic analysis [10–12]. The resolution of FISH is much higher than that of CGH [10–12]. FISH is applicable to both interphase and metaphase chromosomal analysis, but requires the design of sequence-specific probes that can be large (up to a whole chromosome) or small (as low as 1–200 kb), and therefore prior knowledge of the aberrant region is required [10–12].

Loss of heterozygosity (LOH) analysis, although not a cytogenetic technique per se, is often used to confirm and better resolve regions associated with loss of genetic material detected by other methodologies. Highly polymorphic markers or specific genes are used to determine the presence of genetic material in both normal and tumor DNA from an individual, with allelic loss in the tumor tissue correlating with a deleted chromosomal region [13].

More recently, detection of copy number and structural aberrations has reached a new standard with the introduction of array-CGH [14], CNV-microarrays [15], and whole-genome sequencing [16]. In conjunction with other molecular techniques (outlined in Chap. 3), cytogenetic analysis is a powerful means to identify genetic factors that drive tumorigenesis, and has been employed in the study of skin tumors. The determination of numerical and/or structural chromosomal aberrations, accompanied by further refinement of these regions, will facilitate the identification of putative oncogenes and tumor suppressor genes associated with cancer development and progression.

In addition, a number of online databases exist that summarize cytogenetic aberrations in human cancers (for a review see [17]), including the Mitelman Database of Chromosome Aberrations in Cancer [18] and the Atlas of Genetics and Cytogenetics in Oncology and Hematology [19].

Primary Skin Cancers

Cancer of the skin describes the uncontrolled growth of cutaneous cells, arising from an accumulation of inherited and/or sporadic genetic abnormalities, and with the potential for metastatic spread to other organ systems. Classified into two broad categories, melanoma and NMSC, skin cancers are some of the most common human tumors and their incidence is rapidly increasing. NMSC include basal cell carcinoma (BCC), squamous cell carcinoma (SCC), actinic keratosis (AK), SCC in situ, and keratoacanthoma (KA) [20]. Other primary skin cancers include Merkel cell carcinoma (MCC), dermatofibrosarcoma protuberans (DFSP), and cutaneous lymphomas [20, 21]. Extensive cytogenetic analysis of many of these tumors has been conducted, and reveals a number of recurrent aberrations that are likely to be associated with the development or progression of skin cancer.

Melanocytic Skin Tumors

Cutaneous melanoma is the most deadly form of skin cancer [22]. In the USA, it was estimated that in 2009, 68,720 individuals would be diagnosed with melanoma (~22.6 per 100,000 individuals), and that 8,650 individuals would die from this disease (~2.8 per 100,000 individuals) [22].

Summarized information from the Atlas of Genetics and Cytogenetics in Oncology and Hematology indicates that the most common karyotypic aberrations associated with melanoma are: deletions and translocations involving chromosomes 1 and 6q; gain of 6p, which may play a role in cancer progression; and gain of chromosome 7, which is associated with late stages of the disease [19]. Höglund et al. [23] authored a comprehensive review of genetic changes in melanoma, using previously published data obtained from the Mitelman Database of Chromosome Aberrations in Cancer [18]. They noted that the most common aberrations detected were −10 (59%), −6q10-q27 (42%), −9p10-p24, −21 (37%), +7, −16 (36%), −14, +1q24-q44, −4, −15 (33%), −5 (32%), −1p10-p36, −11q23-q25 (28%), −12q13-q24, +20 (27%), −17p, +18 (26%), −8p10-p23, +8q10-q24 (25%), −3 (24%), −22, −X (23%), +6p21-p25, −18 (22%), +3 (18%), −19 (17%), +9q22-q34 (15%), +19 (14%), +13, +17q10-q25 (12%), +2, +15, +21 and +22 (11%) [23]. CGH analysis has also identified a number of chromosomal regions with recurrent aberrations in melanoma. Bastian et al. [24] studied 132 melanomas and identified recurrent gain of 6p (37%), 1q (33%), 7p, 7q (32%), 8q (25%), 17q (24%), and 20q (22%), and recurrent loss of 9p (64%), 9q, 10q (36%), 10p (30%), 6q (26%) and 11q (21%). Genomic imbalance in melanoma is common, with chromosomal gain and loss being reported on all chromosomes [18]. In addition to these aberrations, which result in an imbalance of genetic material, balanced translocations involving regions on 1q, 6q, 14q and 19p have also been identified, although in a smaller percentage of cases [18].

It has been hypothesized that there are key cytogenetic events that drive development and progression of human melanocytic tumors. Karyotypic investigations of benign melanocytic nevi (BMN) have identified translocations as a principal cytogenetic event. Richmond et al. [25] identified single occurrences of reciprocal translocations, involving t(6;15), t(10;15), t(15;20), and t(4;5), in an investigation of eight BMN. In addition, three BMN from a single patient with a family history of melanoma were all found to have simple translocations; one of which included a 6q13 breakpoint [26], a region also implicated (deleted) in melanoma [18]. Bastian et al. [24] performed CGH analysis on 54 BMN and found that only 13% exhibited CNV. Of the seven BMN that exhibited aberrations, six of these showed a gain of 11p (all Spitz nevi) [24]. This aberration was not found in any of the melanomas studied [24]. Early chromosomal instability in BMN could define lesions that have a higher potential for oncogenic transformation, although these chromosomal aberrations

may not necessarily persist during such transformation. Recurrent alterations, predominantly deletions, have been identified on chromosome 9 in both dysplastic melanocytic nevi and metastatic melanoma lesions [19, 27], suggesting that this may be a primary event in melanocytic transformation. Höglund et al. [23] suggested two major karyotypic pathways as early cytogenetic changes in melanoma: (1) one involving +6p, −6q, and possibly −16; and (2) a second involving −3, and either +8q or −8p. A CGH study investigating 16 primary and 12 metastatic melanomas identified gains of 5p, 5q21-q23, 10p, and 18q, as well as losses of 2p21-pter, 11q13-q23, 12q24.1-qter, 19q13.1-qter, and 22qter, in the metastatic lesions, but not in primary tumors [28]. Also, losses involving chromosomes 9p and 17 occurred at a higher frequency in metastatic tumors [28]. In cases where the primary and metastatic lesions ($n = 4$) were excised from the same patient, metastases were associated with the acquisition of additional aberrations, although none of these were determined to be recurrent [28].

FISH and LOH analyses have been used to confirm and further investigate aberrant genomic regions in melanoma, that were previously identified by karyotypic and CGH analysis. Studies have identified: (1) extra copies (89%) and translocations (25%) of chromosome 20 (whole chromosome painting) [29]; (2) extra copies of c-myc (8q24.21) in nodular (61%) and superficial spreading (27%) melanomas [30]; (3) copy number gains of 7 (40.9%), 6, 17 (27%), 9, and 10 (23%), and monosomies of 10 (55%), 9 (37%), 6 (27%), 17 (23%), 1, and 7 (18%) [31]; and (4) polysomy of chromosome 7 (67%), associated with amplification of *EGFR* (epidermal growth factor receptor), in addition to common alterations of +6, +8, −9 and −10 [32]. A higher frequency of LOH on 1p and 9p is found in melanoma (29% and 50% at the most frequently lost loci) compared to dysplastic nevi (12% and 27% at corresponding loci) and BMN (no LOH) [33]. Uribe et al. [34] found loss of 9p21, 17q21, 6q23, and 5q35 more frequently in melanoma (68%), compared to atypical nevi (57%) and BMN (27%). In another study of 13 cases of early-stage melanoma, LOH was detected for at least one locus at 9p22 (31%), 10q11 (31%), and 1p36 (15%) [35]. Udart et al. [36] determined that metastatic melanomas have higher rates of +7 (25%) compared to primary tumors (8%). However, a higher prevalence of additional copies of *CCND1* (11q13) is found in primary (47%) versus metastatic (35%) lesions [37]. Although there is not a clear model to describe the influence of CNV on the development and progression of melanoma, studies to date suggest that a number of chromosomal regions may play a role in the early formation of cancerous cells (9p), as well as a metastatic phenotype (7, 11q). Figure 4.1 summarizes, using Circos [38], the recurrent aberrations detected in melanoma samples by karyotypic, CGH, FISH, and LOH techniques. The application of molecular technologies in the diagnosis, staging, prognostication and pharmacogenetic/pharmacogenomic profiling of melanoma is discussed in Chaps. 5, 6, 9, and 21.

Keratinocytic Skin Tumors

The American Cancer Society estimates that more than one million cases of NMSC arise each year (~300 cases per 100,000 individuals) [39]. BCC accounts for ~80% of all skin cancers, with a lifetime risk of about 28–30% [40]. BCC is locally invasive and destructive, but has a low metastatic potential of 0.0028–0.55% [41]. SCC accounts for almost 20% of all skin cancers, with a lifetime risk of 7–11% [42, 43]. Compared with BCC, it has a much higher and more variable metastatic potential of 3.6–30%, depending on the site and etiology of the lesion [44]. It has been suggested that all SCC are derived from precursor lesions (i.e., AK), although only 0.1–10% of AK lesions are known to progress to SCC [45–47]. AK has a prevalence ranging from 11% to as high as 80% in different populations and age groups [45, 48]. SCC in situ progresses to invasive SCC in only a small number of cases (2–5%), and demonstrates a low metastatic potential [49, 50]. The incidence of SCC in situ can vary from 14.9 to 142 per 100,000 individuals, depending on the ethnic population studied [51]. Keratoacanthoma (KA) has been classified as either a distinct lesion or a subtype of SCC, and its

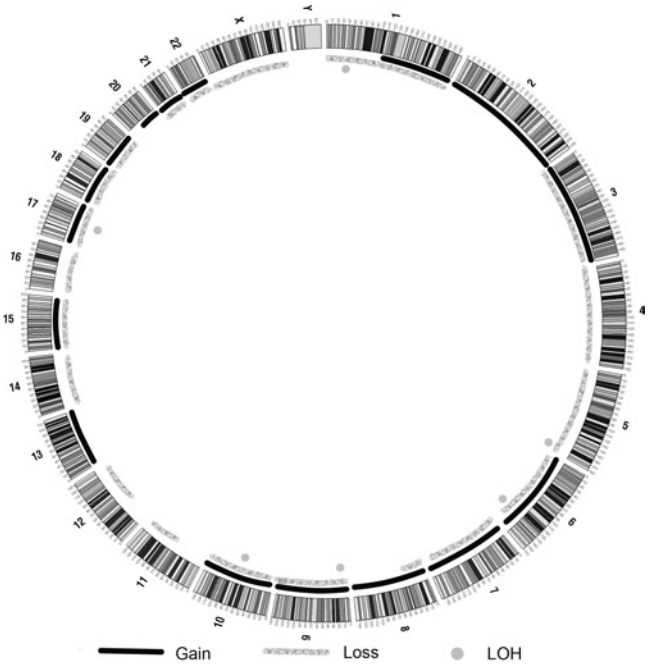

Fig. 4.1 Summary of recurrent chromosomal aberrations in melanoma (Figure generated with the use of Circos [38])

reported incidence varies from 7 to 104 per 100,000 individuals [52]. KA lesions are commonly solitary, demonstrate phases of growth, maturation and spontaneous regression, and rarely metastasize [44]. Given the differing metastatic capabilities of NMSC lesions, the degree of chromosomal instability among these tumors would also be expected to vary.

Jin et al. [53, 54] investigated 69 new and previously published short-term (5–10 days) BCC cultures, and identified recurrent numerical aberrations of +18 (30%), +7, +X (17%) and +9 (14%), structural rearrangements involving 9q (24%), and breakpoints involving 1p32, 1p22, 1q11, 1q21, 4q21, and 4q31 (10%). Other studies have also found structural abnormalities in BCC, such as translocations and inversions involving 9q [7, 55, 56]. Casalone et al. [57] studied 73 BCC samples, harvested within 24 h, as well as short-term cultures (10–28 days). Trisomy of chromosome 6 was found to be the most recurrent change, but only observed in a small number of the samples harvested within 24 h [57]. FISH analysis detected this variation in additional samples, but not in any of the short-term cultures, suggesting that the use of such cultures may lead to erroneous data [57]. CGH analysis of 15 BCC samples identified recurrent CNV, such as gain of 6p (47%), 6q, 9p (20%), 7 and X (13%), as well as loss of 9q (33%) [58]. Follow-up LOH analysis determined loss of 9q22.3 in 53% of cases [58]. Other studies have found LOH at 9q22 (46–60%), 9p21-p22 (55%), 17q21 (34%), 1q (14%), and 17p13 (11%) in BCC [59–62]. In summary, results obtained from karyotypic, CGH, FISH, and LOH analyses indicate that BCC karyotypes typically consist of only one to three aberrations [53, 54], with trisomy 6 [57, 58] and loss of the 9q region [58–61] being the primary numerical changes found. Overall, cytogenetic analyses indicate that BCC is genetically relatively stable compared to other tumors, which may be reflected by its low metastatic capability. Figure 4.2 summarizes recurrent aberrations (>10%) detected in BCC samples by karyotypic, CGH, FISH, and LOH techniques.

A number of studies have investigated if molecular technologies can be employed to distinguish between aggressive and nonaggressive BCC [63–68]. Ansarin et al. [63] reported that elevated p53 protein expression (nuclear staining in >50% of tumor cells) could be used to predict aggressive

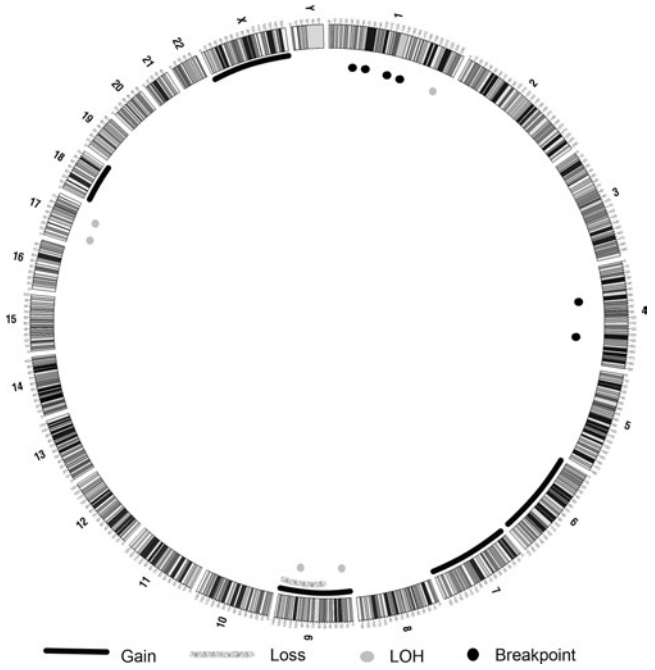

Fig. 4.2 Summary of recurrent chromosomal aberrations in basal cell carcinoma (Figure generated with the use of Circos [38])

behavior in BCC. However, Bolshakov et al. [64] determined that differences in *p53* gene mutation frequency, types of mutations, and hot spots, although present between aggressive and nonaggressive BCC, did not definitively predict tumor behavior. A recent gene expression profiling study by Yu et al. [65] reported that nodular and superficial BCC subtypes demonstrate similar transcriptional profiles, in contrast to morpheiform BCC which shows more diverse gene expression patterns (i.e., upregulation of DNA-damage response transcripts), consistent with its more invasive phenotype. However, Howell et al. [66] suggested that microscopic BCC subtypes (i.e., nodular and sclerosing) are not distinguishable by their gene expression patterns. A FISH-based study by Nangia et al. [67] reported that detection of trisomy 6 in tumor sections identifies subsets of BCC with aggressive and/or metastatic potential. Finally, Fernandes et al. [68] suggested that LOH of *PTCH1* (9q22), in addition to the presence of distinct cytokine gene polymorphisms (TNF-α, -308 SNP and IL-10, -1082 SNP), may be potential markers of aggressive behavior in BCC. Other applications of molecular technologies in BCC are discussed in Chaps. 7, 9, and 21.

Jin et al. [69] also identified recurrent numerical aberrations in short-term cultures from 13 primary cutaneous SCC and 10 previously published cases. These included gains of 7p (32%) and 8q (27%), and losses of 21 (41%), 8p (36%), 4p, 11p, Y (32%), 13, 18q (27%), 10p, X (23%), and 9p (18%) [69]. Isochromosomes (defined as a chromosome that has lost one of its arms and replaced it with an exact copy of the other arm), such as i(1p), i(1q), i(5p), i(8q), i(9p), and i(9q) (all <20%), were also represented in these cases, with i(8q) and i(9q) believed to be early genetic events [69]. By examining direct preparations of three primary cutaneous SCC, Casalone et al. [57] identified aberrations not detected in short-term cultures, including −1, +6, +8, +9, +11, −14, +16, and +21, although due to a limited number of samples, these could not be considered recurrent. Using CGH, gains of 3q (47%), 17q (40%), 14q, Xq (33%), 4p, 8q (27%), 1q, 5p, 7q, 9q, 10q, and 20q (20%), and losses of 3p (53%), 18q (47%), 17p (33%), 4q (27%), 5q, 8p, 11p, 13q, and 18p (20%) were identified in a study of 14 SCC, including five arising from adjacent AK [70]. Although many of the CNVs were shared between SCC and AK, the loss of 18q was specific to SCC ($P = 0.04$) and may be associated with

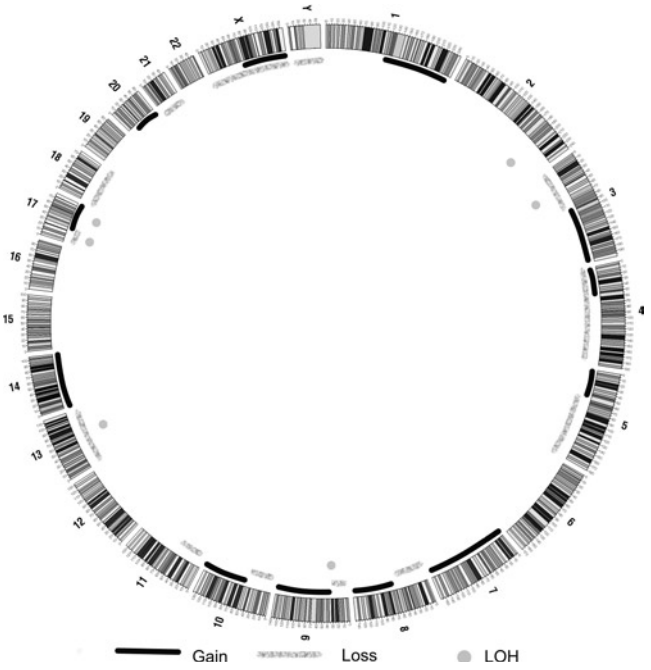

Fig. 4.3 Summary of recurrent chromosomal aberrations in squamous cell carcinoma (Figure generated with the use of Circos [38])

malignant progression [70]. Another study investigated five SCC cell lines and identified recurrent gains on 11q (100%), 7p (60%), and 8q (60%), and recurrent losses on 3 (80%), 9p (80%), and 8p (60%) [71]. LOH analysis of SCC (including in situ lesions) has revealed frequent loss of 17q (43%), 13q (38%), 17p (34%), 9p (32%), 3p (26%), and 2q (20%) [59]. Recurrent aberrations associated with SCC (>10%), as detected by karyotypic, CGH and LOH analysis, can be seen in Fig. 4.3.

Very few cytogenetic studies have been performed on other keratinocyte-derived skin tumors, but do indicate some shared cytogenetic features between premalignant and malignant lesions. These include: (1) gains of chromosome 7 and 20, and structural rearrangements involving chromosomes 1 and 4, identified by short-term cultures of three AK and two SCC in situ lesions [72]; (2) gain of 3q, 4p, 17q (33%), 5p, 9q, and 17p (25%), and loss of 9p, 13q (53%), 3p, 4q, 11p, and 17p (25%), identified by CGH analysis of AK [70]; (3) a similar, but higher frequency of LOH in AK compared to SCC, including loss at 17p (64%), 13q (52%), 17q (46%), 9p (39%), 9q (22%), and 3p (31%) [73]; and (4) recurrent loss of *TP53* (27%) in SCC in situ [74]. LOH of the region encoding *CDKN2A* in both SCC (46%) and AK (21%) [75], in addition to rearrangements involving 3p13 in AK and SCC [72], indicate that these aberrations might be early genetic events that contribute to malignant potential. Furthermore, CGH analysis has detected gain of 7p in metastatic SCC lesions, suggesting *EGFR* as a candidate gene conferring metastatic potential [76]. Cytogenetic abnormalities associated with KA include: (1) alteration of 2p13, identified by karyotypic analysis in two cases [77, 78]; (2) gains of 8q (20%), 1p, and 9q (16%), and losses of 3p, 9p, 19p (20%), and 19q (16%), detected by CGH analysis of lesions mostly derived from immunosuppressed organ transplant recipients [79]; and (3) rare LOH, with isolated cases of loss detected at 9p, 9q, and 10q [80]. Clausen et al. [81] detected significant differences in the frequency of copy number aberrations between SCC and KA, including more frequent gain of 1p, 14q, 16q, and 20q, and loss of 4p, in SCC ($P \leq 0.03$), as well as more frequent loss of 9p in KA ($P = 0.04$), providing further evidence to the theory that SCC and KA are distinct forms of NMSC. Overall, cytogenetic studies suggest that both AK and SCC are more genetically unstable than BCC and KA.

Rare Cancers of the Skin

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine tumor of the skin, affecting about 1,000 individuals in the USA each year (~3.3 per 100,000) [82, 83]. Karyotyping of six MCC cases revealed various structural and numerical rearrangements of chromosome 1, in addition to −13 (67%), +11, and −22 (33%) [84]. CGH analysis has identified recurrent numerical aberrations in MCC, including +19q (63%), +19p (50%), +1p (54%), −3p (46%), +1q, +X (42%), +5p, +8q (38%), −10, +3q, −13q (33%), +20p (29%), +7p, −17p, +20q (25%), −5q, +6q, +7q, −8p, +13q, +18q (21%), −11q, and +21 (17%) [83]. In addition, the average number of imbalances was noted to be different in patients surviving >24 months (6.6) compared with those surviving <24 months (11.2) [83]. In another study, CGH analysis of 19 cases revealed many of the same recurrent aberrations, including +6 (42%), +1q11-q31, +5p (32%), +1q32-qter (26%), +1p33-pter, +12, −13q13-q31 (21%), and −4q (16%) [85]. FISH analysis of 10 cutaneous MCC lesions has also confirmed +6 in 60% of cases [86]. Two independent LOH studies have identified deletion of 10q23 (43% of cases), although this is unlikely to involve *PTEN* [87], in addition to deletion of 1p35-p36 (70% of cases) [88].

Dermatofibrosarcoma protuberans (DFSP) is a rare cutaneous mesenchymal tumor which arises from spindle cells within the dermis [89, 90]. The tumor slowly infiltrates adjacent and deeper subcutaneous tissues, and shows a low propensity for metastases [89, 90]. The annual incidence of DFSP is estimated at 4.2 per million people in the USA [89]. The tumor is associated with a signature reciprocal translocation involving chromosomes 17 and 22 [t(17;22)(q22;q13)], with fusion of the *COL1A1* and *PDGFB* genes [90–92]. This translocation involves the formation of supernumerary ring chromosomes in about 70% of cases [90–92]. CGH analyses of DFSP have revealed consistent recurrent aberrations, including: 11 cases showing +17q21-qter (100%), +22pter-q13 (82%), and +5 (27%) [93]; and 12 cases showing aberrations of +17q22-qter (83%), +22q13 (75%), and +8q24.1-qter (25%) [94]. The application of molecular technologies in the diagnosis of DFSP is discussed in Chaps. 8 and 9.

Cutaneous lymphomas, including cutaneous T-cell lymphoma (CTCL) and cutaneous B-cell lymphoma (CBCL) subtypes, affect approximately 3 in 1,000,000 people annually [21]. CTCL is the more common of the two subtypes, with about 1,500 new cases per year in the USA [95]. It includes mycosis fungoides (MF) and Sezary syndrome (SS) [96]. CBCL accounts for only 10–25% of cutaneous lymphomas [21], and includes follicle center lymphoma (FCL), marginal zone B-cell lymphoma, and large B-cell lymphoma subtypes [97]. Karyotypic testing of 18 SS lesions revealed structural aberrations of chromosomes 10, 17 (28%), 1p (22%), 6q, and 14q (17%) [98]. FISH analysis verified rearrangements of 1p, 17p (33%), and 10 (27%) [98]. CGH analysis of MF lesions has revealed recurrent numerical aberrations of 1p (38%), −17p (21%), +4/4q (18%), −10q/10, +18, −19 (15%), and +17q/17 (12%) [98]. A review of 166 SS samples revealed recurrent losses of chromosomes 1, 2, 6q, 9, 10, 13, 16, and 17, as well as structural rearrangement of 17q [99]. In another study, CGH analysis of three MF and four SS cases identified recurrent copy number changes of −10q23 (29%) and +17q11.2 (71%), suggesting that the duplication at 17q seen in both MF and SS may be an early clonal event [100]. LOH analysis has detected recurrent deletions at 9p (46%), 17p (42%), 10q, and 2p (14%) in 15 cases of SS, and at 9p (16%), 10q (12%), 1p, and 17p (10%) in 51 cases of MF [96]. Molecular testing of CTCL is discussed in Chaps. 10 and 11.

CGH analysis of CBCL subtypes has identified: (1) recurrent numerical alterations of +2q, +7q, +12, +13, −17p, +18, and −19 in nine primary cutaneous large B-cell lymphoma lesions; (2) a sole deletion of −17p (60%) in five secondary lesions; and (3) aberrations of +3q, +4, and +7q in four cases of primary cutaneous FCL [101]. In another study, FISH analysis of 27 cases of FCL identified recurrent translocations involving the immunoglobulin heavy chain gene *(IGH)* on chromosome 14 (52%) [102]. These were associated with *BCL2* [t(14;18)(q32;q31)] in 41% of cases and

BCL6 [t(3;14)(q27;q32)] in 7% of cases [102]. FISH analysis of 14 cases of primary cutaneous large B-cell lymphoma also showed translocations involving *IGH* (50%), associated with *MYC* [t(8;14)(q24;q32)] in 36% of cases and *BCL6* [t(3;14)(q27;q32)] in 14% of cases [103]. Translocations involving *MYC* and *BCL6*, independent of the *IGH* locus, were detected in an additional 7% and 21% of lesions, respectively [103]. Molecular testing of CBCL is discussed in Chap. 12.

Recent Advances in Cytogenetic Detection Methods

While cytogenetic methods have predominantly been employed as research tools for the genetic characterization of diseases, recent advances in these technologies has prompted interest in their use in the diagnostic setting. However, their routine clinical implementation has been hampered by high cost, prolonged turnaround time, and the expertise required for most cytogenetic detection methods. Nonetheless, efforts have been undertaken to increase their application by reducing time and cost, and increasing the convenience and automation of these methods.

Array-based CGH (aCGH) utilizes microarray chips dotted with thousands of positionally-defined specific probes, to which differentially labeled normal and tumor DNA are co-hybridized and scanned, using computerized fluorescence imaging and analysis systems [14]. This has allowed CGH resolution to vastly improve, from >5–20 Mb (for traditional CGH) to <100 kb, with some high-resolution tiling arrays (HR-CGH) now able to resolve to 50–200 bp [104]. In addition, single nucleotide polymorphism (SNP)-based CNV arrays can detect microscopic CNVs, polyploidy, mosaicisms, and uniparental disomy [105, 106]. A number of studies using aCGH and CNV-arrays have been performed on skin cancers. These techniques have confirmed and better resolved many of the genomic aberrations seen in previous traditional CGH and LOH studies, as well as identifying novel alterations. Next-generation sequencing has recently been implemented as a cytogenetic tool, in an effort to more easily detect structural abnormalities, such as translocations and inversions [16]. No studies to date have utilized this methodology to detect genomic aberrations in skin cancers.

aCGH analysis of five cell lines derived from different melanoma metastases in a single patient identified a primary cluster of genomic imbalances, including +3p, +7, −9p, −10p, −14q, −16q, −17p, and +17q [107]. Another study, investigating two formalin-fixed paraffin-embedded (FFPE) melanoma samples, confirmed known aberrations of +1q and −9p [108]. More recently, two large aCGH studies of melanoma samples (126 and 102 samples, 80 shared cases) were undertaken [109, 110]. In these reports, Curtin et al. [109, 110] aimed to investigate possible differences in chromosomal aberrations between tumors derived from different sites or associated with variable sun-induced damage. Amongst the different subtypes of melanoma (i.e., those with chronic sun-induced damage; those without chronic sun-induced damage; and at mucosal and acral sites), gains of 6p, 17q and 20q, as well as loss of 9p and 21q, are common aberrations [110]. Gain involving the *CCND1* locus (11q13) and regions of chromosome 22, and loss of 4q, are significantly more common in tumors associated with chronic sun-induced damage ($p = 0.001$, 0.004 and 0.004, respectively) [110], suggesting that these aberrations may arise as a result of long-term exposure to ultraviolet radiation. Copy number amplifications of 4q are shared between lesions, both with and without chronic sun-induced damage, and appear to involve the *KIT* oncogene (which is essential for melanocyte survival and development) [109]. CNV-array analysis of 76 melanoma cell lines detected: (1) frequent LOH at 6q, 9p, 9q, 10p, 10q, 11q, and 17p; (2) 174 homozygous deletions, primarily associated with *CDKN2A*, *PTEN*, *PTPRD*, and *HDAC4*; and (3) 197 focal amplifications, many of which were associated with *BRAF*, *CCND1*, *MDM2*, *MITF*, *NRAS*, and *PIK3CA* [105].

Ma et al. [111] detected loss of the *CSMD1* (8p23) tumor suppressor gene in SCC (29%) and BCC (17%) using aCGH. In an effort to support the applicability of aCGH in the clinical setting,

a study on archival FFPE SCC, SCC precursors, and normal skin has also been undertaken [112]. The most common aberration detected was loss of 10q-ter, containing the *INPP5A* (10q26.3) tumor suppressor gene, which was reported in both SCC in situ and SCC, but not in non-tumorous sun-damaged skin [112]. CNV-array analysis of 16 primary SCC and 2 metastatic SCC identified common LOH at 2q, 3p, 8p, 9p and 13, as well as gains at 3q and 8q [113]. Analysis of paired primary and metastatic SCC (to lymph nodes) from two of these patients suggested that the primary and metastatic lesions shared common genetic aberrations (although these were different for the two patients), and that the acquisition of additional genetic aberrations is associated with metastatic spread in SCC [113]. LOH at 9q21-q31 was detected in ~93% of BCC [114], suggesting a much higher rate of 9q loss than previously reported with other techniques [58–61]. In addition, loss of a novel region, 6q23-q27, was identified in 36% of BCC [114]. These studies support CNV-arrays as a powerful tool for the identification of novel cytogenetic aberrations.

aCGH has also been used to investigate MCC, DFSP, and MF lesions. In a study of 25 MCC samples, oligonucleotide aCGH was used to detect recurrent aberrations of +1, −3p, +3q, −4, +5p, −5q, +6, −7, −10, and −13 [115]. In addition, increasing numbers of genomic aberrations correlated with decreased patient survival ($p = 0.04$) [115]. Another aCGH study of ten cases of MCC detected recurrent aberrations, including +1q, +6p, +11, and −17p [116]. In the case of DFSP, aCGH analysis (using a pooled approach) identified recurrent copy number gains of +8q24.3, +17q21.33-qter, and +22cen-q13.1 [90]. Finally, oligonucleotide aCGH detected common chromosomal imbalances of −6q21.3, +7q33.3-q35, +8q24.21, −9p21.3, −9q31.2, +9q34-qter, −10p11.22, +10p14, −13q14.11, −16q23.2, −16q24, −17p13.1, and +17q21.1 in 41 skin biopsies from MF patients [117]. Interestingly, aberrations at 8q24.21, 9p21.3, and 10q26-qter correlated with overall patient survival ($p = 0.017$, 0.042, and 0.022, respectively) [117].

Implications of Cytogenetic Findings

The employment of cytogenetic methods, in concert with other molecular techniques (outlined in Chap. 3), has greatly increased our ability to identify putative genes or genetic regions associated with tumorigenesis. Genomic aberrations associated with skin tumors include: (1) those that are shared amongst a number of different lesions; and (2) those that are specific to a particular lesion, or even present at a distinct stage of skin cancer development. In addition, the degree of genomic instability appears to be a measure of the potential aggressiveness of a tumor.

A number of recurrent genomic aberrations are shared between different forms of skin cancer, including: rearrangements and numerical abnormalities of chromosome 1 (melanoma, BCC, SCC, AK, KA, MCC, DFSP, CTCL); −3p (SCC, AK, KA, MCC); +3q (SCC, AK, MCC, CBCL); trisomy of all or part of chromosome 6 (melanoma, BCC, SCC, MCC); trisomy of all or part of chromosome 7 (melanoma, BCC, SCC, AK, MCC, CBCL); +8q (melanoma, SCC, KA, MCC, DFSP); −9p (dysplastic nevi, melanoma, BCC, SCC, AK, KA, CTCL); +9q (melanoma, SCC, AK, KA); loss of part or all of chromosome 10 (melanoma, SCC, MCC, CTCL); −17p (melanoma, BCC, SCC, SCC in situ, AK, MCC, CTCL, CBCL); +17q (melanoma, SCC, AK, DFSP); and gain of all or part of chromosome 20 (melanoma, SCC, AK, MCC, DFSP). Within these regions, a number of potential tumor suppressor genes and oncogenes exist, including: (1) *FHIT* (3p14.2), which is involved in cell cycle control [118], and whose loss has been associated with MCC [83] and many other malignancies [118]; (2) *BCL6* (3q27), a breakpoint region implicated in cutaneous lymphomas [102, 103], that mediates transcriptional repression [119]; (3) *E2F3* (6p22), an essential component of *RB*- and *MYC*-mediated cell cycle progression pathways [120, 121], and whose overexpression is seen in retinoblastoma and bladder cancer [122]; (4) *CDK6* (7q21-q22), involved in *RB*-mediated cell cycle progression [123], and *NRCAM* (7q31.1-q31.2), both of which are overexpressed in melanoma cell

lines [124]; (5) *MYC* (8q24.12-q24.13), which promotes cell proliferation [121], and is known to further destabilize the genome [125, 126]; (6) *CDKN2A* (9p21), which encodes two major proteins p16(INK4A) and p14(ARF) that regulate the *RB* and *TP53* cell cycle progression pathways, respectively (for a review see reference [127]); (7) *PTEN* (10q23.31), which suppresses tumorigenicity by blocking cell cycle progression [128], and whose loss is seen in various human cancers [129]; (8) *TP53* (17p13.1), which is disrupted in about 50–60% of all human cancers [130, 131], and plays a role in multiple cellular functions, including regulation of cell cycle arrest, apoptosis, glycolysis, autophagy, oxidative stress, invasion, motility, angiogenesis, and differentiation (for a review see reference [132]); and (9) *E2F1* (20q11.2), an essential component of *RB*-mediated cell cycle progression and *MYC*-induced apoptosis [120, 121].

It is also evident that amplifications and deletions in some regions are detected at similar frequencies within the same types of skin lesions, including chromosomes 9q, 15, 18, 19, 21, and 22 in melanoma, and 4q and 9q in AK. Some of these changes may reflect background genomic instability rather than being directly associated with tumorigenesis. Additionally, it has also been shown that regions of amplification contain genes that are both over- and under expressed, indicating that chromosomal aberrations do not always reflect changes at the gene level [133].

Some aberrations are found to be more prevalent in specific skin cancers. For example, loss of 18q in SCC as compared to AK. 18q harbors potential candidate genes *SMAD2* (18q21) and *SMAD4* (18q21.1), that mediate TGF-beta signaling and cell growth regulation [134], and which have been implicated in malignant progression in other non-cutaneous tumors [135, 136]. Another example is loss of 9q22 in sporadic BCC. 9q22 contains the *PTCH1* (9q22.3) tumor suppressor gene, that is part of the hedgehog developmental signaling pathway, and which has been implicated in the pathogenesis of hereditary BCC [137]. In DFSP, translocation involving 17q22 and 22q13 creates a gene fusion between *COL1A1* (17q21.31-q22) and *PDGFB* (22q12.3-13.1). This results in aberrant expression of *PDGFB* [90], which promotes cellular proliferation, disorganized growth, and inhibition of apoptosis [138, 139]. Other studies have implicated aberrations in specific regions as early events in skin tumor development, such as loss of 9p (*CDKN2A* locus), which has been detected in dysplastic nevi, and primary and metastatic melanoma [19, 27, 28, 33–35], as well as in SCC and AK [75].

Cytogenetic analysis is a powerful means to detect aberrant chromosomal regions that may harbor tumor suppressor genes and oncogenes. Analyses of melanoma and NMSC have identified a number of consistently aberrant regions. These regions contain genes, which when altered have been implicated in cancer pathogenesis and progression pathways, including cell growth, signaling, differentiation, and apoptosis. In addition, some aberrant regions appear to be specific to only one type of skin tumor, possibly containing genes more relevant to the morphological characteristics and/or biologic behavior of that particular cancer. The implementation of newer cytogenetic techniques with higher resolutions of detection will continue to improve our understanding of the genetic basis of skin tumors.

References

1. Ford CE. Human cytogenetics: its present place and future possibilities. Am J Hum Genet. 1960;12:104–17.
2. Glassman AB. Cytogenetics, in situ hybridization and molecular approaches in the diagnosis of cancer. Ann Clin Lab Sci. 1998;28:324–30.
3. Haferlach C, Bacher U, Tiu R, et al. Myelodysplastic syndromes with del(5q): indications and strategies for cytogenetic testing. Cancer Genet Cytogenet. 2008;187:101–11.
4. Folpe AL, Goldblum JR, Rubin BP, et al. Morphologic and immunophenotypic diversity in Ewing family tumors: a study of 66 genetically confirmed cases. Am J Surg Pathol. 2005;29:1025–33.
5. Nowell P, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science. 1960;132:1497.
6. Rowley JD. Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature. 1973;243:290–3.

7. Mertens F, Heim S, Mandahl N, et al. Cytogenetic analysis of 33 basal cell carcinomas. Cancer Res. 1991;51:954–7.
8. Lotem M, Yehuda-Gafni O, Butnaryu E, et al. Cytogenetic analysis of melanoma cell lines: subclone selection in long-term melanoma cell cultures. Cancer Genet Cytogenet. 2003;142:87–91.
9. Kallioniemi OP, Kallioniemi A, Piper J, et al. Optimizing comparative genomic hybridization for analysis of DNA sequence copy number changes in solid tumors. Genes Chromosom Cancer. 1994;10:231–43.
10. Kallioniemi A, Visakorpi T, Karhu R, et al. Gene copy number analysis by fluorescence in situ hybridization and comparative genomic hybridization. Methods. 1996;9:113–21.
11. Thompson CT, Gray JW. Cytogenetic profiling using fluorescence in situ hybridization (FISH) and comparative genomic hybridization (CGH). J Cell Biochem Suppl. 1993;17G:139–43.
12. Varella-Garcia M. Molecular cytogenetics in solid tumors: laboratorial tool for diagnosis, prognosis, and therapy. Oncologist. 2003;8:45–58.
13. Happle R. Loss of heterozygosity in human skin. J Am Acad Dermatol. 1999;41:143–64.
14. Pinkel D, Segraves R, Sudar D, et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet. 1998;20:207–11.
15. Redon R, Ishikawa S, Fitch KR, et al. Global variation in copy number in the human genome. Nature. 2006;444:444–54.
16. Chen W, Kalscheuer V, Tzschach A, et al. Mapping translocation breakpoints by next-generation sequencing. Genome Res. 2008;18:1143–9.
17. Knutsen T, Gobu V, Knaus R, et al. The interactive online SKY/M-FISH & CGH database and the Entrez cancer chromosomes search database: linkage of chromosomal aberrations with the genome sequence. Genes Chromosom Cancer. 2005;44:52–64.
18. Mitelman F, Johansson B, Mertens F, editors. Mitelman database of chromosome aberrations in cancer. 2009. http://cgap.nci.nih.gov/Chromosomes/Mitelman. Accessed 25 Nov 2009.
19. Dorkeld F, Bernheim A, Dessen P, Huret JL. A database on cytogenetics in haematology and oncology. Nucleic Acids Res. 1999;27:353–354. http://atlasgeneticsoncology.org/. Accessed 27 Nov 2009.
20. Weinstock MA. Epidemiology of nonmelanoma skin cancer: clinical issues, definitions, and classification. J Invest Dermatol. 1994;102:4S–5S.
21. Burg G, Kempf W. Cutaneous B-cell lymphoma. eMedicine 2008. http://emedicine.medscape.com/article/1099540-overview. Accessed 12 Apr 2009.
22. American Cancer Society. Cancer facts and figures. Atlanta: American Cancer Society. http://www.cancer.org/downloads/STT /500809web.pdf (2009). Accessed 27 Nov 2009.
23. Hoglund M, Gisselsson D, Hansen GB, et al. Dissecting karyotypic patterns in malignant melanomas: temporal clustering of losses and gains in melanoma karyotypic evolution. Int J Cancer. 2004;108:57–65.
24. Bastian BC, Olshen AB, LeBoit PE, et al. Classifying melanocytic tumors based on DNA copy number changes. Am J Pathol. 2003;163:1765–70.
25. Richmond A, Fine R, Murray D, et al. Growth factor and cytogenetic abnormalities in cultured nevi and malignant melanomas. J Invest Dermatol. 1986;86:295–302.
26. Marras S, Faa G, Dettori T, et al. Chromosomal changes in dysplastic nevi. Cancer Genet Cytogenet. 1999;113:177–9.
27. Cowan JM, Francke U. Cytogenetic analysis in melanoma and nevi. Cancer Treat Res. 1991;54:3–16.
28. Balazs M, Adam Z, Treszl A, et al. Chromosomal imbalances in primary and metastatic melanomas revealed by comparative genomic hybridization. Cytometry. 2001;46:222–32.
29. Barks JH, Thompson FH, Taetle R, et al. Increased chromosome 20 copy number detected by fluorescence in situ hybridization (FISH) in malignant melanoma. Genes Chromosom Cancer. 1997;19:278–85.
30. Treszl A, Adany R, Rakosy Z, et al. Extra copies of c-myc are more pronounced in nodular melanomas than in superficial spreading melanomas as revealed by fluorescence in situ hybridisation. Cytom B Clin Cytom. 2004;60:37–46.
31. Matsuta M, Imamura Y, Matsuta M, et al. Detection of numerical chromosomal aberrations in malignant melanomas using fluorescence in situ hybridization. J Cutan Pathol. 1997;24:201–5.
32. Rakosy Z, Vizkeleti L, Ecsedi S, et al. EGFR gene copy number alterations in primary cutaneous malignant melanomas are associated with poor prognosis. Int J Cancer. 2007;121:1729–37.
33. Hussein MR, Roggero E, Tuthill RJ, et al. Identification of novel deletion Loci at 1p36 and 9p22-21 in melanocytic dysplastic nevi and cutaneous malignant melanomas. Arch Dermatol. 2003;139:816–7.
34. Uribe P, Wistuba II, Gonzalez S. Allelotyping, microsatellite instability, and BRAF mutation analyses in common and atypical melanocytic nevi and primary cutaneous melanomas. Am J Dermatopathol. 2009;31:354–63.
35. Hussein MR, Sun M, Roggero E, et al. Loss of heterozygosity, microsatellite instability, and mismatch repair protein alterations in the radial growth phase of cutaneous malignant melanomas. Mol Carcinog. 2002;34:35–44.

36. Udart M, Utikal J, Krahn GM, et al. Chromosome 7 aneusomy. A marker for metastatic melanoma? Expression of the epidermal growth factor receptor gene and chromosome 7 aneusomy in nevi, primary malignant melanomas and metastases. Neoplasia. 2001;3:245–54.
37. Utikal J, Udart M, Leiter U, et al. Additional Cyclin D(1) gene copies associated with chromosome 11 aberrations in cutaneous malignant melanoma. Int J Oncol. 2005;26:597–605.
38. Krzywinski M, Schein J, Birol I, et al. Circos: an information aesthetic for comparative genomics. Genome Res. 2009;19:1639–45.
39. American Cancer Society. Overview: skin cancer – basal and squamous cell. How many people get basal and squamous cell skin cancers? 2008. http://www.cancer.org/docroot/CRI/content/CRI_2_2_1X_How_many_people_get_nonmelanoma_skin_cancer_51.asp?sitearea=. Accessed 27 Nov 2009.
40. Miller DL, Weinstock MA. Nonmelanoma skin cancer in the United States: incidence. J Am Acad Dermatol. 1994;30:774–8.
41. Wong CS, Strange RC, Lear JT. Basal cell carcinoma. BMJ. 2003;327:794–8.
42. Bernstein SC, Lim KK, Brodland DG, et al. The many faces of squamous cell carcinoma. Dermatol Surg. 1996;22:243–54.
43. Diepgen TL, Mahler V. The epidemiology of skin cancer. Br J Dermatol. 2002;146 Suppl 61:1–6.
44. Skidmore Jr RA, Flowers FP. Nonmelanoma skin cancer. Med Clin North Am. 1998;82:1309–23. vi.
45. Salasche SJ. Epidemiology of actinic keratoses and squamous cell carcinoma. J Am Acad Dermatol. 2000;42:4–7.
46. Marks R, Rennie G, Selwood TS. Malignant transformation of solar keratoses to squamous cell carcinoma. Lancet. 1988;1:795–7.
47. Evans C, Cockerell CJ. Actinic keratosis: time to call a spade a spade. S Med J. 2000;93:734–6.
48. Frost C, Williams G, Green A. High incidence and regression rates of solar keratoses in a queensland community. J Invest Dermatol. 2000;115:273–7.
49. Ramrakha-Jones VS, Herd RM. Treating Bowen's disease: a cost-minimization study. Br J Dermatol. 2003;148:1167–72.
50. Cohen PR. Bowen's disease: squamous cell carcinoma in situ. Am Fam Physician. 1991;44:1325–9.
51. Reizner G, Chuang T, Elpern D, et al. Bowen's disease (squamous cell carcinoma *in situ*) in Kauai, Hawaii. A population-based incidence report. J Am Acad Dermatol. 1994;31:596–600.
52. Reizner GT, Chuang TY, Elpern DJ, et al. Keratoacanthoma in Japanese Hawaiians in Kauai, Hawaii. Int J Dermatol. 1995;34:851–3.
53. Jin Y, Mertens F, Persson B, et al. Nonrandom numerical chromosome abnormalities in basal cell carcinomas. Cancer Genet Cytogenet. 1998;103:35–42.
54. Jin Y, Martins C, Salemark L, et al. Nonrandom karyotypic features in basal cell carcinomas of the skin. Cancer Genet Cytogenet. 2001;131:109–19.
55. Jin Y, Merterns F, Persson B, et al. The reciprocal translocation t(9;16)(q22;p13) is a primary chromosome abnormality in basal cell carcinomas. Cancer Res. 1997;57:404–6.
56. Kawasaki-Oyama RS, Andre FS, Caldeira LF, et al. Cytogenetic findings in two basal cell carcinomas. Cancer Genet Cytogenet. 1994;73:152–6.
57. Casalone R, Mazzola D, Righi R, et al. Cytogenetic and interphase FISH analyses of 73 basal cell and three squamous cell carcinomas: different findings in direct preparations and short-term cell cultures. Cancer Genet Cytogenet. 2000;118:136–43.
58. Ashton KJ, Weinstein SR, Maguire DJ, et al. Molecular cytogenetic analysis of basal cell carcinoma DNA using comparative genomic hybridization. J Invest Dermatol. 2001;117:683–6.
59. Quinn AG, Sikkink S, Rees JL. Basal cell carcinomas and squamous cell carcinomas of human skin show distinct patterns of chromosome loss. Cancer Res. 1994;54:4756–9.
60. Shen T, Park WS, Boni R, et al. Detection of loss of heterozygosity on chromosome 9q22.3 in microdissected sporadic basal cell carcinoma. Hum Pathol. 1999;30:284–7.
61. Shanley SM, Dawkins H, Wainwright BJ, et al. Fine deletion mapping on the long arm of chromosome 9 in sporadic and familial basal cell carcinomas. Hum Mol Genet. 1995;4:129–33.
62. Saridaki Z, Koumantaki E, Liloglou T, et al. High frequency of loss of heterozygosity on chromosome region 9p21-p22 but lack of p16INK4a/p19ARF mutations in Greek patients with basal cell carcinoma of the skin. J Invest Dermatol. 2000;115:719–25.
63. Ansarin H, Daliri M, Soltani-Arabshahi R. Expression of p53 in aggressive and non-aggressive histologic variants of basal cell carcinoma. Eur J Dermatol. 2006;16:543–7.
64. Bolshakov S, Walker CM, Strom SS, et al. p53 mutations in human aggressive and nonaggressive basal and squamous cell carcinomas. Clin Cancer Res. 2003;9:228–34.
65. Yu M, Zloty D, Cowan B, et al. Superficial, nodular, and morpheiform basal-cell carcinomas exhibit distinct gene expression profiles. J Invest Dermatol. 2008;128:1797–805.

66. Howell BG, Solish N, Lu C, et al. Microarray profiles of human basal cell carcinoma: insights into tumor growth and behavior. J Dermatol Sci. 2005;39:39–51.
67. Nangia R, Sait SN, Block AW, et al. Trisomy 6 in basal cell carcinomas correlates with metastatic potential: a dual color fluorescence in situ hybridization study on paraffin sections. Cancer. 2001;91:1927–32.
68. Fernandes H, Fernandes N, Bhattacharya S, et al. Molecular signatures linked with aggressive behavior in basal cell carcinoma: a report of 6 cases. Am J Dermatopathol. 2010;32(6):550–6.
69. Jin Y, Martins C, Jin C, et al. Nonrandom karyotypic features in squamous cell carcinomas of the skin. Genes Chromosom Cancer. 1999;26:295–303.
70. Ashton KJ, Weinstein SR, Maguire DJ, et al. Chromosomal aberrations in squamous cell carcinoma and solar keratoses revealed by comparative genomic hybridization. Arch Dermatol. 2003;139:876–82.
71. Popp S, Waltering S, Herbst C, et al. UV-B-type mutations and chromosomal imbalances indicate common pathways for the development of Merkel and skin squamous cell carcinomas. Int J Cancer. 2002;99:352–60.
72. Jin Y, Jin C, Salemark L, et al. Clonal chromosome abnormalities in premalignant lesions of the skin. Cancer Genet Cytogenet. 2002;136:48–52.
73. Rehman I, Takata M, Wu YY, et al. Genetic change in actinic keratoses. Oncogene. 1996;12:2483–90.
74. Lee HJ, Kim JS, Ha SJ, et al. p53 gene mutations in Bowen's disease in Koreans: clustering in exon 5 and multiple mutations. Cancer Lett. 2000;158:27–33.
75. Mortier L, Marchetti P, Delaporte E, et al. Progression of actinic keratosis to squamous cell carcinoma of the skin correlates with deletion of the 9p21 region encoding the p16(INK4a) tumor suppressor. Cancer Lett. 2002;176:205–14.
76. Shimizu T, Izumi H, Oga A, et al. Epidermal growth factor receptor overexpression and genetic aberrations in metastatic squamous-cell carcinoma of the skin. Dermatology. 2001;202:203–6.
77. Kim DK, Kim JY, Kim HT, et al. A specific chromosome aberration in a keratoacanthoma. Cancer Genet Cytogenet. 2003;142:70–2.
78. Mertens F, Heim S, Mandahl N, et al. Clonal chromosome aberrations in a keratoacanthoma and a basal cell papilloma. Cancer Genet Cytogenet. 1989;39:227–32.
79. Clausen OP, Beigi M, Bolund L, et al. Keratoacanthomas frequently show chromosomal aberrations as assessed by comparative genomic hybridization. J Invest Dermatol. 2002;119:1367–72.
80. Waring AJ, Takata M, Rehman I, et al. Loss of heterozygosity analysis of keratoacanthoma reveals multiple differences from cutaneous squamous cell carcinoma. Br J Cancer. 1996;73:649–53.
81. Clausen OP, Aass HC, Beigi M, et al. Are keratoacanthomas variants of squamous cell carcinomas? A comparison of chromosomal aberrations by comparative genomic hybridization. J Invest Dermatol. 2006;126:2308–15.
82. Merkel cell carcinoma, information for patients and their physicians. What is Merkel cell carcinoma. 2009. http://www.merkelcell.org/aboutDisease/index.php. Accessed 30 Nov 2009.
83. Van Gele M, Speleman F, Vandesompele J, et al. Characteristic pattern of chromosomal gains and losses in Merkel cell carcinoma detected by comparative genomic hybridization. Cancer Res. 1998;58:1503–8.
84. Leonard JH, Leonard P, Kearsley JH. Chromosomes 1, 11, and 13 are frequently involved in karyotypic abnormalities in metastatic Merkel cell carcinoma. Cancer Genet Cytogenet. 1993;67:65–70.
85. Larramendy ML, Koljonen V, Bohling T, et al. Recurrent DNA copy number changes revealed by comparative genomic hybridization in primary Merkel cell carcinomas. Mod Pathol. 2004;17:561–7.
86. Vasuri F, Magrini E, Foschini MP, et al. Trisomy of chromosome 6 in Merkel cell carcinoma within lymph nodes. Virchows Arch. 2008;452:559–63.
87. Van Gele M, Leonard JH, Van Roy N, et al. Frequent allelic loss at 10q23 but low incidence of PTEN mutations in Merkel cell carcinoma. Int J Cancer. 2001;92:409–13.
88. Vortmeyer AO, Merino MJ, Boni R, et al. Genetic changes associated with primary Merkel cell carcinoma. Am J Clin Pathol. 1998;109:565–70.
89. Chen CJ, Siegel DM. Dermatofibrosarcoma protuberans. eMedicine 2009. http://emedicine.medscape.com/article/1100203-overview. Accessed 30 Nov 2009.
90. Kaur S, Vauhkonen H, Bohling T, et al. Gene copy number changes in dermatofibrosarcoma protuberans - a fine-resolution study using array comparative genomic hybridization. Cytogenet Genome Res. 2006;115:283–8.
91. Pedeutour F, Simon MP, Minoletti F, et al. Translocation, t(17;22)(q22;q13), in dermatofibrosarcoma protuberans: a new tumor-associated chromosome rearrangement. Cytogenet Cell Genet. 1996;72:171–4.
92. Pedeutour F, Coindre JM, Sozzi G, et al. Supernumerary ring chromosomes containing chromosome 17 sequences. A specific feature of dermatofibrosarcoma protuberans? Cancer Genet Cytogenet. 1994;76:1–9.
93. Kiuru-Kuhlefelt S, El-Rifai W, Fanburg-Smith J, et al. Concomitant DNA copy number amplification at 17q and 22q in dermatofibrosarcoma protuberans. Cytogenet Cell Genet. 2001;92:192–5.
94. Nishio J, Iwasaki H, Ohjimi Y, et al. Overrepresentation of 17q22-qter and 22q13 in dermatofibrosarcoma protuberans but not in dermatofibroma: a comparative genomic hybridization study. Cancer Genet Cytogenet. 2002;132:102–8.

95. The Leukemia and Lymphoma Society. Cutaneous T-cell lymphoma, vol 5. 2006. http://www.leukemia-lymphoma.org/attachments/National/br_ 1163608564.pdf. Accessed 12 Apr 2009.
96. Scarisbrick JJ, Woolford AJ, Russell-Jones R, et al. Allelotyping in mycosis fungoides and Sezary syndrome: common regions of allelic loss identified on 9p, 10q, and 17p. J Invest Dermatol. 2001;117:663–70.
97. Hoefnagel JJ, Dijkman R, Basso K, et al. Distinct types of primary cutaneous large B-cell lymphoma identified by gene expression profiling. Blood. 2005;105:3671–8.
98. Mao X, Lillington D, Scarisbrick JJ, et al. Molecular cytogenetic analysis of cutaneous T-cell lymphomas: identification of common genetic alterations in Sezary syndrome and mycosis fungoides. Br J Dermatol. 2002;147:464–75.
99. Mao X, Lillington DM, Czepulkowski B, et al. Molecular cytogenetic characterization of Sezary syndrome. Genes Chromosom Cancer. 2003;36:250–60.
100. Barba G, Matteucci C, Girolomoni G, et al. Comparative genomic hybridization identifies 17q11.2 approximately q12 duplication as an early event in cutaneous T-cell lymphomas. Cancer Genet Cytogenet. 2008;184:48–51.
101. Gimenez S, Costa C, Espinet B, et al. Comparative genomic hybridization analysis of cutaneous large B-cell lymphomas. Exp Dermatol. 2005;14:883–90.
102. Streubel B, Scheucher B, Valencak J, et al. Molecular cytogenetic evidence of t(14;18)(IGH;BCL2) in a substantial proportion of primary cutaneous follicle center lymphomas. Am J Surg Pathol. 2006;30:529–36.
103. Hallermann C, Kaune KM, Gesk S, et al. Molecular cytogenetic analysis of chromosomal breakpoints in the IGH, MYC, BCL6, and MALT1 gene loci in primary cutaneous B-cell lymphomas. J Invest Dermatol. 2004;123:213–9.
104. Urban AE, Korbel JO, Selzer R, et al. High-resolution mapping of DNA copy alterations in human chromosome 22 using high-density tiling oligonucleotide arrays. Proc Natl Acad Sci USA. 2006;103:4534–9.
105. Stark M, Hayward N. Genome-wide loss of heterozygosity and copy number analysis in melanoma using high-density single-nucleotide polymorphism arrays. Cancer Res. 2007;67:2632–42.
106. Gijsbers AC, Lew JY, Bosch CA, et al. A new diagnostic workflow for patients with mental retardation and/or multiple congenital abnormalities: test arrays first. Eur J Hum Genet. 2009;17:1394–402.
107. Sabatino M, Zhao Y, Voiculescu S, et al. Conservation of genetic alterations in recurrent melanoma supports the melanoma stem cell hypothesis. Cancer Res. 2008;68:122–31.
108. Harvell JD, Kohler S, Zhu S, et al. High-resolution array-based comparative genomic hybridization for distinguishing paraffin-embedded Spitz nevi and melanomas. Diagn Mol Pathol. 2004;13:22–5.
109. Curtin JA, Busam K, Pinkel D, et al. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol. 2006;24:4340–6.
110. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005;353:2135–47.
111. Ma C, Quesnelle KM, Sparano A, et al. Characterization CSMD1 in a large set of primary lung, head and neck, breast and skin cancer tissues. Cancer Biol Ther. 2009;8:907–16.
112. Hostetter G, Kim SY, Savage S, et al. Random DNA fragmentation allows detection of single-copy, single-exon alterations of copy number by oligonucleotide array CGH in clinical FFPE samples. Nucleic Acids Res. 2010;38:e9.
113. Purdie KJ, Lambert SR, Teh MT, et al. Allelic imbalances and microdeletions affecting the PTPRD gene in cutaneous squamous cell carcinomas detected using single nucleotide polymorphism microarray analysis. Genes Chromosom Cancer. 2007;46:661–9.
114. Teh MT, Blaydon D, Chaplin T, et al. Genomewide single nucleotide polymorphism microarray mapping in basal cell carcinomas unveils uniparental disomy as a key somatic event. Cancer Res. 2005;65:8597–603.
115. Paulson KG, Lemos BD, Feng B, et al. Array-CGH reveals recurrent genomic changes in Merkel cell carcinoma including amplification of L-Myc. J Invest Dermatol. 2009;129:1547–55.
116. Sastre-Garau X, Peter M, Avril MF, et al. Merkel cell carcinoma of the skin: pathological and molecular evidence for a causative role of MCV in oncogenesis. J Pathol. 2009;218:48–56.
117. Salgado R, Servitje O, Gallardo F, et al. Oligonucleotide Array-CGH Identifies Genomic Subgroups and Prognostic Markers for Tumor Stage Mycosis Fungoides. J Invest Dermatol. 2010;130:1126–35.
118. Pekarsky Y, Garrison PN, Palamarchuk A, et al. Fhit is a physiological target of the protein kinase Src. Proc Natl Acad Sci USA. 2004;101:3775–9.
119. Deweindt C, Albagli O, Bernardin F, et al. The LAZ3/BCL6 oncogene encodes a sequence-specific transcriptional inhibitor: a novel function for the BTB/POZ domain as an autonomous repressing domain. Cell Growth Differ. 1995;6:1495–503.
120. Nevins JR. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ. 1998;9:585–93.
121. Leone G, Sears R, Huang E, et al. Myc requires distinct E2F activities to induce S phase and apoptosis. Mol Cell. 2001;8:105–13.
122. Santos GC, Zielenska M, Prasad M, et al. Chromosome 6p amplification and cancer progression. J Clin Pathol. 2007;60:1–7.

123. Meyerson M, Harlow E. Identification of G1 kinase activity for cdk6, a novel cyclin D partner. Mol Cell Biol. 1994;14:2077–86.
124. Okamoto I, Pirker C, Bilban M, et al. Seven novel and stable translocations associated with oncogenic gene expression in malignant melanoma. Neoplasia. 2005;7:303–11.
125. Felsher DW, Bishop JM. Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc Natl Acad Sci USA. 1999;96:3940–4.
126. Karlsson A, Deb-Basu D, Cherry A, et al. Defective double-strand DNA break repair and chromosomal translocations by MYC overexpression. Proc Natl Acad Sci USA. 2003;100:9974–9.
127. Sharpless NE. INK4a/ARF: a multifunctional tumor suppressor locus. Mutat Res. 2005;576:22–38.
128. Li DM, Sun H. PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. Proc Natl Acad Sci USA. 1998;95:15406–11.
129. Li J, Yen C, Liaw D, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997;275:1943–7.
130. Hickman ES, Moroni MC, Helin K. The role of p53 and pRB in apoptosis and cancer. Curr Opin Genet Dev. 2002;12:60–6.
131. Bourdon JC. p53 and its isoforms in cancer. Br J Cancer. 2007;97:277–82.
132. Vousden KH, Lane DP. p53 in health and disease. Nat Rev Mol Cell Biol. 2007;8:275–83.
133. Platzer P, Upender MB, Wilson K, et al. Silence of chromosomal amplifications in colon cancer. Cancer Res. 2002;62:1134–8.
134. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature. 1997;390:465–71.
135. Miyaki M, Kuroki T. Role of Smad4 (DPC4) inactivation in human cancer. Biochem Biophys Res Commun. 2003;306:799–804.
136. Tian F, DaCosta SB, Parks WT, et al. Reduction in Smad2/3 signaling enhances tumorigenesis but suppresses metastasis of breast cancer cell lines. Cancer Res. 2003;63:8284–92.
137. Bale AE, Yu KP. The hedgehog pathway and basal cell carcinomas. Hum Mol Genet. 2001;10:757–62.
138. Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature. 1999;401:86–90.
139. Shimizu A, O'Brien KP, Sjoblom T, et al. The dermatofibrosarcoma protuberans-associated collagen type Ialpha1/platelet-derived growth factor (PDGF) B-chain fusion gene generates a transforming protein that is processed to functional PDGF-BB. Cancer Res. 1999;59:3719–23.

Chapter 5
Melanocytic Neoplasms I: Molecular Diagnosis

Pedram Gerami, Bryan Gammon, and Michael J. Murphy

There are many possible indications and potential uses for molecular diagnostic techniques in the evaluation and management of melanocytic neoplasms. These include: (a) the identification of better diagnostic, staging, and prognostic markers; (b) the discovery of novel therapeutic targets; (c) the development of a molecular classification scheme, with the potential to stratify melanomas into subtypes which have similar pathogenesis, prognosis, and treatment responses; (d) the determination of patterns of genetic deletions and/or gains that are associated with different clinical outcomes in patients with metastatic melanoma; (e) an improvement in our ability to accurately classify melanocytic lesions that are currently considered morphologically ambiguous, such as atypical Spitz tumors; (f) the identification of individuals and populations at high-risk for melanoma development; and (g) the application of genetic testing to help identify candidates requiring more comprehensive clinical screening.

The incidence and mortality rates of melanoma have been increasing over the last number of decades. The American Cancer Society now estimates that the lifetime risk of developing melanoma is approximately 1 in 50 for Caucasians, 1 in 200 for Hispanics, and 1 in 1,000 for African-Americans [1]. Overall, it is the sixth most common cancer in men and seventh most common cancer in women. In 2008, 68,720 new cases of invasive melanoma and 8,650 deaths were reported in the USA [1]. Although the number of melanoma-related deaths continues to increase, and results of treatment for metastatic melanoma remain dismal, there have been several significant breakthroughs in the past decade. These advances are based on the recognition of discrete subsets of melanoma – each with distinct chromosomal aberrations, gene mutations, and oncogenic pathway activation [2]. There are several obvious benefits that may be realized from these discoveries. Firstly, melanomas can be classified into cohorts that may have similar clinical course and treatment responses. Secondly, if the pathogenic mechanisms within the subgroup are well understood, potential targeted therapies may be developed. Pharmacogenetic and pharmacogenomic strategies in melanoma are further discussed in Chap. 21. Importantly, the identification of key melanoma-associated somatic mutations is likely to play a significant role in the development of a molecular classification scheme for this tumor.

In this chapter, a number of clinically relevant applications for molecular strategies in the setting of melanocytic tumors will be described. Methodologies discussed include comparative genomic hybridization (CGH), fluorescence in situ hybridization (FISH), DNA microarray technologies, and epigenetic profiling tools. Karyotypic investigations of melanocytic tumors are discussed in Chap. 4.

P. Gerami (✉)
Department of Dermatology, Northwestern University Feinberg School of Medicine,
676 North St. Clair Street, Suite 1600, Chicago, IL 60611, USA
e-mail: Pedram.Gerami@nmff.org

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_5,

Common Somatic Mutations in Melanoma and Melanocytic Nevi

BRAF Mutations

The mitogen-activated protein kinase (MAPK) pathway is comprised of RAS (HRAS, KRAS and NRAS), RAF (ARAF, BRAF and CRAF), MEK-1/2 and ERK-1/2 members, which sequentially relay proliferative signals from cell surface receptors through a cytoplasmic signaling cascade into the nucleus (Fig. 5.1) [3]. This signaling pathway plays a critical role in cellular proliferation, differentiation, and survival. One of the most significant developments in our understanding of the molecular basis for melanocytic neoplasms was the discovery of frequent somatic BRAF mutations in melanoma (Table 5.1) [3]. In a seminal study, Davies et al. [3] identified mutations of this gene in 66% of melanomas. All activating mutations (over 65 types reported to date) represent acquired (somatic) events and are found within the kinase domain of BRAF. A single-base missense T to A substitution (at nucleotide 1799 in exon 15), which results in a change of valine to glutamic acid at amino acid 600 (V600E), is responsible for ~97% of the observed mutations. Subsequently, it was noted that the same $BRAF^{V600E}$ mutation was also present in a majority of melanocytic nevi (Table 5.2) [4]. In this regard, BRAF mutations may be seen in up to 87.5% of common acquired nevi, 52–62% of dysplastic nevi, and 0–12% of blue nevi [4]. Most investigators have reported that Spitz nevi do not contain BRAF mutations, although a small number of studies dispute these findings [4, 5]. Discordant results could be due to differences in patient selection and diagnostic histopathological criteria between studies [5]. The $BRAF^{V600E}$ mutation results in the production of a protein with serine/threonine kinase activity that is 10.7 times higher than its wild-type BRAF counterpart [3]. Constitutive activation of the MAPK pathway regulates key processes that are involved in melanoma biology, including cell proliferation, invasion, angiogenesis, and metastasis.

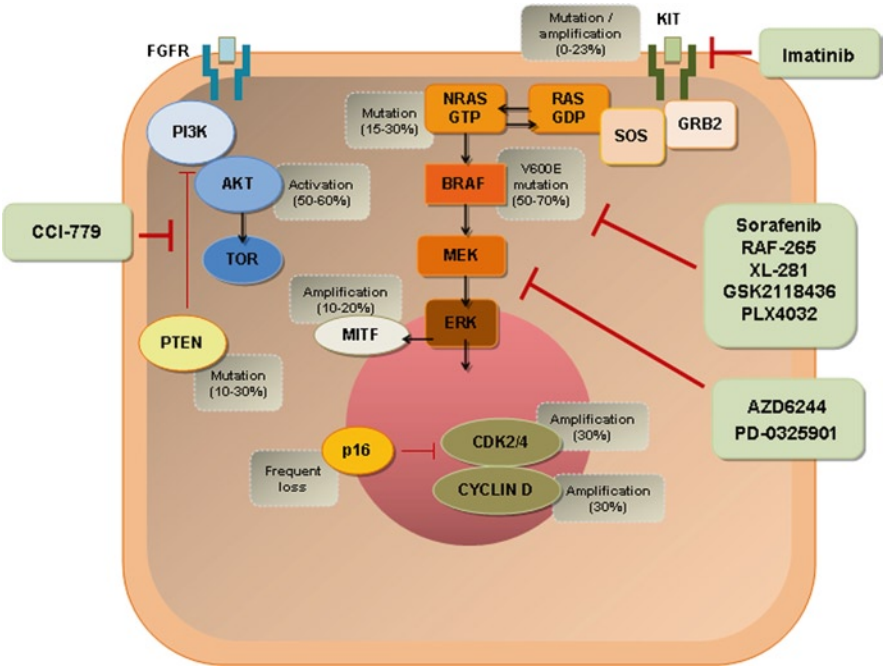

Fig. 5.1 Molecular pathways in melanoma. The identification of recurrent aberrations in signaling pathways, such as mitogen-activated protein kinase (MAPK; RAS/RAF/MEK/ERK), KIT, and PI3K-AKT, is promoting the development of targeted therapies for melanoma (*green boxes*). (Courtesy of Dr. Zendee Elaba, Department of Pathology, Hartford Hospital, Hartford, CT, USA)

Table 5.1 Genetic aberrations in melanoma according to anatomical location

Melanoma subtype	Predominant histopathological subtype	BRAF mutation (%)	NRAS mutation (%)	KIT mutation/ amplification (%)	Chromosomal alterations
Melanoma on NCSD skin	SSM	59	22	0	Gain on 6p,7,8q,17q,20q Loss on 9p,10,21q
Melanoma on CSD skin	LMM	11	15	2–17	Gain on 6p,11q13,17q,20q Loss on 6q,8p,9p,13,21q
Acral melanoma	ALM	23	10	7–23	Gain on 6p,7,8q,17q,20q Amplification on 5p13,5p15,11q13,12q14 Loss on 6q,9p,10,11q,21q
Mucosal melanoma	Unspecified	11	5	8–21	Gain on 1q,6p,7,8q,11q13,17q,20q Amplification on 1q31,4q12,12q14 Loss on 3q,4q,6q,8p,9p,10,11p,11q,21q

NCSD non-chronic sun-damaged, *CSD* chronic sun-damaged, *SSM* superficial spreading melanoma, *LMM* lentigo maligna melanoma, *ALM* acral lentiginous melanoma

Table 5.2 Genetic alterations in different melanocytic tumors

Tumor	Chromosomal aberrations	Oncogenic events
Primary melanoma	Multiple copy number alterations	BRAF and NRAS mutations common, particularly on NCSD skin
Common acquired nevus	None	BRAF mutations common, up to 87.5%
Dysplastic nevus	None	BRAF mutations in 52–62%; NRAS mutations in 71%
Blue nevus	None	GNAQ mutations in 83%; BRAF mutations in 0–12%
Spitz nevus	+11p in 10–20%; +7q rare	HRAS mutations in up to 29%
Small and medium-sized congenital nevus	None	BRAF mutations in 0–80%; NRAS mutations in 64–81%
Giant congenital nevus	None or a few aberrations	BRAF mutations absent; NRAS mutations in up to 81%
Proliferative nodule	Whole chromosome loss of 7, 9 and 10	Malfunction of chromosomal segregation; NRAS mutations in 70%

NCSD non-chronic sun-damaged

Current evidence suggests that an oncogene-induced premature senescent mechanism, promoted by mutant BRAF, is responsible for the cellular senescence (a permanently nondividing state) seen in common acquired nevi [6–8]. Recent studies have identified a number of pathways and cell mediators that may potentially play a role in this senescence pathway. It has been reported that normal melanocytes express low levels of insulin growth factor binding protein 7 (IGFBP7), restraining proliferation. However, expression of IGFBP7 is found to increase with $BRAF^{V600E}$, promoting cellular senescence through autocrine/paracrine pathways [7]. Interestingly, in cell culture studies, melanocytes that are wild-type for both BRAF and RAS are found to be relatively insensitive to IGFBP7-induced growth inhibition. In contrast, those with mutant RAS are weakly responsive, whereas those with $BRAF^{V600E}$ are highly sensitive, with resultant cellular apoptosis and senescence. This is likely related, at least in part, to the ability of IGFBP7 to block BRAF-MEK-ERK signaling, with resultant decreased levels of phosphorylated ERK. In one study, IGFBP7 expression was not detectable in $BRAF^{V600E}$-positive melanomas or melanoma cell lines, suggesting that loss of IGFBP7 and escape from cellular senescence may be critical for the development of melanoma from $BRAF^{V600E}$ melanocytes [7]. However, this hypothesis has been recently challenged [8]. Studies have also concluded that $BRAF^{V600E}$-induced senescence can be either dependent on, or occur independently of, $p16^{INK4a}$-, β-Gal- and/or p53-related mechanisms [6–8]. Theoretically, additional genetic events, such as loss of PTEN, p53 and/or $p16^{INK4a}$, or upregulation of AKT3, would be required for arrested melanocytes to circumvent the $BRAF^{V600E}$-induced senescence and re-enter the cell cycle.

Viros et al. [9] have reported that melanomas with $BRAF^{V600E}$ are associated with distinct morphologic and clinical features. In their recent study of 302 archival melanomas, $BRAF^{V600E}$ tumors were more likely to occur in patients <55 years of age; show increased upward migration and nest formation of intraepidermal melanocytes; thickening of the involved epidermis; sharper demarcation from the surrounding skin; and have larger, rounder, and more pigmented tumor cells [9]. In another study, Edlundh-Rose et al. [10] agreed that BRAF-mutant melanomas were significantly associated with a lower age at diagnosis, in addition to a more frequent moderate to marked host lymphocytic response. However, Akslen et al. [11] found that the presence of $BRAF^{V600E}$ was not associated with tumor cell proliferation, tumor thickness, microvessel density, or vascular invasion. In addition, from a study of 69 primary melanomas, Kannengiesser et al. [12] reported that no significant differences in clinical-histopathological parameters were observed between patients with and without BRAF mutations. Nonetheless, most studies do agree that $BRAF^{V600E}$ is most frequently found in melanomas that arise on areas of the body which experience intermittent intense sun exposure (i.e., trunk and extremities), and less frequently in areas of chronic sun exposure or sun protection (i.e., head and neck, and acral and mucosal surfaces) (Table 5.1) [2]. BRAF mutations have been identified in 59% of melanomas on non-chronic sun-damaged skin, but in only 23% of acral lentiginous melanomas, 11% of mucosal melanomas, and 11% of melanomas from skin with chronic sun damage [2]. A number of issues have yet to be resolved regarding BRAF mutations in melanoma. One issue is whether they are homogeneously present within melanomas or, for instance, increase in frequency during the progression from radial growth phase [RGF] to vertical growth phase [VGF] disease. Of note, some authors have proposed that BRAF and NRAS mutations are present in only 40% of in situ melanomas [13]. Secondly, it is unclear whether BRAF-mutations significantly influence clinical outcome in patients with melanoma [9–12]. The study by Viros et al. [9] suggested that BRAF-mutant melanomas show a pattern of metastases (i.e., progression via regional lymph nodes) which is different to that of BRAF-negative tumors (which demonstrate satellite, in-transit and visceral metastases). In this study, patients with BRAF-mutant melanomas also demonstrated better overall survival compared with their BRAF-negative counterparts [9]. Other authors, however, have found no correlation between BRAF mutational status and patient outcome [10–12]. Currently, it appears that this mutation is most important for tumor initiation/early progressive disease rather than associated with metastatic potential [10–12].

Importantly, recognition of the $BRAF^{V600E}$ mutation in melanomas does appear to be associated with significant therapeutic consequence (see Chap. 21). Over the last number of years, several targeted multi kinase inhibitors which decrease MAPK activity have been developed (Fig. 5.1). Early clinical studies using sorafenib in melanoma patients, as a single agent or in combination with chemotherapy, demonstrated little benefit beyond disease stabilization [14–16]. Clinical trials are now ongoing with second generation selective and nonselective RAF inhibitors, such as PLX4032, SB-590885/GSK2118436, XL-281, and RAF-265 (www.clinicaltrials.gov). In recent Phase I and Phase II trials, XL-281 (ARAF, BRAF and CRAF inhibitor) and PLX4032 ($BRAF^{V600E}$ inhibitor) were shown to have single-agent antitumor activity in melanoma patients, with the achievement of objective responses [14–16]. These studies indicate the potential therapeutic value of single-agent therapy against a mutated oncogene in melanoma. However, not all patients respond to this treatment; and dose-limiting toxicities, primary and secondary drug resistance with disease progression, and the development of therapy-related cutaneous squamous cell carcinomas/keratoacanthomas represent compounding issues [14–16]. The targeting of MEK, the downstream substrate of BRAF, could also prove to be an effective strategy in the treatment of advanced melanomas with BRAF or RAS mutations [14–16]. However, in addition to common somatic aberrations, such as $BRAF^{V600E}$, melanomas may demonstrate genomic changes involving PTEN, CDKN2A, TP53, EGFR, NOTCH2, MDM2, CCND1, CCNE1, CDK2, CDK4, MITF, AKT3, ERBB4, and ETV_1, and loss of chromosomes 13q and 16q, which could impact responses to current therapeutic strategies [14–20]. Therefore, it is likely that combination therapy regimens against multiple targets will be required for many patients with metastatic melanoma. Importantly, the successful identification of mutations in melanoma, such as $BRAF^{V600E}$, will help to stratify patients for potential targeted therapy, clinical trials, and outcome measures.

NRAS Mutations

Mutations in NRAS, another key regulator of the MAPK pathway upstream of BRAF, can also be found in melanoma (Fig. 5.1), although at a significantly lower frequency than $BRAF^{V600E}$ (Table 5.1). Overall, 15–30% of melanomas show activating NRAS mutations that are located predominantly within exon 1 (codons 12 and 13) or exon 2 [codons 59 and 61 (90%)] of the gene [2, 16]. NRAS mutations have been identified in 22% of melanomas on non-chronic sun-damaged skin, 10% of acral lentiginous melanomas, 5% of mucosal melanomas, and 15% of melanomas from skin with chronic sun damage [2]. Similar to $BRAF^{V600E}$, it appears that NRAS mutations are infrequently found in melanomas on chronically sun-damaged skin, and are more commonly seen in melanomas on skin with intermittent sun exposure (i.e., trunk and extremities) [11, 21–26]. In general, NRAS and BRAF mutations are mutually exclusive, although rare double-mutant cases have been reported [5]. Together, they account for MAPK pathway activation in >80% of melanomas. However, in contrast to BRAF-mutant melanomas, which typically require a synchronous mutation in a member of the phosphatidylinositol 3′ kinase (PI3K) pathway, the upstream location of NRAS allows for mutant forms to simultaneously activate both MAPK and PI3K-AKT signaling [11, 21–26]. In melanomas lacking BRAF or NRAS mutations, the signaling cascade can be triggered by autocrine mechanisms, that include the downregulation of RAF-1 or SPRY-2 (MAPK inhibitory proteins), or upregulation of C-MET. Of note, mutations in the other RAS genes, KRAS and HRAS, occur in only ~2% and ~1% of melanomas, respectively.

Congenital melanocytic nevi show mutations in NRAS in 64–81% of cases (Table 5.2) [27, 28]. In addition, BRAF mutations are seen in 88% and 30% of small (<1.5 cm in diameter) and medium-sized (1.5–20 cm in diameter) lesions, respectively, but are noticeably absent in giant congenital nevi (>20 cm in diameter) [27, 28]. Interestingly, nevi with microscopic evidence of adnexal involvement and splaying of the deep collagen (i.e., histopathological characteristics of congenital nevi), but which are not congenital in origin by history, commonly show BRAF rather than NRAS mutations [28].

KIT Mutations

Mutations of KIT (receptor tyrosine kinase) have been identified in 17% of chronic sun-damaged cutaneous, 11% of acral, and 21% of mucosal melanomas, but not in any melanomas on skin without chronic sun damage – supporting a role for KIT as an oncogene in a subset of tumors (Fig. 5.1 and Table 5.1) [29–31]. In addition, KIT gene amplification has been found to be present in 6% of chronic sun-damaged, 7% of acral, and 8% of mucosal melanomas [29–31]. Similar rates of KIT alterations in acral and mucosal melanomas, but lower rates (~2%) in chronic sun-damaged cutaneous tumors are reported by other studies [30]. Point mutations in KIT result in constitutive activation of the c-KIT protein in melanoma cells, and the activation of downstream proliferative and pro-survival signaling pathways [30]. At the protein level, immunohistochemical studies have shown c-KIT expression in 81% of mucosal and acral melanomas [32]. Interestingly, cases with activating mutations are commonly positive for c-KIT protein expression, although this is not uniformly the case. Furthermore, many tumors that do not have detectable gene mutation or amplification show high c-KIT protein expression levels [32–34].

Inhibition of KIT signaling has been shown to inhibit the proliferation of cultured melanoma cells [35, 36]. In addition, several anecdotal case reports have noted remarkable responses to small molecule KIT inhibitors (imatinib, sorafenib, and dasatinib) in patients with widely metastatic melanoma [30, 37–39]. However, recent Phase II trials of imatinib reported that, among 63 patients with melanoma, only one clinical response was seen (in a patient with an acral tumor) [30, 40].

Importantly, these patients' melanomas were not tested for the presence of a KIT (or PDGFRA) mutation, with only c-KIT (and PDGFRA) immunohistochemistry being performed. C-KIT receptor protein expression, in the absence of downstream signaling activity, has not been shown to be highly predictive of clinical response to imatinib [30, 32, 41, 42]. More specifically, KIT mutations, and not gene amplifications, appear to be associated with drug response in melanoma patients [30]. These findings clearly illustrate the importance of proper patient selection prior to imatinib treatment, including the performance of KIT and PDGFRA gene mutational analysis.

In summary, somatic BRAF, NRAS, and KIT gene mutations are now recognized as frequent events associated with melanoma development (Table 5.1). Whereas KIT mutations are most common in acral, mucosal, and chronically sun-damaged skin melanomas, BRAF and NRAS mutations seem to predominate in melanomas that arise on skin without chronic sun damage. Furthermore, BRAF and NRAS aberrations are largely mutually exclusive. A further distinction of BRAF and NRAS mutant melanomas is the requirement for co-activation of a member of the PI3K-AKT pathway in the former [43, 44]. Of note, somatic inactivating mutations of PTEN and constitutive activation of AKT3 are found in 10–30% and 50–60% of melanomas, respectively. PTEN loss usually occurs in melanomas with BRAF mutations [30]. Conversely, NRAS is located upstream of BRAF, and mutations in NRAS can simultaneously activate both the MAPK and PI3K-AKT pathways. Other common genomic aberrations include amplification of MITF, which is found in 10–20% of tumors. A recent study of metastatic melanoma samples identified 30 somatic mutations in 19 protein tyrosine kinase genes, including ERBB4 (19%), FLT1 (10%), and PTK2B (10%) [30]. Therefore, the identification of somatic gene mutations in melanoma may serve as the basis for a future integrated genomic-morphologic classification scheme for this tumor, in addition to the rationale for drug development and more effective targeted therapy.

HRAS Mutations

Copy number alterations and/or mutations in HRAS are characteristic of 10–29% of Spitz nevi (Table 5.2) [45, 46]. Using CGH- or FISH-based testing, isolated increased copies of 11p, the site of the HRAS gene, can be identified in these lesions. Approximately 67% of Spitz nevi with 11p gain show a coexisting mutation in HRAS. In contrast, only 1 of 21 Spitz nevi without 11p gain is found to be associated with HRAS mutation [45, 46]. As HRAS mutations and/or distal 11p gain are rarely present in melanomas [5, 31, 45, 46], their isolated detection without other chromosomal copy number alterations appear to be highly diagnostic of Spitz nevi. A histopathological study of Spitz nevi with 11p gain found that these tumors typically were large in size, predominantly intradermal, and characterized by marked desmoplasia and an infiltrative growth pattern [46]. Importantly, most evidence suggests that Spitz nevi are distinct melanocytic tumors, which arise through BRAF- and NRAS-independent pathways, and with a minority showing HRAS alterations. However, molecular findings in other spitzoid lesions (i.e., atypical Spitz nevus/tumor and spitzoid melanoma) are inconclusive, possibly due to variable patient selection criteria and diagnostic parameters (i.e., age and histopathological features) employed by different investigators [5].

GNAQ Mutations

GNAQ is a heterotrimeric G-protein-coupled receptor. Somatic mutations in GNAQ (Q209 residue), resulting in constitutive activation of this oncogene, have been found in up to 83% of blue nevi,

46–49% of uveal melanomas, and 27% of uveal melanoma cell lines (Table 5.2) [30, 47]. The frequency of GNAQ mutations in other blue nevus-like proliferations, such as nevus of Ota, seems to be significantly lower, with only 1 of 16 cases being positive [47]. This finding could be the result of technical difficulties in detecting mutations in pauci-cellular processes, such as nevus of Ota. However, a recent report evaluating a highly cellular proliferation of melanocytes, evolving to melanoma from a nevus of Ota, also failed to show evidence of GNAQ mutations [48]. GNAQ mutations are rarely found in melanomas on chronic sun-damaged skin, but have not been identified in acral or mucosal melanomas, or cutaneous tumors on sites without chronic sun damage [47]. Point mutations in another G-protein-coupled receptor, GNA11, have also been reported in uveal melanoma [30].

Germline Mutations Leading to Increased Melanoma Susceptibility

Molecular testing for germline mutations in melanoma may have several benefits [49, 50]. Firstly, it could help to identify patients requiring intensive skin cancer screening. Secondly, by determining those kindreds with known germline mutations and studying their melanomas for the presence of additional somatic mutations, frequent cooperating oncogenic pathways in the development and progression of melanoma may be revealed. Subtypes of melanoma with common pathogenesis and clinical behavior could also be uncovered. Additionally, specific sites for targeted therapy may be identified.

High-Risk Melanoma Genes

CDKN2A Mutations

The majority of melanomas are sporadic, but 5–10% of cases occur in familial clusters. Approximately 20–40% of highly penetrant familial melanoma is the result of germline alterations in the CDKN2A gene [49, 50]. In addition, somatic mutations in CDKN2A (predominantly involving exons 1 and 2) and/or chromosomal deletions of 9p21 (where CDKN2A resides) are extremely frequent events in melanoma [49, 50]. CDKN2A polymorphisms (C500G and C540T) and mutations in cyclin-dependent kinase inhibitor 2A/p14 alternate reading frame (CDKN2A/ARF) are also associated with increased melanoma risk [49, 50]. CDKN2A encodes $p16^{INK4a}$ and $p14^{ARF}$, both of which are known tumor suppressors and involved in cell cycle arrest, apoptosis, and melanocyte senescence.

Penetrance estimates (i.e., the likelihood of melanoma development when an individual carries a CDKN2A mutation) depend on several factors. These include the coexistence of common genetic polymorphisms in DNA repair, apoptosis, and immune response pathways, or other co-inherited predisposing genetic variants (MC1R), as well as geography and ethnicity [49–51]. The likelihood of melanoma development by age 80 in a patient with a germline mutation in CDKN2A is 58% in Europe, 76% in the USA, and 91% in Australia. CDKN2A mutations also predispose to pancreatic cancer, with a 25% risk of developing this tumor by age 80 [49, 50]. The role of CDKN2A testing within melanoma genetics is controversial. There are now at least five commercial laboratories which offer serum- or buccal swab-based testing for germline mutations in CDKN2A (http://www.genetests.org). Mutations in this gene are identified in only ~1% of unselected melanoma cases, and routine genetic analysis in all melanoma patients is inappropriate. The incidence of CDKN2A mutation is quite low when using single criteria, such as the presence of clinically atypical nevi (4%), two or more primary melanomas (2%), or early onset (<40 years old) melanomas (1%), although

combinations thereof may increase the rate of detection significantly. In a recent study, which reviewed the likelihood of finding CDKN2A germline mutations, it was proposed that in moderate-to-high melanoma incidence areas, (a) individuals with three or more primary cutaneous melanomas or (b) families with at least one invasive melanoma and two or more other diagnoses of melanoma and/or pancreatic cancer among first- or second-degree relatives on the same side of the family may be ideal candidates for evaluation [49]. Based on current evidence, CDKN2A testing of patients with clinically atypical nevi and/or dysplastic nevi does not appear to be useful [49]. Currently, the primary benefit of testing for CDKN2A mutations is the identification of patients and family members who may benefit from increased surveillance and intensive skin cancer screening, with the possible earlier detection of melanoma in carriers [49–51]. The patient's ethnicity, age at diagnosis, and other risk factors, such as sun exposure history, are important considerations. Informed consent and proper counseling must also be incorporated into any genetic testing strategy. Useful resources for familial melanoma testing are GenoMEL, an international melanoma genetics research consortium (www.genomel.org), and the Huntsman Cancer Institute Melanoma and Skin Cancer Program (http://www.huntsmancancer.org/group/melanomaProgram/overview.jsp).

CDK4 Mutations

The interaction between CDK4 and cyclin D regulates passage through the G1-S checkpoint of the cell cycle [50]. $p16^{INK4a}$ is known to selectively inhibit CDK4. Both germline and somatic mutations of CDK4 have been found in familial melanoma and melanoma cell lines [50]. Two common mutations, $p.Arg24Cys^{5}$ and $p.Arg24His^{51}$, which occur in the p16-binding region, result in constitutive activation of CDK4 and cellular proliferation [50].

Low-Risk Melanoma Genes

Epidemiological studies have directly linked specific phenotypic traits, such as skin pigmentation, eye color, and tanning ability to melanoma predisposition [50, 52]. Melanocortin-1-receptor (MC1R), a gene involved in skin pigmentation, has been recently implicated in melanoma susceptibility [50, 52]. Activation of MC1R, through α-melanocyte stimulating hormone (α-MSH) binding, results in increased cAMP production with upregulation of downstream melanosomal enzymes, such as tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1). Activation of this pathway stimulates melanin synthesis and a switch from basal pheomelanogenesis to eumelanogenesis, resulting in darker skin pigmentation and increased protection from ultraviolet radiation [50, 52]. MC1R is extremely polymorphic, with over 60 variant alleles identified to date. Importantly, the MC1R allelotype can influence skin and hair color, as well as susceptibility to melanoma. One of the earliest studies noted a relative risk of 3.9 for melanoma in carriers of MC1R variants compared with normal homozygotes [53]. Interestingly, the influence of MC1R on melanoma susceptibility appears to go beyond its effect on pigmentary phenotype [54]. MC1R may play a role as a modifier gene in melanoma risk among CDKN2A mutation carriers. One study has found that co-inheritance of CDKN2A mutations and MC1R red-hair variants increases the risk of melanoma from 50% to 80% [55]. Investigations have also suggested that MC1R variants increase the risk for development of BRAF-mutant melanomas [56, 57].

Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) or genetic variants in other pigmentation-related genes, including TPCN2, ASIP, KITLG, NCKX5, IRF4, OCA2, SLC24A4, TYR, and TYRP1, that are associated with variable melanoma risk and

confirming the importance of gene-environment interactions in tumor pathogenesis [50, 52]. BSM1, a vitamin D receptor variant, is also associated with elevated melanoma susceptibility [50]. In addition, individuals with hereditary retinoblastoma (resulting from germline RB mutations) and xeroderma pigmentosum (resulting from defects in nucleotide excision repair) are at increased risk of melanoma development [50].

Molecular Diagnostic Strategies

Although we are likely on the verge of some considerable breakthroughs in the management of metastatic melanoma, the clinical and histopathological identification of skin-localized tumors with subsequent surgical intervention remain the cornerstone of therapy for most patients. With such an emphasis placed on early detection, the number of biopsies of pigmented lesions, that are performed to exclude melanoma, continues to grow at an overwhelming rate. A recent study, addressing this issue in skin cancer clinics in Australia, estimated an average "number needed to treat" (NNT) of 30; that is, for every melanoma detected, approximately thirty biopsies to exclude melanoma are performed [58]. Based on an annual incidence of 68,720 new cases of invasive melanoma in the USA and a potential NNT of 30, it can be estimated that over 2,000,000 biopsies are performed yearly in the USA alone to exclude melanoma.

Entire monographs are devoted to the histopathological distinction between various types of melanocytic nevi and melanoma [59–61]. In the vast majority of cases, light microscopic examination can reliably distinguish benign and malignant melanocytic tumors. However, there are subsets of nevi that histopathologically simulate melanoma, in addition to variants of melanoma that resemble nevi. Importantly, there is significant interobserver variability among pathologists in the light microscopic interpretation of melanocytic tumors. A recent study of 5,136 melanocytic lesions, that underwent confirmatory re-review in a multidisciplinary pigmented lesion clinic setting, reported a critical change in diagnosis from malignant to benign in 1.2% of cases, and from benign to malignant in 1.1% of cases [62]. Smaller series, focusing on consult level cases, have reported discrepancies in the range of 15–25% [63]. In another study, a panel of ten expert pathologists evaluated 30 spitzoid neoplasms from 28 patients [64]. No clear consensus was obtained in 17 of the 30 cases. Furthermore, some lesions that were categorized by the majority of pathologists as Spitz nevus or atypical Spitz tumor proved to have fatal outcomes [64]. Based on an estimate of ~2,000,000 biopsies performed annually in the USA to exclude melanoma, approximately 20,000–40,000 cases (1–2%) may have some level of diagnostic ambiguity.

Among the potential roles of molecular diagnostic testing in the setting of melanocytic tumors, the ability to improve on the classification of microscopically ambiguous spitzoid melanocytic neoplasms is unquestionably among the most paramount. While classic Spitz nevi and melanomas with some spitzoid features are less problematic, intermediate lesions categorized as atypical Spitz tumors are a source of significant controversy and diagnostic dilemma for dermatopathologists. The term atypical Spitz tumor, sometimes referred to as STUMP (spitzoid tumor of uncertain malignant potential) or MELTUMP (melanocytic tumor with uncertain malignant potential), has been adopted for cases which cannot be classified into a traditional framework of clearly benign or clearly malignant by histopathology. Importantly, there may exist a category of melanocytic tumors with an intermediate level of malignancy. The latter not only includes some spitzoid melanocytic tumors, but also cases classified as pigmented epithelioid melanocytoma. Recent studies on both of these two tumor types have shown a high tendency for lymph node (LN) involvement, including parenchymal disease, but without any evidence of further tumor progression on long-term clinical follow-up. Ludgate et al. [65] identified LN involvement in 47% of adult patients with atypical Spitz tumors, although none of these patients showed any adverse outcomes (average follow-up,

43.8 months). Similarly, a case series by Mandal et al. [66] of pigmented epithelioid melanocytoma identified 8 of 26 (31%) patients with LN disease, but no further tumor progression or death (average follow-up, 67 months). Advances in our understanding of the molecular similarities and differences between various melanocytic tumor types will undoubtedly improve the classification of these lesions.

Immunohistochemistry

The most common ancillary laboratory technique used for the diagnosis of melanocytic lesions is immunohistochemistry. There is a long list of immunohistochemical stains that have been evaluated for their diagnostic and prognostic potential in this setting [67–71]. S-100 protein is the most sensitive marker of melanocytic differentiation. Other less sensitive and perhaps more specific markers include MART-1, HMB-45, MITF, and tyrosinase. MIB1 (anti-Ki-67, a marker of cellular proliferation) is also commonly used in the work-up of melanocytic lesions. Immunohistochemical studies with these markers have been employed in a number of settings, including the distinction between invasive melanoma versus compound/intradermal benign melanocytic nevus, and desmoplastic melanoma versus desmoplastic nevus or scar [67]. In addition to quantification of absolute numbers of cells staining, a consideration of the pattern (patchy *vs.* diffuse) and localization (maturational gradient) of reactivity for some antibodies (i.e., HMB-45, Ki-67) is employed [67]. Immunohistochemistry is also routinely used in the staging of melanoma, such as to detect microscopic foci of melanoma cells in LNs and/or distinguish between metastatic melanoma and capsular nevus (see Chap. 6). However, there is no single marker or panel of markers that unequivocally proves the diagnosis of melanoma or benign melanocytic nevus [67, 68]. In addition, numerous immunohistochemical studies have assessed the potential prognostic value of protein expression analysis in melanomas [69–71]. This is because the stratification of patients with localized (stages I-II) disease based on histopathological variables (i.e., depth of invasion, ulceration) results in a wide range of 10-year melanoma-specific survival rates. With the possible exception of Ki-67, no immunohistochemical marker is routinely used in this setting. A recent review by Gould Rothberg and Rimm [70] identified 101 proteins that may be good candidates for prognostic discrimination in melanoma. However, the authors point out that evaluations of these proteins in methodologically robust prognostic studies will be required to determine their clinical potential as independent prognostic markers in patients with skin-localized tumors [70].

Comparative Genomic Hybridization

Both classic metaphase chromosome-based and array-based comparative genomic hybridization (CGH) methods have been used to analyze melanocytic neoplasms for the presence of copy number alterations (gains or losses) in all chromosomal segments (Figs. 5.2–5.4). Using these techniques, Bastian et al. [72] have shown that ~96% of melanomas harbor chromosomal copy number aberrations (Table 5.1). Frequent copy number alterations in melanoma include deletions of chromosomes 9p (82%), 10q (63%), 6q (28%), and 8p (22%), and gains of chromosomes 7 (50%), 8 (34%), 6p (28%), and 1q (25%). In contrast, benign melanocytic nevi rarely show copy number alterations by CGH (or karyotyping) (Table 5.2). Spitz nevi may demonstrate isolated gains in 11p (locus of the HRAS gene) in ~10–20% of cases and gains of 7q in a small percentage of examples, using classic and array CGH [45, 73, 74]. 11p gain has been confirmed by FISH analysis of a Spitz nevus [73]. Typical Spitz nevi rarely show copy number gains involving both 7q and 11p in the same

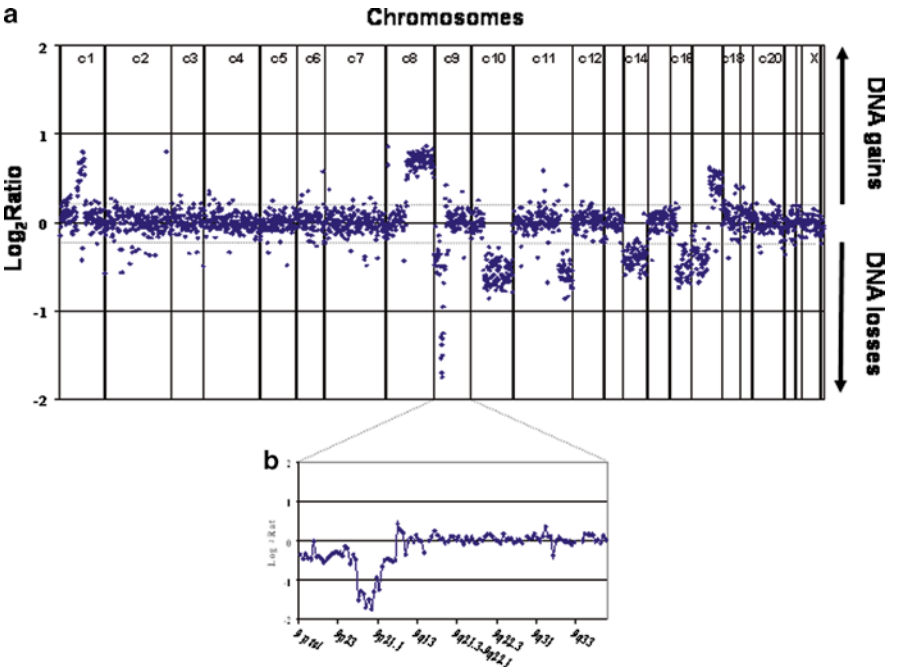

Fig. 5.2 (**a**) Array comparative genomic hybridization (*CGH*) profile of a primary melanoma. *Dashed lines* represent the upper and lower thresholds. Clear homozygous deletions are detected at chromosome 9p, including the 9p21.3 region covering the CDKN2A gene. DNA gains are seen on chromosomes 1, 8q, and 17q. (**b**) *Zoom-in* on chromosome 9 displaying deletion of the 9p21.1-9p23 region (Courtesy of Prof. Margit Balázs, Department of Preventive Medicine, University of Debrecen, Debrecen, Hungary)

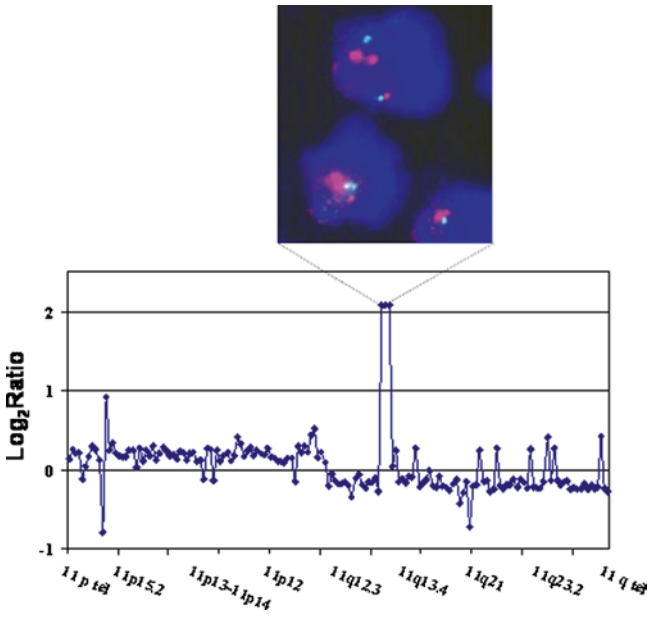

Fig. 5.3 Array comparative genomic hybridization (*CGH*) result on chromosome 11 of a primary melanoma. High-level amplification of the 11q13 sequence covering the CCND1 gene is clearly visible. Color image shows high-level amplification of CCND1 by fluorescence in situ hybridization (*FISH*) (*green* = centromere 11; *red* = the CCND1 gene). DNA for array CGH and tumor cells for FISH analysis were obtained from the same patient (Courtesy of Prof. Margit Balázs, Department of Preventive Medicine, University of Debrecen, Debrecen, Hungary)

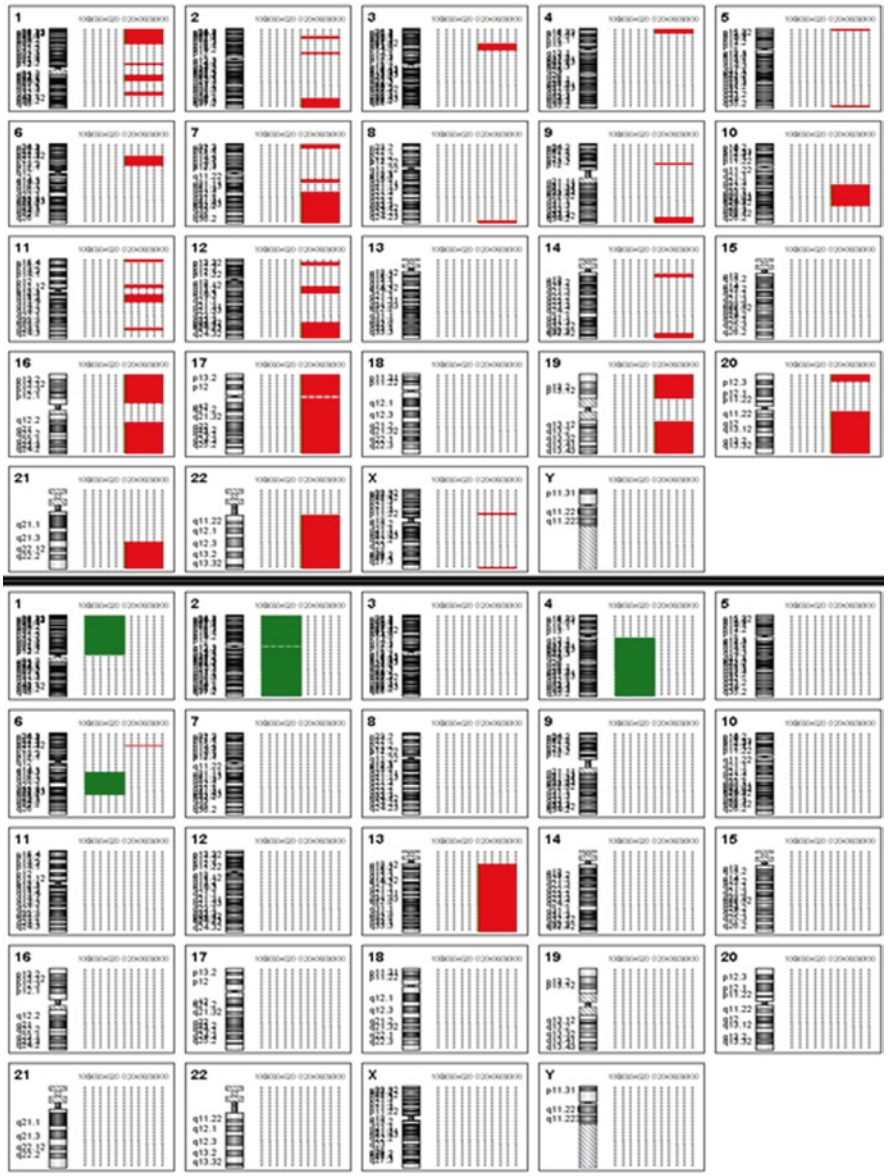

Fig. 5.4 Array comparative genomic hybridization (*CGH*) demonstrating that melanocytic skin lesions associated with the development of metastases ($n = 3$; *upper panel*) show significantly more chromosomal aberrations compared with melanocytic skin lesions without the development of metastases ($n = 5$; *lower panel*). All three melanocytic lesions with malignant clinical behavior showed gains of chromosomes 7p22, 9q34, 11p15, 11q13, 14q32, 16q13, 17q25, 19p13, and 20q13. *Red bars* indicate chromosomal gains; *green bars* indicate chromosomal losses (Courtesy of Drs. Timo Gaiser, Maria R. Becker, Heinz Kutzner and Thomas Wiesner, University of Heidelberg, Heidelberg, Germany; Dermatopathologie Friedrichshafen, Friedrichshafen, Germany; and Medical University of Graz, Graz, Austria)

tumor [73]. Studies by Takata et al. [75] and Harvell et al. [76] have confirmed the utility of both classic and array CGH in the distinction of formalin-fixed paraffin-embedded (FFPE) spitzoid melanocytic neoplasms and melanoma. In another study, Ali et al. [73] demonstrated gain of 19p in an atypical Spitz tumor by array CGH. This 1 cm nodule arose in a 6-year-old boy, and was

characterized by focal ulceration, a sheet-like proliferation of spitzoid cells in the dermis, minimal maturation, and mitotic activity (including near the base of the lesion). The patient's sentinel LN (SLN) showed bulky involvement by tumor [73]. Therefore, the presence of single or limited copy number aberrations may be characteristic of some intermediate or borderline malignant tumors. In contrast, melanomas with spitzoid features show multiple chromosomal copy number aberrations (i.e., +6p, +17q, −1p, −15p), as found in conventional melanomas [73]. In this regard, Mihic-Probst et al. [77] used CGH to re-evaluate a spitzoid primary tumor which later metastasized – demonstrating 6q and 9p deletions in the primary lesion, with additional 10p and 10q deletions, and chromosome 7 gain, in the metastasis. The authors suggested that the malignant nature of this lesion might have been determined if CGH was employed in the initial work-up [77]. Vincek et al. [74] used array CGH to analyze DNA extracted from laser-capture microdissected level I (in situ) and level III (invasive) melanomas. In situ melanomas demonstrated deletions (13q and 16q) and duplications that were limited in size compared with invasive melanomas. Balázs et al. [78] also confirmed that the average number of chromosomal copy number alterations was less in melanomas of <4 mm in thickness (4.4 ± 4.5) compared with those of >4 mm in thickness (7.4 ± 3.7), although the difference was not statistically significant (Figs. 5.2 and 5.3). Maize et al. [79] used CGH to show that unequivocally benign or malignant blue nevus-like proliferations show non-overlapping patterns of chromosomal aberrations, and that ambiguous tumors could be separated into lesions with and without genomic changes. CGH has also been employed in the evaluation of proliferative nodules (PNs) arising in congenital nevi [80, 81]. These lesions can show strikingly concerning features, such as expansile nodular proliferations of atypical melanocytes with many mitotic figures, but are typically benign and resolve spontaneously within a number of months. PNs frequently demonstrate whole copy number aberrations by CGH, particularly losses of chromosomes 7, 9, and 10 [80]. This is in contrast to melanoma, which is characterized by aberrations involving chromosomal fragments. Although whole chromosomal aberrations can also be found in melanoma, they are almost always accompanied by chromosomal fragment alterations. True melanomas arising in congenital nevi show aberrations in chromosomal fragments that are identical to those seen in conventional melanomas. Chromosomal fragment changes are typically associated with abnormalities in the handling of double-stranded DNA breaks, whereas aberrations involving whole chromosomes (as seen in PNs) may result from a malfunction of chromosomal segregation, with intact DNA check-points eventually halting the proliferation of aneuploid cells [45, 80].

CGH assays could also be potentially used to stratified patients into prognostically relevant groups. Balázs et al. [78] reported that the number of genetic alterations detected by CGH was significantly higher in primary melanomas which metastasized within the first year after surgery (7.8 ± 4.1) compared to tumors without metastasis during the same time period (2.0 ± 1.4). Gaiser et al. [82] demonstrated that histopathologically ambiguous melanocytic tumors which metastasized had significantly more chromosomal aberrations by array CGH than those lesions that did not develop metastasis (Fig. 5.4). These studies confirm that aggressive behavior among melanocytic tumors is associated with the accumulation of multiple genetic events. In addition, chromosomal aberrations, which have been found to differ between primary and metastatic lesions, could represent potential targets to uncover metastases-related genomic changes [78].

The introduction of CGH was a major advancement in the diagnostic analysis of melanocytic neoplasms, with this technique being used as a clinical tool to distinguish benign from malignant tumors. One advantage of CGH is its ability to screen the entire genome for areas of copy number alterations. Additionally, CGH has high specificity; in order to meet the lower limits of resolution and be detected by this methodology, the aberration must be highly characteristic of the cell population studied. However, CGH is cumbersome and time-consuming, and DNA must be extracted from dissected tumor cells for analysis. Therefore, one cannot directly visualize the population(s) of cells with abnormal copy number changes. Additionally, the limits of resolution for the identification of copy number alterations are less than with some other molecular assays, such as FISH.

This could be an issue if, for example, several melanocytic cell populations exist in a lesion, with only one of them being malignant and having chromosomal aberrations (i.e., melanoma arising in a melanocytic nevus). Significant, but focal, chromosomal gains or losses could be masked as a result of testing procedures (i.e., analysis of DNA from both benign and malignant cells in one reaction). Finally, CGH requires a significant tumor cell population to be present for adequate DNA extraction; therefore, superficial and/or smaller lesions may not yield informative results.

Fluorescence In situ Hybridization

The development of a FISH assay for melanoma diagnostics was initiated by combinatorial analysis of CGH data, in an effort to identify the chromosomal loci most frequently altered in melanoma, but not in melanocytic nevi [83]. The first commercially available probe set for FISH analysis of melanocytic tumors began with 14 chromosomal loci as potential targets. For the loci that are commonly gained, a well-recognized oncogene from that region was used as a FISH target. Similarly, for those loci that are commonly deleted, a well-known tumor suppressor gene from that region was selected. Fluorescent probes to all 14 targets were obtained. The probes were arranged in multiple four-probe panels and applied to a series of 97 melanomas and 95 melanocytic nevi. After examining a large number of potential parameters, probes targeting 6p25 (RREB1), 6q23 (MYB), 11q13 (CCND1), and centromere 6 (CEP6) were selected as the best combination for distinguishing melanoma from melanocytic nevi [83]. RREB1 (also known as RAS-responsive element binding protein 1 and Raf responsive zinc finger protein) is a transcription factor which binds specifically to the distal RAS-responsive element (RRE) in the calcitonin gene promoter, leading to an increase in the RAS/RAF-mediated transcriptional response of that promoter; MYB encodes another transcription factor; and CCND1 (cyclin D1 proto-oncogene) plays a role in G1-S phase check-point transition. The CEP6 probe is included as a control for the ploidy status of chromosome 6. These probes were then applied to an additional cohort of melanoma and melanocytic nevi to determine ideal cut-off values for distinguishing these groups. A test result was considered positive for melanoma if any of the following criteria were met: (a) >29% of enumerated cells had gains in RREB1; (b) >55% of enumerated cells had more copies of RREB1 than CEP6; (c) >40% of enumerated cells had fewer copies of MYB than CEP6; or (d) >38% of enumerated cells had gains in CCND1. These criteria were later validated on a third set of melanocytic tumors, demonstrating a sensitivity of 86.7% and specificity of 95.4% for these four probes in distinguishing melanoma from benign melanocytic nevi [83]. The four selected probes and the predetermined criteria were then applied to a set of 27 melanocytic neoplasms with conflicting/ambiguous histopathological features [83]. Among these 27 cases, 6 resulted in bulky LN metastasis, distant metastasis, or death. The remaining 21 cases showed no evidence of progression with 5-year follow-up. The test successfully identified all six of the metastasizing cases, although 6 of 21 (29%) non-metastasizing lesions also tested positive with the four-probe set [83].

This four-probe assay has been subsequently employed in a number of interesting and clinically challenging scenarios (Figs. 5.5 and 5.6), including the distinction of mitotically active nevi from nevoid melanoma [84], epithelioid blue nevi from blue nevus-like cutaneous melanoma metastases [85], and intranodal melanocytic nevi from melanoma metastases to LNs (see Chap. 6) [86]. Furthermore, the assay has been used in the evaluation of superficial melanocytic neoplasms with pagetoid melanocytosis (i.e., pagetoid Spitz nevi, *de novo* epithelioid melanocytic dysplasia, and melanoma) [87]; to assess a nevus of Ota which showed progressive evolution to melanoma with intermediate stages resembling a cellular blue nevus [46]; and to objectively determine Breslow thickness and microstage melanoma (i.e., distinguish malignant *vs.* benign components of a tumor, in which a melanoma arose in association with a melanocytic nevus) [88]. In addition, this four-probe test has provided insights into some recently recognized subtypes of melanocytic neoplasms,

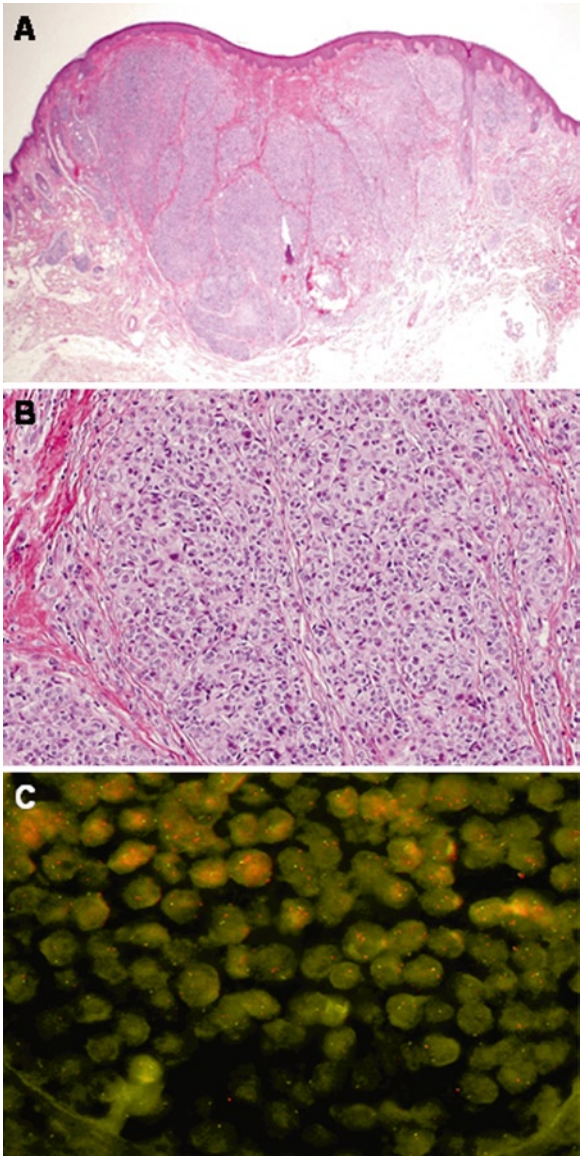

Fig. 5.5 (**a**) Low-power view of hematoxylin and eosin (*H&E*) stained section of an atypical intradermal melanocytic tumor. The lesion is large, multinodular and lacks definitive evidence of "maturation," concerning for melanoma. However, no associated epidermal component is identified. (**b**) High-power view demonstrating enlarged monomorphous epithelioid cells without significant mitotic activity. (**c**) Fluorescence in situ hybridization (*FISH*) analysis with 6p25 probe (RREB1; red signal) and 6q23 probe (MYB; gold signal). This tumor shows clear gains of 6p25 relative to 6q23, with significant chromosome 6 imbalance likely a result of an isochromosome 6

such as lentiginous junctional melanoma of the elderly [89]. These latter lesions show chromosomal copy number aberrations by FISH, with the same frequency as other subtypes of melanomas, supporting their classification as malignant tumors [89]. A number of other groups have also demonstrated the value of FISH analysis, using the four-probe set, in the diagnosis and stratification of melanocytic tumors that are difficult to classify by conventional light microscopy (Figs. 5.7 and 5.8) [82, 90–95]. A further application of FISH is the use of 11q13- and 5p15-directed probes to evaluate for the presence of morphologically normal, but genetically aberrant melanocytes at acral sites; and therefore, define either (a) histopathologically unrecognizable early lesions of acral lentiginous melanoma in situ or (b) the surgical margins of acral lentiginous melanoma excisions (see Chap. 9) [96, 97]. These genetically aberrant epidermal melanocytes, or "field cells," are thought to progress through the same evolutionary phases as the primary tumor, and lead to recurrence at the excision site if not completely removed (Fig. 9.1) [97].

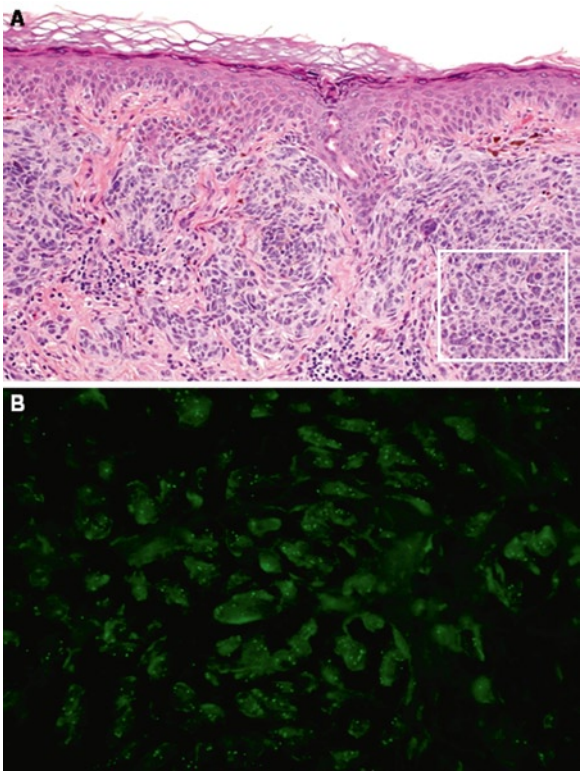

Fig. 5.6 (**a**) Hematoxylin and eosin (*H&E*) stained section of a changing melanocytic neoplasm. (**b**) The expansile nodular area in the dermis (*inset*) shows uniform copy number gains of 11q13 (CCND1; green signal)

A number of issues must be considered when using FISH as an adjunct tool in the work-up of melanocytic lesions. Firstly, 5 to 10% of benign Spitz nevi may have sufficient numbers of tetraploid cells to result in a false-positive fish test, if not recognized by the evaluating pathologist [98]. One must be aware of this potential pitfall to avoid an overdiagnosis of melanoma. Future studies may determine if polyploid Spitz nevi differ in their biological behavior compared with their diploid counterparts. In addition, because melanocytic tumors are heterogeneous in the molecular pathways that lead to melanoma development, it is likely that different clinical-histopathological subtypes show variable propensities for specific chromosomal aberrations. In this regard, the diagnostic sensitivity of FISH, using probes targeting 6p25, 6q23, 11q13, and CEP6, has been shown to vary among melanoma subtypes [99]. The four-probe assay shows the greatest sensitivity in the acral lentiginous (100%) and nodular (91%) subgroups of melanoma, and the least sensitivity in the lentigo maligna (82%) and superficial spreading (81%) subtypes [99]. Of note, 11q13 gain is more commonly identified in melanocytic lesions from chronically sun-damaged skin compared with those from non-chronically sun-damaged skin [99]. Gain of 6p25 shows the highest sensitivity both overall and in each subtype [99]. Another FISH study by Glatz-Krieger et al. [100] also identified anatomic site-specific patterns of gene copy number gains (CCND1, MDM2 and MYC) in melanomas.

Gerami et al. [83] demonstrated a significant difference in metastasis-free survival between test-positive and test-negative cases with ambiguous pathology, using the four-probe assay. The criteria employed by Gerami et al. [83] were formulated using 4 cohorts of data in a multi-institutional effort, which included researchers from Northwestern University, Chicago and the University of California at San Francisco. With a different set of diagnostic criteria, Gaiser et al. [82] found less concordance between FISH results and clinical outcome. Additional multi-institutional studies

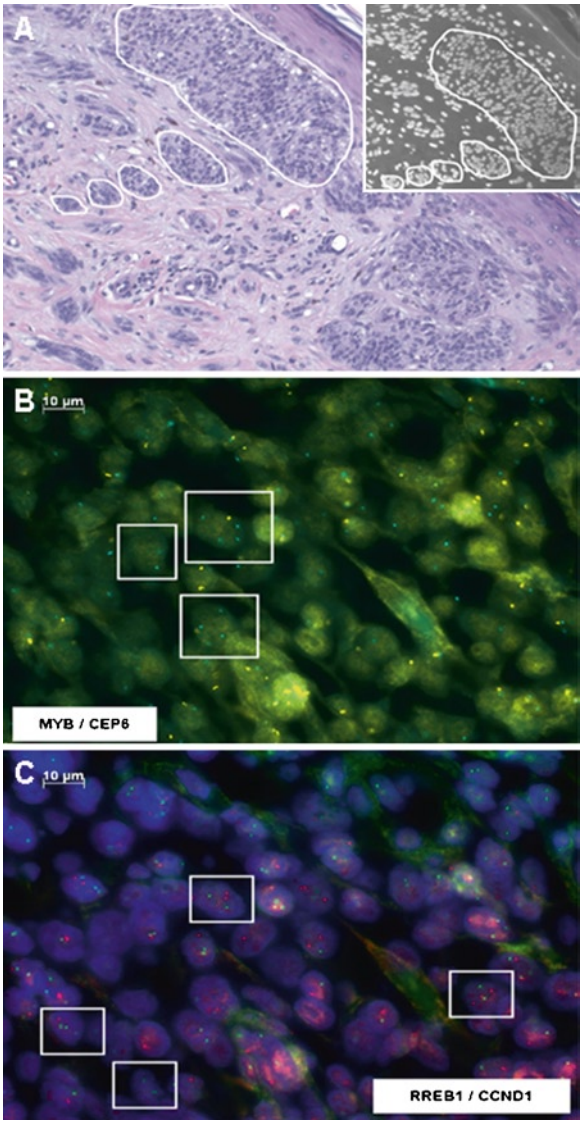

Fig. 5.7 (**a**) Nests of melanoma cells are identified by comparing the hematoxylin and eosin (*H&E*) image and 4′,6-diamidino-2-phenylindole (*DAPI*)-pattern (*inset*). (**b**) Loss of 6q23 (MYB; gold signal) relative to centromere 6 (CEP6; aqua signal) in ~80% of melanoma cells. (**c**) Gain of 11q13 (CCND1; green signal, average 2.5); with gain or loss of 6p25 (RREB1; red signal) in ~75% of melanoma cells (Courtesy of Drs. Anne-Katrin Zimmermann and Joachim Diebold, Department of Pathology, University Hospital Zurich, Zurich; and Institute of Pathology, Cantonal Hospital Lucerne, Switzerland)

with larger cohorts are needed to further evaluate the use of FISH in separating ambiguous melanocytic neoplasms into distinct prognostic categories. A possible modification of the probe composition and greater standardization could lead to more sensitive and specific diagnostic testing strategies. It is important to interpret the results of fish analyses within the context of the clinical-histopathological features of any lesion.

It has yet to be determined whether the current four-probe set offers prognostic information in conventional melanomas. Anecdotally, we have previously noted that subsets of melanomas may contain extensive amplification of CCND1, in the form of either double-minute chromatin bodies (dmins) or homogeneous staining regions (HSRs) [101, 102]. It is hypothesized that these changes may be associated with increased tumorigenicity and possibly portend a worse prognosis, although this matter requires further study.

FISH has also been used to test for targets on chromosomes 1, 3, 4, 6, 7, 8, 9, 10, 11, 12, 17, 18, and 20 to distinguish different subtypes of melanocytic tumors, and to determine genetic alterations

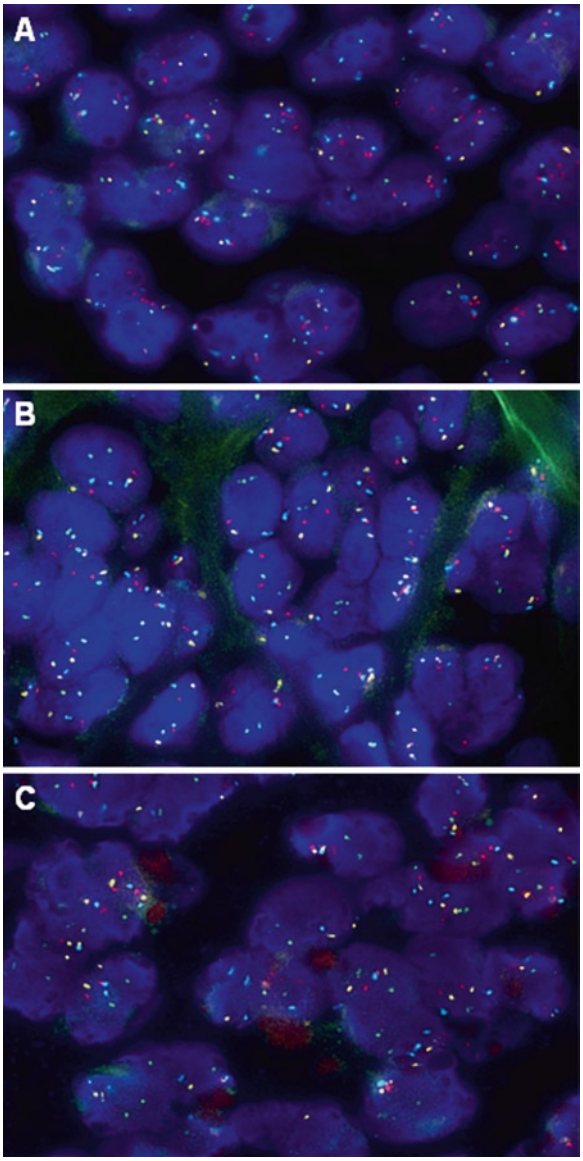

Fig. 5.8 (**a**) Aneuploidy: fluorescence in situ hybridization (*FISH*) positive for 6p25-RREB1, CEP6, and 6q23-MYB; the number of red, aqua, and gold signals suggests an aneuploid pattern, supporting the diagnosis of a malignant melanocytic proliferation. (**b**) Aneuploidy: FISH positive for CEP6 and 6q23-MYB; the number of aqua and gold signals suggests an aneuploid pattern, supporting the diagnosis of a malignant melanocytic proliferation. (**c**) Diploid: FISH negative; the number of signals suggests a diploid pattern (Courtesy of Drs. Sebastiana Boi and Silvia Fasanella, Department of Pathology, Santa Chiara Hospital, Trento, Italy)

associated with melanoma progression [103–109]. In some studies, findings were correlated with the results of immunohistochemistry, CGH, or other technologies [104, 108, 109]. For example, deletion of 9p21 is reported in the majority (84%) of melanomas, and is detected at similar frequencies in both early- and late-stage tumors [103]. In addition, partial heterozygous deletions of 9p21 have been found by FISH in subsets of spitzoid melanocytic lesions [104]. Alterations of 9p21 were reported as useful in the differentiation of atypical spitzoid lesions from Spitz nevi, but not in the prediction of their biological behavior [104]. Further studies are needed to determine if higher levels of heterozygous deletions or homozygous deletions can be seen in spitzoid tumors, and whether such alterations can determine prognosis in these lesions. Another recent FISH study identified topoisomerase 1 (TOP1; 20q12-q13) amplification in 32–38% of melanomas – a finding associated with thicker tumors and poor prognosis [105]. In addition, a variant of the in situ hybridization

methodology, known as chromogenic in situ hybridization (CISH), has been used to determine melastatin (MLSN) expression, a marker of disease progression/aggressiveness, in melanomas (Fig. 3.8) [110]. FISH and other technologies have also demonstrated some utility in the molecular staging of melanoma, and the distinction of a secondary primary tumor from a cutaneous metastasis (see Chap. 6). Finally, molecular detection of EWS gene rearrangements by FISH and reverse transcription-polymerase chain reaction (RT-PCR) can be used to distinguish melanoma from clear cell sarcoma (CCS) (see Chap. 8) [111–116]. This distinction is important due to different treatment regimens and prognoses (i.e., 5-year survival rates of 48–67% for CCS). However, the differentiation of these two tumors can be extremely difficult, since they are usually indistinguishable by morphologic (i.e., presence of melanin), histochemical, immunohistochemical (i.e., S100+/HMB-45+), and ultrastructural (i.e., presence of pre-melanosomes) features. The t(12;22)(q13;q12) translocation is the hallmark of CCS, present in >90% of cases, and resulting in the production of an EWS-ATF1 fusion gene (Fig. 8.3) [111–116]. The t(2;22)(q33;q12) translocation is present in <10% of CCS cases, resulting in an EWS-CREB1 fusion gene [111–116]. These translocations and/or their resulting fusion genes have not been documented in cutaneous melanoma [115, 116]. Consequently, the presence of EWS-ATF1 or EWS-CREB1 fusion genes can be used to definitively differentiate CCS from melanoma.

Several studies in multiple cancer types have now noted an association between activating gene mutations and local copy number aberrations of the same chromosomal locus [20, 117]. For example, BRAF-mutant melanomas frequently show gains of 7q (the site of BRAF) [20]. The recognition of specific patterns of mutations and related copy number alterations could reveal homogeneous subtypes of melanoma, further enhancing the classification of these tumors (Table 5.1).

DNA Microarray Technology

Over the past decade, a number of studies have employed cDNA/oligonucleotide microarray-based gene expression analysis to investigate melanoma pathogenesis and progression [118–140]. This technology has been used to determine molecular alterations between benign nevomelanocytes and melanoma, subsets of dysplastic melanocytic nevi, RGP and VGP melanoma, and primary and metastatic tumors. In addition, microarray-based studies have assessed changes in transcriptome patterns as a function of therapeutic intervention, guided treatment strategies for in-transit metastatic melanoma, and identified markers related to prognosis. An in-depth review of all gene expression profiling investigations in melanocytic tumors is beyond the scope of this chapter. However, from the vast amount of data collected, a number of interesting discoveries have been made and hypotheses generated. For example, gene expression profiling has determined that individual lesions can show altered biological processes (i.e., apoptosis and/or transcription regulation), that are not associated with specific histopathological subtypes, but rather with subgroups of samples without apparent relationship (i.e., two classes of dysplastic nevi appear to exist with transcriptional profiles similar to either RGP or VGP melanoma) [121]. Other studies have suggested that transcript levels among primary melanomas of different Breslow thickness are dynamic, and that a transition point of tumor progression exists where a distinct set of gene expression change occurs [120, 122]. In addition, a phenotype-switching model for melanoma progression has been proposed, in which cells can transition back-and-forth between proliferative and invasive states [123]. However, results have been largely inconsistent and not reproducible across transcriptome investigations. This is likely the result of different study parameters employed (i.e., experimental platforms and protocols, cell lines *vs.* tissue samples, fresh/frozen *vs*. FFPE tissue, and statistical analyses) [118–140]. Nonetheless, a number of studies will be discussed vis-à-vis the application of microarray technology for diagnostic and prognostic purposes in the clinical setting.

In an early study of 45 primary melanomas and 18 benign melanocytic nevi, Talantov et al. [124] identified novel melanoma-specific genes, and confirmed the utility of a two-gene (PLAB and L1CAM) RT-PCR assay to distinguish between benign and malignant melanocytes in skin and LN samples. Importantly, these two markers showed superior performance compared with those commonly employed to determine melanocytic differentiation, such as TYR, gp100, and MART-1 [124]. Koh et al. [125] successfully used archival FFPE tissues for microarray analysis, and also identified genes that were differentially expressed in melanomas and melanocytic nevi. However, in contrast to the study by Talantov et al. [124], expression of L1CAM was found to be decreased in melanomas [125]. In another report, Kashani-Sabet et al. [126] described an immunohistochemistry-based diagnostic assay for melanocytic tumors, using five markers [ARPC2, FN1, RGS1, WNT2, and SPP1 (osteopontin)] whose transcripts were found to be overexpressed in melanomas by prior gene expression profiling [127]. Both the intensity and pattern of expression of each marker were noted to be significantly different between melanomas and melanocytic nevi [127]. Based on comparison with the actual histopathological diagnoses, this commercially available multimarker assay is reported to show 95% specificity and 97% sensitivity for diagnosing melanomas arising in melanocytic nevi, 95% accuracy in identifying both Spitz nevi and dysplastic nevi, and 75% accuracy in correctly diagnosing previously misinterpreted melanocytic lesions [126, 128]. Finally, another company is offering a patented combined noninvasive/tape-stripping and gene-based assay (MelDTect™) for the detection of melanoma [129]. RNA is harvested from the surface layer of the skin, without the need for biopsy, and analyzed using a 19-gene classifier. This test is purported to demonstrate a sensitivity of 100% and a specificity of 88% in discriminating melanomas from melanocytic nevi (Sherman Chang, Ph.D., personal communication, 2010) [129].

DNA microarray technology has also been used to identify supplemental prognostic indicators to the Breslow thickness, as well as biomarkers of patient survival and treatment response. The most extensive study of this type was conducted on behalf of the Melanoma Group of the European Organization for the Research and Treatment of Cancer (EORTC) [130]. This group collected 83 primary melanomas from 58 patients, and used an oligonucleotide-based array to identify 254 genes that were associated with distant metastasis-free survival. Twenty three of these genes were studied at the protein level by immunohistochemistry, with the expression of five markers (MCM4, MCM3, MCM6, KPNA2, and geminin) found to be statistically associated with overall survival [130]. In multivariate regression analysis adjusted for tumor thickness, ulceration, age and sex, the expression of MCM4 and MCM6 were still significantly associated with overall survival in these patients [130]. A follow-up investigation by Kauffmann et al. [131] on 60 fresh/frozen primary melanomas (with and without metastases) determined that differential expression of 48 genes (predominantly overexpression of DNA repair genes) was associated with metastatic progression and poor prognosis. Gene expression profiling studies by Conway et al. [132] and Jewell et al. [133], on FFPE primary melanomas, confirmed that upregulation of SPP1 and DNA repair genes (predominantly those involved in double-strand break repair, RAD51, RAD52, and TOP2A) were associated with poor prognostic histopathological features and predicted reduced relapse-free survival. These data support the hypothesis that maintenance of genomic stability (via intact DNA repair pathways) is required for melanoma progression, and influences response to chemotherapeutic agents and radiotherapy [131, 133, 134]. From their prior cDNA microarray studies [128], Kashani-Sabet et al. [135] also described a three-marker (NCOA3, SPP1, and RGS1) immunohistochemistry-based assay with independent prognostic significance vis-à-vis SLN status and disease-specific survival in patients with primary melanoma. In another study, Mandruzzato et al. [136] correlated gene expression to survival in a cohort of 38 melanoma patients with metastatic disease (stages III and IV). A 30-probe-set survival prediction model was generated. Transcripts overexpressed in patients with longer survival included those associated with innate and acquired immunity (i.e., IL-4R, TNFAIP3, CD2), confirming the interplay between immunological mechanisms and the biological behavior of melanoma. In contrast, the

poor-survival group was characterized by the expression of genes related to cellular proliferation and tissue invasion (i.e., GJB2, CSPG4, MCM3) [136]. Investigations by John et al. [137], Bogunovic et al. [138], and Jönsson et al. [139] have also determined that transcriptome profiles are capable of distinguishing clinical outcomes in patients with metastatic melanoma. These studies have included both treated (i.e., radiotherapy, immunotherapy, and/or chemotherapy) and untreated patients with metastatic disease [136-139]. Finally, Augustine et al. [140] reported that gene expression signatures can be used to predict response to chemotherapy in patients with in-transit metastatic melanoma. Results of microarray-based studies indicate that metastatic melanoma is biologically diverse, and reiterate the importance of tailoring clinical trials to the molecular and cellular profiles of tumors in individual patients.

Epigenetic Biomarkers in Melanoma

Epigenetics refers to heritable changes in gene expression without an alteration in the primary sequence of genomic DNA. Modifications of genomic DNA methylation patterns (i.e., gene-specific hypermethylation and genome-wide hypomethylation), post-translational modifications of histones, and microRNA (miRNA) profiles are epigenetic alterations that are associated with melanoma pathobiology (reviewed in references [141–145]). Methylation usually takes place at so-called CpG islands, or regions of DNA rich in cytosine-guanine repeats, that are commonly located near the 5′ promoter regions of genes. CpG hypermethylation often results in an inhibition of tumor suppressor gene transcription. To date, ~80 genes have been shown to be associated with altered DNA methylation status in melanoma [141]. In addition, ~29 genes potentially regulated by histone modifications have also been described for this tumor [141]. MiRNAs are small, noncoding RNAs (~21–25 nucleotides in length) which inhibit RNA translation or promote RNA degradation. The list of miRNAs known to be altered in melanoma continues to grow [144, 145]. These epigenetic modifications play an important role in tumor development and progression by affecting key cellular pathways, including cell cycle regulation, signaling mechanisms, differentiation, DNA repair, apoptosis, invasion, and immune recognition.

Similar to transcriptomic-based strategies, epigenetic investigations are identifying changes associated with different stages of the melanoma progression pathway. For example, some epigenetic alterations are found with similar frequencies in primary and metastatic tumors (i.e., RAR-β2 methylation), suggesting that their modification is an early event in melanoma. Others show higher frequencies in advanced disease (i.e., MGMT, RASSF1A and DAPK methylation, ↑ miR-182, ↑ miR-122/222), supporting their role in melanoma progression [141]. Importantly, studies are identifying diagnostic, prognostic, and predictive markers that could have future implementation in the clinical management of melanoma patients. In addition, investigations continue to uncover novel therapeutic targets and promote the development of drugs with more specific epigenetic effects (i.e., pharmacologic inhibition of DNA methyltransferases and/or histone deacetylases) [141–145].

There have been relatively few attempts to exploit epigenetic changes as diagnostic markers of melanocytic tumors. Takata et al. [146] used methylation-specific multiplex ligation-dependent probe amplification (MLPA) to assess the promoter CpG methylation status of 25 known tumor suppressor genes in a series of melanomas and spitzoid melanocytic tumors. CpG methylation of multiple genes, including RARB, CDKN2A, CDKN2B, PTEN, RASSF1, TIMP3, and GSTP1, was identified in 10 of 24 primary melanomas, but in none of the Spitz nevi or atypical Spitz tumors examined. Because CpG methylation can be detected in archival FFPE tissue using rapid and sensitive methylation-specific PCR, the authors suggested that it may be promising adjunct diagnostic tool for melanocytic tumors [146].

Other studies have proposed that methylation patterns in melanoma may be used to identify biomarkers for diagnosis, prediction of disease outcome, and/or response to therapy [141]. For example, assessments of the methylation status of TSPY1, CYBA, MX1, MT2A, RPL37A, HSPB1, FABP5, and BAGE have been promoted as diagnostic and/or prognostic tools for melanoma [141]. In a study of 230 primary melanomas, Lahtz et al. [147] determined that PTEN methylation status is an independent predictor of impaired patient survival, although its prognostic relevance was not superior to histopathological parameters (i.e., tumor thickness and ulceration). In contrast, aberrant hypermethylation of the MINT31 locus was recently found to correlate with improved overall survival in melanoma patients with stage III disease [141, 148]. As discussed in Chap. 6, the methylation status of a number of genes in the peripheral blood of melanoma patients has also been studied. Serum methylated ER-α is noted to be a negative predictor of overall and progression-free survival in stage IV melanoma patients treated with biochemotherapy (dacarbazine or temozolomide, cisplatin, vinblastine, IFN-α2b, IL-2, and tamoxifen) [141, 149]. Circulating methylated RASSF1A is also found to inversely correlate with overall survival and response to biochemotherapy in melanoma patients [141, 150]. With regard to biomarkers that predict therapeutic response, the methylation status of TP73 is reported to be associated with increased *in vitro* sensitivity of melanoma cells to alkylating agents, including cisplatin [141, 151]. Finally, a trend toward a positive correlation is found between MGMT promoter methylation levels of ≥25% and the achievement of partial clinical responses to the alkylating agent temozolomide in patients with melanoma [141, 152].

In addition to identifying specific miRNAs that contribute to tumor pathogenesis, a number of studies have investigated the diagnostic and/or prognostic utility of miRNA signatures in melanocytic lesions [144, 145]. Lu et al. [153] and Rosenfeld et al. [154] reported that poorly differentiated tumors and metastatic tumors of unknown primary origin, including melanoma samples, could be accurately classified on the basis of their miRNA profiles. Satzger et al. [155] reported differential miRNA expression between benign and malignant melanocytic proliferations. Of note, miR-15b and miR-210 were significantly upregulated, and miR-34a was significantly downregulated, in melanomas compared with melanocytic nevi. In addition, elevated expression of miR-15b correlated with poor recurrence-free survival and overall survival in melanoma patients [155]. Jukic et al. [156] and Schultz et al. [157] have also identified ~72 miRNAs that are differentially expressed between melanoma and melanocytic nevi. Of note, members of the let-7 family of miRNAs are significantly downregulated in primary melanomas as compared with benign nevi, suggesting possible tumor suppressor roles for these molecules in melanoma [157]. Segura et al. [158] identified an 18-miRNA signature in FFPE melanoma metastases whose overexpression was significantly correlated with longer survival. A focused 6-miRNA signature (miR-150, miR-342-3p, miR-455-3p, miR-145, miR-155, and miR-497) was shown to significantly stratify stage III patients into “better” and “worse” prognostic categories. This signature was found to be an independent predictor of survival by multivariate Cox regression analysis [158]. Differentially expressed miRNAs were also observed in the corresponding primary tumors, suggesting that the miRNA signature could have a role in the prognostication of early cutaneous lesions [158]. Analyzing LN metastases, Caramuta et al. [159] determined that melanomas from patients with poor survival showed low expression of miR-191, whereas miR-193b, miR-365, miR-338, let-7i, and miR-193a were overexpressed (Fig. 5.9). Interestingly, low expression of specific miRNAs was noted to be associated with mutations of either BRAF (miR-193a, miR-338, miR-565) or NRAS (miR-663). However, differentially expressed miRNAs did not significantly correlate with age at diagnosis, gender, or the Breslow thickness of primary melanomas in their study [159]. Jukic et al. [156] also demonstrated that the miRNA expression profiles of primary melanomas differ among older and younger patients. Despite a limited set of tumor samples, LN disease in both age groups was

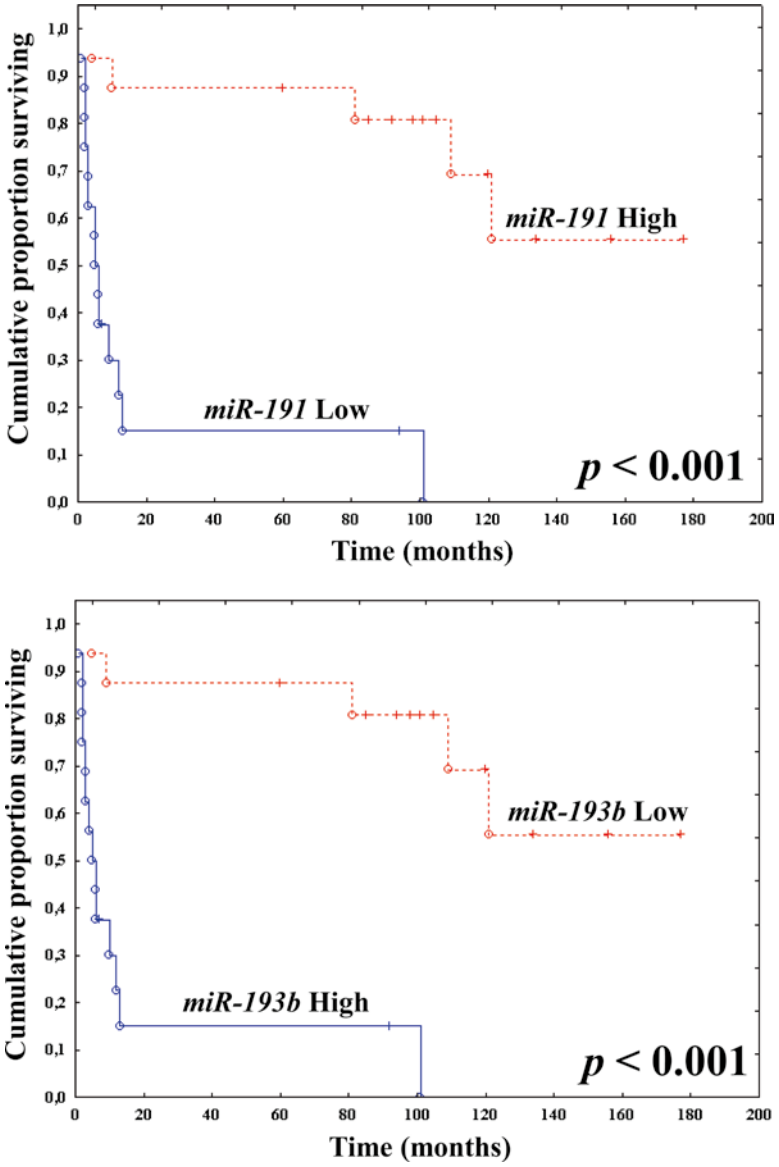

Fig. 5.9 Associations between microRNA expression levels and survival in melanoma. Kaplan-Meier plots illustrating significant associations between low miR-191 (*top panel*) and high miR-193b (*bottom panel*) expression and poor melanoma-specific survival in a cohort of 32 melanoma cases. High or low expression levels of the two microRNAs were determined by quantitative reverse transcription-polymerase chain reaction (*qRT-PCR*) analyses. Differences in survival were calculated using log-rank test (Courtesy of Dr. Stefano Caramuta, Department of Molecular Medicine and Surgery, Karolinska University Hospital, Stockholm, Sweden)

characterized by statistically significant upregulation of miR-204 and miR-30a* (* = derived from opposing arm of pre-miRNA) [156]. Finally, miRNAs have been shown to be stable in serum, and may potentially serve as biomarkers of disease in such samples (see Chap. 6) [160, 161].

Other Molecular Technologies

Analyses of loss of heterozygosity (LOH) or allelic imbalance (AI) reveal the presence of deletions or gains of specific alleles. PCR amplification of microsatellite polymorphic markers followed by gel electrophoresis is typically used for this analysis, and easily performed with DNA obtained from FFPE tissues. Healy et al. [162] allelotyped 41 primary cutaneous melanomas and 32 benign melanocytic nevi using 45 microsatellite markers that spanned all autosomal arms. Frequent AI on several chromosomal arms, including 9p, 10q, 6q, and 18q, was identified in primary melanomas. In contrast, 30 of 32 melanocytic nevi showed no AI. Two nevi with atypical histopathological features demonstrated AI, including a loss of 9p in one case. Two of 27 Spitz nevi also showed interstitial deletions of 9p. Thus, AI of 9p is not confined to melanoma; however, loss of 10q, 6q, and 18q could be markers of a malignant phenotype. Van Dijk et al. [163] tested the diagnostic utility of AI analysis in a series of 55 melanocytic tumors, including benign Spitz nevi, spitzoid melanocytic tumors of indeterminate biological potential (atypical Spitz tumors), and spitzoid melanomas. Twelve microsatellite markers that mapped to chromosomal arms 1p, 3p, 6q, 8q, 9p, 10q, and 11q were selected for testing. AI was identified in 2 of 12 (17%) typical Spitz nevi, 3 of 9 (33%) atypical Spitz tumors, 12 of 17 (65%) atypical Spitz tumors suspected of being melanomas, and 15 of 17 (88%) spitzoid melanomas [163]. The authors concluded that this approach appeared to have no clinical role in distinguishing between benign and malignant spitzoid tumors.

MLPA is a novel technique that can be used to measure the copy number of up to 45 nucleic acid sequences in a single reaction [104, 164, 165]. This method relies on sequence-specific probe hybridization to genomic DNA, followed by multiplex-PCR amplification of the hybridized probe, and semiquantitative analysis of the resulting PCR products. Takata et al. [164] examined copy number alterations in 55 FFPE melanocytic tumors (24 primary melanomas, 14 Spitz nevi, and 17 banal nevi), using commercially available MLPA kits against 76 target genes spanning almost all chromosomal arms. Multiple (>3) copy number gains and losses were found in all but two primary melanomas. In contrast, all of the Spitz nevi and banal nevi showed copy number changes at less than two loci. The threshold value of copy number aberrations corresponding to 98% specificity for melanoma was determined to be 2.42, with a sensitivity using this threshold value of 92.5% [164]. The results of this study suggested that MLPA could be used as an adjunct diagnostic tool for melanocytic tumors [164]. Importantly, MLPA shows high concordance with both FISH and CGH results, in addition to being more sensitive and less cumbersome than the latter technique [104, 165]. Using MLPA (and FISH), Cesinaro et al. [104] reported deletions of CDKN2A, CDKN2B and MTAP in atypical spitzoid tumors, but not in conventional Spitz nevi. However, these alterations did not appear to correlate with metastasis or prognosis in patients with spitzoid tumors [104].

Studies by Kauffmann et al. [166] and Lewis et al. [167] determined that standard and real-time RT-PCR-based approaches may also have diagnostic utility in the molecular classification of melanocytic tumors. Using standard RT-PCR on a sample set of 194 lesions, Kauffmann et al. [166] identified five markers (me20m, PLAB, SPP1, CAPG, and CTSB) that could reliably differentiate between melanoma and melanocytic nevi, with two of these markers (me20m and CTSB) capable of distinguishing melanoma from atypical or borderline nevi.

It is envisioned that newer technologies, such as SNP-based arrays, DNA sequencing methods (Fig. 6.7), and mass spectrometry-based proteomic strategies (Fig. 5.10), will be increasingly employed in the evaluation of melanocytic tumors, with results of molecular studies incorporated into current morphological-based diagnostic and prognostic classification systems (i.e., clinical findings and light microscopic changes) [168–170]. In addition to the wider use and acceptance of CGH, FISH, DNA microarrays, and epigenetic profiling tools, these genomic/proteomic technologies will: (a) facilitate more accurate diagnosis and classification of melanocytic tumors;

(b) improve on current staging criteria, leading to better stratification of melanoma patients into prognostically relevant groups; and (c) promote the individualization of melanoma therapy ("personalized medicine"), based on a patient's germline genetic variation, somatic genomic aberrations that arise during tumor development, and protein abundance, structure, stability, and function in established tumors.

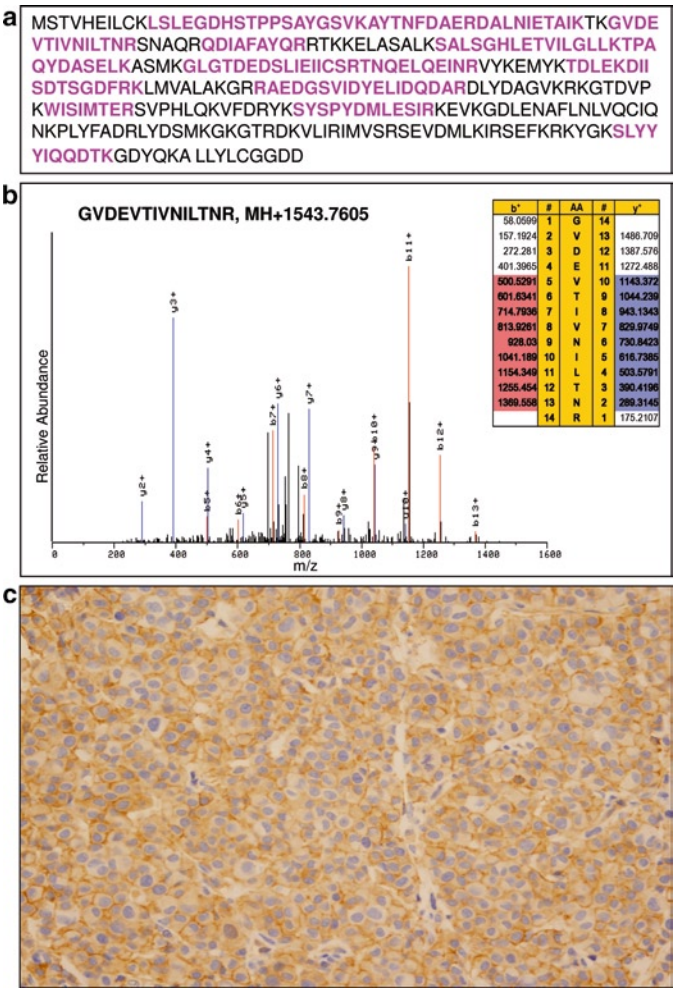

Fig. 5.10 Identification of Annexin A2 (ANXA2) protein in melanoma using a combined mass spectrometry (*MS*)- and immunohistochemistry (*IHC*)-based approach. (**a**) A total of 15 tryptic peptides matched the ANXA2 protein sequence (ending with R or K, and shown in bold and pink colored). (**b**) Tandem MS/MS spectrum from m/z 1543.7605 $[M+H]^+$, corresponding to the peptide GVDEVTIVNILTNR from ANXA2. Detected *b*- and *y*-series ions are highlighted in red and blue colors, respectively. (**c**) Validation of ANXA2 expression by IHC (Courtesy of Drs. David Han and Karim Rezaul, Department of Cell Biology, Center for Vascular Biology, University of Connecticut Health Center, Farmington, CT, USA)

References

1. American Cancer Society. Available at http://www.cancer.org/Cancer/SkinCancer-Melanoma/DetailedGuide/melanoma-skin-cancer-key-statistics. Accessed 24 Augt 2010.
2. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005;353:2135–47.
3. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417:949–54.
4. Pollock PM, Harper UL, Hansen KS, et al. High frequency of BRAF mutations in nevi. Nat Genet. 2003;33:19–20.
5. Da Forno PD, Fletcher A, Pringle JH, et al. Understanding spitzoid tumours: new insights from molecular pathology. Br J Dermatol. 2008;158:4–14.
6. Michaloglou C, Vredeveld LC, Soengas MS, et al. BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature. 2005;436:720–4.
7. Wajapeyee N, Serra RW, Zhu X, et al. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. Cell. 2008;132:363–74.
8. Scurr LL, Pupo GM, Becker TM, et al. IGFBP7 is not required for B-RAF-induced melanocyte senescence. Cell. 2010;141:717–27.
9. Viros A, Fridlyand J, Bauer J, et al. Improving melanoma classification by integrating genetic and morphologic features. PLoS Med. 2008;5:e120.
10. Edlundh-Rose E, Egyházi S, Omholt K, et al. NRAS and BRAF mutations in melanoma tumours in relation to clinical characteristics: a study based on mutation screening by pyrosequencing. Melanoma Res. 2006;16:471–8.
11. Akslen LA, Angelini S, Straume O, et al. BRAF and NRAS mutations are frequent in nodular melanoma but are not associated with tumor cell proliferation or patient survival. J Invest Dermatol. 2005;125:312–7.
12. Kannengiesser C, Spatz A, Michiels S, et al. Gene expression signature associated with BRAF mutations in human primary cutaneous melanomas. Mol Oncol. 2008;1:425–30.
13. Greene VR, Johnson MM, Grimm EA, et al. Frequencies of NRAS and BRAF mutations increase from the radial to the vertical growth phase in cutaneous melanoma. J Invest Dermatol. 2009;129:1483–8.
14. Ji Z, Flaherty KT, Tsao H. Molecular therapeutic approaches to melanoma. Mol Aspects Med. 2010;31:194–204.
15. Flaherty KT, Hodi FS, Bastian BC. Mutation-driven drug development in melanoma. Curr Opin Oncol. 2010;22:178–83.
16. Flaherty KT, McArthur G. BRAF, a target in melanoma: implications for solid tumor drug development. Cancer. 2010;116:4902–13.
17. Celebi JT, Ward KM, Wanner M, et al. Evaluation of germline CDKN2A, ARF, CDK4, PTEN, and BRAF alterations in atypical mole syndrome. Clin Exp Dermatol. 2005;30:68–70.
18. Gast A, Scherer D, Chen B, et al. Somatic alterations in the melanoma genome: a high-resolution array-based comparative genomic hybridization study. Genes Chromosomes Cancer. 2010;49:733–45.
19. Daniotti M, Oggionni M, Ranzani T, et al. BRAF alterations are associated with complex mutational profiles in malignant melanoma. Oncogene. 2004;23:5968–77.
20. Jönsson G, Dahl C, Staaf J, et al. Genomic profiling of malignant melanoma using tiling-resolution arrayCGH. Oncogene. 2007;26:4738–48.
21. Eskandarpour M, Kiaii S, Zhu C, et al. Suppression of oncogenic NRAS by RNA interference induces apoptosis of human melanoma cells. Int J Cancer. 2005;115:65–73.
22. Eskandarpour M, Huang F, Reeves KA, et al. Oncogenic NRAS has multiple effects on the malignant phenotype of human melanoma cells cultured in vitro. Int J Cancer. 2009;124:16–26.
23. Goel VK, Lazar AJ, Warneke CL, et al. Examination of mutations in BRAF, NRAS, and PTEN in primary cutaneous melanoma. J Invest Dermatol. 2006;126:154–60.
24. Platz A, Egyhazi S, Ringborg U, et al. Human cutaneous melanoma; a review of NRAS and BRAF mutation frequencies in relation to histogenetic subclass and body site. Mol Oncol. 2008;1:395–405.
25. Poynter JN, Elder JT, Fullen DR, et al. BRAF and NRAS mutations in melanoma and melanocytic nevi. Melanoma Res. 2006;16:267–73.
26. Rosso R, Romagosa Y, Kirsner RS. Progression of NRAS and BRAF mutations in cutaneous melanoma. J Invest Dermatol. 2009;129:1318.
27. Ichii-Nakato N, Takata M, Takayanagi S, et al. High frequency of BRAFV600E mutation in acquired nevi and small congenital nevi, but low frequency of mutation in medium-sized congenital nevi. J Invest Dermatol. 2006;126:2111–8.
28. Bauer J, Curtin JA, Pinkel D, et al. Congenital melanocytic nevi frequently harbor NRAS mutations but no BRAF mutations. J Invest Dermatol. 2007;127:179–82.
29. Curtin JA, Busam K, Pinkel D, et al. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol. 2006;24:4340–6.

30. Davies MA, Samuels Y. Analysis of the genome to personalize therapy for melanoma. Oncogene. 2010;29:5545–55.
31. Blokx WA, van Dijk MC, Ruiter DJ. Molecular cytogenetics of cutaneous melanocytic lesions – diagnostic, prognostic and therapeutic aspects. Histopathology. 2010;56:121–32.
32. Torres-Cabala CA, Wang WL, Trent J, et al. Correlation between KIT expression and KIT mutation in melanoma: a study of 173 cases with emphasis on the acral-lentiginous/mucosal type. Mod Pathol. 2009;22:1446–56.
33. Ashida A, Takata M, Murata H, et al. Pathological activation of KIT in metastatic tumors of acral and mucosal melanomas. Int J Cancer. 2009;124:862–8.
34. Rivera RS, Nagatsuka H, Gunduz M, et al. C-kit protein expression correlated with activating mutations in KIT gene in oral mucosal melanoma. Virchows Arch. 2008;452:27–32.
35. Fisher DE. Microphthalmia: a signal responsive transcriptional regulator in development. Pigment Cell Res. 2000;13 Suppl 8:145–9.
36. Jiang X, Zhou J, Yuen NK, et al. Imatinib targeting of KIT-mutant oncoprotein in melanoma. Clin Cancer Res. 2008;14:7726–32.
37. Hodi FS, Friedlander P, Corless CL, et al. Major response to imatinib mesylate in KIT-mutated melanoma. J Clin Oncol. 2008;26:2046–51.
38. Lutzky J, Bauer J, Bastian BC. Dose-dependent, complete response to imatinib of a metastatic mucosal melanoma with a K642E KIT mutation. Pigment Cell Melanoma Res. 2008;21:492–3.
39. Quintás-Cardama A, Lazar AJ, Woodman SE, et al. Complete response of stage IV anal mucosal melanoma expressing KIT Val560Asp to the multikinase inhibitor sorafenib. Nat Clin Pract Oncol. 2008;5:737–40.
40. Wyman K, Atkins MB, Prieto V, et al. Multicenter Phase II trial of high-dose imatinib mesylate in metastatic melanoma: significant toxicity with no clinical efficacy. Cancer. 2006;106:2005–11.
41. Hofmann UB, Kauczok-Vetter CS, Houben R, et al. Overexpression of the KIT/SCF in uveal melanoma does not translate into clinical efficacy of imatinib mesylate. Clin Cancer Res. 2009;15:324–9.
42. Ivan D, Niveiro M, Diwan AH, et al. Analysis of protein tyrosine kinases expression in the melanoma metastases of patients treated with Imatinib Mesylate (STI571, Gleevec). J Cutan Pathol. 2006;33:280–5.
43. Dankort D, Curley DP, Cartlidge RA, et al. Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. Nat Genet. 2009;41:544–52.
44. Madhunapantula SV, Robertson GP. The PTEN-AKT3 signaling cascade as a therapeutic target in melanoma. Pigment Cell Melanoma Res. 2009;22:400–19.
45. Bastian BC, Wesselmann U, Pinkel D, et al. Molecular cytogenetic analysis of Spitz nevi shows clear differences to melanoma. J Invest Dermatol. 1999;113:1065–9.
46. Bastian BC, LeBoit PE, Pinkel D. Mutations and copy number increase of HRAS in Spitz nevi with distinctive histopathological features. Am J Pathol. 2000;157:967–72.
47. Van Raamsdonk CD, Bezrookove V, Green G, et al. Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature. 2009;457:599–602.
48. Gerami P, Pouryazdanparast P, Vemula S, et al. Molecular analysis of a case of nevus of Ota showing progressive evolution to melanoma with intermediate stages resembling cellular blue nevus. Am J Dermatopathol. 2010;32:301–5.
49. Leachman SA, Carucci J, Kohlmann W, et al. Selection criteria for genetic assessment of patients with familial melanoma. J Am Acad Dermatol. 2009;61:677.e1–14.
50. Udayakumar D, Tsao H. Melanoma genetics: an update on risk-associated genes. Hematol Oncol Clin North Am. 2009;23:415–29.vii.
51. Kohlmann W, Dunn K, Leachman S. Role of genetic testing in hereditary melanoma. Expert Rev Dermatol. 2008;3:639–43.
52. Pho LN, Leachman SA. Genetics of pigmentation and melanoma predisposition. G Ital Dermatol Venereol. 2010;145:37–45.
53. Valverde P, Healy E, Sikkink S, et al. The Asp84Glu variant of the melanocortin 1 receptor (MC1R) is associated with melanoma. Hum Mol Genet. 1996;5:1663–6.
54. Kennedy C, ter Huurne J, Berkhout M, et al. Melanocortin 1 receptor (MC1R) gene variants are associated with an increased risk for cutaneous melanoma which is largely independent of skin type and hair color. J Invest Dermatol. 2001;117:294–300.
55. Box NF, Duffy DL, Chen W, et al. MC1R genotype modifies risk of melanoma in families segregating CDKN2A mutations. Am J Hum Genet. 2001;69:765–73.
56. Landi MT, Bauer J, Pfeiffer RM, et al. MC1R germline variants confer risk for BRAF-mutant melanoma. Science. 2006;313:521–2.
57. Fargnoli MC, Pike K, Pfeiffer RM, et al. MC1R variants increase risk of melanomas harboring BRAF mutations. J Invest Dermatol. 2008;128:2485–90.
58. Hansen C, Wilkinson D, Hansen M, et al. How good are skin cancer clinics at melanoma detection? Number needed to treat variability across a national clinic group in Australia. J Am Acad Dermatol. 2009;61:599–604.

59. Piepkorn M, Busam KJ, Barnhill R, editors. Pathology of melanocytic nevi and malignant melanoma. 2nd ed. New York: Springer; 2004.
60. Massi D, LeBoit P, editors. Histological diagnosis of nevi and melanoma. Wurzburg, Germany: Steinkopff Verlag Darmstadt; 2004.
61. Mooi WJ, Krausz T, editors. Pathology of melanocytic disorders. 2nd ed. New York: Oxford University Press; 2007.
62. McGinnis KS, Lessin SR, Elder DE, et al. Pathology review of cases presenting to a multidisciplinary pigmented lesion clinic. Arch Dermatol. 2002;138:617–21.
63. Lodha S, Saggar S, Celebi JT, et al. Discordance in the histopathologic diagnosis of difficult melanocytic neoplasms in the clinical setting. J Cutan Pathol. 2008;35:349–52.
64. Barnhill RL, Argenyi ZB, From L, et al. Atypical Spitz nevi/tumors: lack of consensus for diagnosis, discrimination from melanoma, and prediction of outcome. Hum Pathol. 1999;30:513–20.
65. Ludgate MW, Fullen DR, Lee J, et al. The atypical Spitz tumor of uncertain biologic potential: a series of 67 patients from a single institution. Cancer. 2009;115:631–41.
66. Mandal RV, Murali R, Lundquist KF, et al. Pigmented epithelioid melanocytoma: favorable outcome after 5-year follow-up. Am J Surg Pathol. 2009;33:1778–82.
67. Prieto VG, Shea CR. Use of immunohistochemistry in melanocytic lesions. J Cutan Pathol. 2008;35 Suppl 2:1–10.
68. Horst B, Ohsie S, Binder SW. Protein biomarkers: differentiation of melanocytic tumors. In: Murphy M, editor. Diagnostic and prognostic biomarkers and therapeutic targets in melanoma. New York: Springer; in press.
69. Horst B, Ohsie S, Binder SW. Protein biomarkers: prognostication of melanoma. In: Murphy M, editor. Diagnostic and prognostic biomarkers and therapeutic targets in melanoma. New York: Springer; in press.
70. Gould Rothberg BE, Rimm DL. Biomarkers: the useful and the not so useful – an assessment of molecular prognostic markers for cutaneous melanoma. J Invest Dermatol. 2010;130:1971–87.
71. Gould BER, Bracken MB, Rimm DL. Tissue biomarkers for prognosis in cutaneous melanoma: a systematic review and meta-analysis. J Natl Cancer Inst. 2009;101:452–74.
72. Bastian BC, LeBoit PE, Hamm H, et al. Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization. Cancer Res. 1998;58:2170–5.
73. Ali L, Helm T, Cheney R, et al. Correlating array comparative genomic hybridization findings with histology and outcome in spitzoid melanocytic neoplasms. Int J Clin Exp Pathol. 2010;3:593–9.
74. Vincek V, Xu S, Fan YS. Comparative genome hybridization analysis of laser-capture microdissected in situ melanoma. J Cutan Pathol. 2010;37:3–7.
75. Takata M, Maruo K, Kageshita T, et al. Two cases of unusual acral melanocytic tumors: illustration of molecular cytogenetics as a diagnostic tool. Hum Pathol. 2003;34:89–92.
76. Harvell JD, Kohler S, Zhu S, et al. High-resolution array-based comparative genomic hybridization for distinguishing paraffin-embedded Spitz nevi and melanomas. Diagn Mol Pathol. 2004;13:22–5.
77. Mihic-Probst D, Zhao J, Saremaslani P, et al. Spitzoid malignant melanoma with lymph-node metastasis. Is a copy-number loss on chromosome 6q a marker of malignancy? Virchows Arch. 2001;439:823–6.
78. Balázs M, Adám Z, Treszl A, et al. Chromosomal imbalances in primary and metastatic melanomas revealed by comparative genomic hybridization. Cytometry. 2001;46:222–32.
79. Maize Jr JC, McCalmont TH, Carlson JA, et al. Genomic analysis of blue nevi and related dermal melanocytic proliferations. Am J Surg Pathol. 2005;29:1214–20.
80. Bastian BC, Xiong J, Frieden IJ, et al. Genetic changes in neoplasms arising in congenital melanocytic nevi: differences between nodular proliferations and melanomas. Am J Pathol. 2002;161:1163–9.
81. Murphy MJ, Jen M, Chang MW, et al. Molecular diagnosis of a benign proliferative nodule developing in a congenital melanocytic nevus in a 3-month-old infant. J Am Acad Dermatol. 2008;59:518–23.
82. Gaiser T, Kutzner H, Palmedo G, et al. Classifying ambiguous melanocytic lesions with FISH and correlation with clinical long-term follow up. Mod Pathol. 2010;23:413–9.
83. Gerami P, Jewell SS, Morrison LE, et al. Fluorescence in situ hybridization (FISH) as an ancillary diagnostic tool in the diagnosis of melanoma. Am J Surg Pathol. 2009;33:1146–56. Erratum in: Am J Surg Pathol. 2010;34:688.
84. Gerami P, Wass A, Mafee M, et al. Fluorescence in situ hybridization for distinguishing nevoid melanomas from mitotically active nevi. Am J Surg Pathol. 2009;33:1783–8.
85. Pouryazdanparast P, Newman M, Mafee M, et al. Distinguishing epithelioid blue nevus from blue nevus-like cutaneous melanoma metastasis using fluorescence in situ hybridization. Am J Surg Pathol. 2009;33:1396–400.
86. Dalton SR, Gerami P, Kolaitis NA, et al. Use of fluorescence in situ hybridization (FISH) to distinguish intranodal nevus from metastatic melanoma. Am J Surg Pathol. 2010;34:231–7.
87. Gerami P, Barnhill RL, Beilfuss BA, et al. Superficial melanocytic neoplasms with pagetoid melanocytosis: a study of interobserver concordance and correlation with FISH. Am J Surg Pathol. 2010;34:816–21.
88. Newman MD, Lertsburapa T, Mirzabeigi M, et al. Fluorescence in situ hybridization as a tool for microstaging in malignant melanoma. Mod Pathol. 2009;22:989–95.

89. Newman MD, Mirzabeigi M, Gerami P. Chromosomal copy number changes supporting the classification of lentiginous junctional melanoma of the elderly as a subtype of melanoma. Mod Pathol. 2009;22:1258–62.
90. Busam KJ, Fang Y, Jhanwar SC, et al. Distinction of conjunctival melanocytic nevi from melanomas by fluorescence in situ hybridization. J Cutan Pathol. 2010;37:196–203.
91. Clemente C, Bettio D, Venci A, et al. A fluorescence in situ hybridization (FISH) procedure to assist in differentiating benign from malignant melanocytic lesions. Pathologica. 2009;101:169–74.
92. Morey AL, Murali R, McCarthy SW, et al. Diagnosis of cutaneous melanocytic tumours by four-colour fluorescence in situ hybridisation. Pathology. 2009;41:383–7.
93. Zimmermann AK, Hirschmann A, Pfeiffer D, et al. FISH analysis for diagnostic evaluation of challenging melanocytic lesions. Histol Histopathol. 2010;25:1139–47.
94. Boi S, Leonardi E, Fasanella S, et al. The four-color FISH probe in the diagnosis of melanocytic lesions. J Eur Acad Dermatol Venereol. 2010;24:1235–6.
95. Battistella M, Prochazkova-Carlotti M, Berrebi D, et al. Two congenital cases of pigmented epithelioid melanocytoma studied by fluorescent in situ hybridization for melanocytic tumors: case reports and review of these recent topics. Dermatology. 2010;221:97–106.
96. Yamaura M, Takata M, Miyazaki A, et al. Specific dermoscopy patterns and amplifications of the cyclin D1 gene to define histopathologically unrecognizable early lesions of acral melanoma in situ. Arch Dermatol. 2005;141:1413–8.
97. North JP, Kageshita T, Pinkel D, et al. Distribution and significance of occult intraepidermal tumor cells surrounding primary melanoma. J Invest Dermatol. 2008;128:2024–30.
98. Isaac AK, Lertsburapa T, Pathria JM, et al. Polyploidy in Spitz nevi: a not uncommon karyotypic abnormality identifiable by fluorescence in situ hybridization. Am J Dermatopathol. 2010;32:144–8.
99. Gerami P, Mafee M, Lurtsbarapa T, et al. Sensitivity of fluorescence in situ hybridization for melanoma diagnosis using RREB1, MYB, Cep6, and 11q13 probes in melanoma subtypes. Arch Dermatol. 2010;146:273–8.
100. Glatz-Krieger K, Pache M, Tapia C, et al. Anatomic site-specific patterns of gene copy number gains in skin, mucosal, and uveal melanomas detected by fluorescence in situ hybridization. Virchows Arch. 2006;449:328–33.
101. Pouryazdanparast P, Newman M, Mafee M, et al. Malignant melanoma with monster cells showing massive cyclin D1 amplification. Am J Dermatopathol. 2009;31:402–3.
102. Gerami P, Guitart J, Martini M, et al. Cyclin D1 homogeneous staining regions by fluorescent in situ hybridization: a possible indicator of aggressive behavior in melanomas. Arch Dermatol. 2008;144:1235–6.
103. Rákosy Z, Vízkeleti L, Ecsedi S, et al. Characterization of 9p21 copy number alterations in human melanoma by fluorescence in situ hybridization. Cancer Genet Cytogenet. 2008;182:116–21.
104. Cesinaro AM, Schirosi L, Bettelli S, et al. Alterations of 9p21 analysed by FISH and MLPA distinguish atypical spitzoid melanocytic tumours from conventional Spitz's nevi but do not predict their biological behaviour. Histopathology. 2010;57:515–27.
105. Ryan D, Rafferty M, Hegarty S, et al. Topoisomerase I amplification in melanoma is associated with more advanced tumours and poor prognosis. Pigment Cell Melanoma Res. 2010;23:542–53.
106. Casorzo L, Luzzi C, Nardacchione A, et al. Fluorescence in situ hybridization (FISH) evaluation of chromosomes 6, 7, 9 and 10 throughout human melanocytic tumorigenesis. Melanoma Res. 2005;15:155–60.
107. Wettengel GV, Draeger J, Kiesewetter F, et al. Differentiation between Spitz nevi and malignant melanomas by interphase fluorescence in situ hybridization. Int J Oncol. 1999;14:1177–83.
108. Moore SR, Persons DL, Sosman JA, et al. Detection of copy number alterations in metastatic melanoma by a DNA fluorescence in situ hybridization probe panel and array comparative genomic hybridization: a southwest oncology group study (S9431). Clin Cancer Res. 2008;14:2927–35.
109. Sini MC, Manca A, Cossu A, et al. Molecular alterations at chromosome 9p21 in melanocytic naevi and melanoma. Br J Dermatol. 2008;158:243–50.
110. Hammock L, Cohen C, Carlson G, et al. Chromogenic in situ hybridization analysis of melastatin mRNA expression in melanomas from American Joint Committee on Cancer stage I and II patients with recurrent melanoma. J Cutan Pathol. 2006;33:599–607.
111. Song JS, Choi J, Kim JH, et al. Diagnostic utility of EWS break-apart fluorescence in situ hybridization in distinguishing between non-cutaneous melanoma and clear cell sarcoma. Pathol Int. 2010;60:608–13.
112. Hisaoka M, Ishida T, Kuo TT, et al. Clear cell sarcoma of soft tissue: a clinicopathologic, immunohistochemical, and molecular analysis of 33 cases. Am J Surg Pathol. 2008;32:452–60.
113. Wang WL, Mayordomo E, Zhang W, et al. Detection and characterization of EWSR1/ATF1 and EWSR1/CREB1 chimeric transcripts in clear cell sarcoma (melanoma of soft parts). Mod Pathol. 2009;22:1201–9.
114. Antonescu CR, Nafa K, Segal NH, et al. EWS-CREB1: a recurrent variant fusion in clear cell sarcoma–association with gastrointestinal location and absence of melanocytic differentiation. Clin Cancer Res. 2006;12:5356–62.
115. Antonescu CR, Tschernyavsky SJ, Woodruff JM, et al. Molecular diagnosis of clear cell sarcoma: detection of EWS-ATF1 and MITF-M transcripts and histopathological and ultrastructural analysis of 12 cases. J Mol Diagn. 2002;4:44–52.

116. Covinsky M, Gong S, Rajaram V, et al. EWS-ATF1 fusion transcripts in gastrointestinal tumors previously diagnosed as malignant melanoma. Hum Pathol. 2005;36:74–81.
117. Modrek B, Ge L, Pandita A, Lin E, et al. Oncogenic activating mutations are associated with local copy gain. Mol Cancer Res. 2009;7:1244–52.
118. Chiorino G, Scatolini M. mRNA biomarkers: gene expression profiling studies. In: Murphy M, editor. Diagnostic and prognostic biomarkers and therapeutic targets in melanoma. New York: Springer; in press.
119. Hoek KS. DNA microarray analyses of melanoma gene expression: a decade in the mines. Pigment Cell Res. 2007;20:466–84.
120. Ren S, Liu S, Howell Jr P, et al. The impact of genomics in understanding human melanoma progression and metastasis. Cancer Control. 2008;15:202–15.
121. Scatolini M, Grand MM, Grosso E, et al. Altered molecular pathways in melanocytic lesions. Int J Cancer. 2010;126:1869–81.
122. Riker AI, Enkemann SA, Fodstad O, et al. The gene expression profiles of primary and metastatic melanoma yields a transition point of tumor progression and metastasis. BMC Med Genomics. 2008;1:13.
123. Hoek KS, Eichhoff OM, Schlegel NC, et al. In vivo switching of human melanoma cells between proliferative and invasive states. Cancer Res. 2008;68:650–6.
124. Talantov D, Mazumder A, Yu JX, et al. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res. 2005;11:7234–42.
125. Koh SS, Opel ML, Wei JP, et al. Molecular classification of melanomas and nevi using gene expression microarray signatures and formalin-fixed and paraffin-embedded tissue. Mod Pathol. 2009;22:538–46.
126. Kashani-Sabet M, Rangel J, Torabian S, et al. A multi-marker assay to distinguish malignant melanomas from benign nevi. Proc Natl Acad Sci USA. 2009;106:6268–72.
127. Haqq C, Nosrati M, Sudilovsky D, et al. The gene expression signatures of melanoma progression. Proc Natl Acad Sci USA. 2005;102:6092–7.
128. Melanoma Diagnostics. http://www.melanomadiagnostics.com/clinical/. Accessed 2 Sept 2010.
129. DermTech International. http://dermtech.com/technology/melanoma-detection/index.php. Accessed 2 Sept 2010.
130. Winnepenninckx V, Lazar V, Michiels S, et al. Melanoma Group of the European Organization for Research and Treatment of Cancer. Gene expression profiling of primary cutaneous melanoma and clinical outcome. J Natl Cancer Inst. 2006;98:472–82.
131. Kauffmann A, Rosselli F, Lazar V, et al. High expression of DNA repair pathways is associated with metastasis in melanoma patients. Oncogene. 2008;27:565–73.
132. Conway C, Mitra A, Jewell R, et al. Gene expression profiling of paraffin-embedded primary melanoma using the DASL assay identifies increased osteopontin expression as predictive of reduced relapse-free survival. Clin Cancer Res. 2009;15:6939–46.
133. Jewell R, Conway C, Mitra A, et al. Patterns of expression of DNA repair genes and relapse from melanoma. Clin Cancer Res. 2010;16:5211–21.
134. Sarasin A, Kauffmann A. Overexpression of DNA repair genes is associated with metastasis: a new hypothesis. Mutat Res. 2008;659:49–55.
135. Kashani-Sabet M, Venna S, Nosrati M, et al. A multimarker prognostic assay for primary cutaneous melanoma. Clin Cancer Res. 2009;15:6987–92.
136. Mandruzzato S, Callegaro A, Turcatel G, et al. A gene expression signature associated with survival in metastatic melanoma. J Transl Med. 2006;4:50.
137. John T, Black MA, Toro TT, et al. Predicting clinical outcome through molecular profiling in stage III melanoma. Clin Cancer Res. 2008;14:5173–80.
138. Bogunovic D, O'Neill DW, Belitskaya-Levy I, et al. Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. Proc Natl Acad Sci USA. 2009;106:20429–34.
139. Jönsson G, Busch C, Knappskog S, et al. Gene expression profiling-based identification of molecular subtypes in stage IV melanomas with different clinical outcome. Clin Cancer Res. 2010;16:3356–67.
140. Augustine CK, Jung SH, Sohn I, et al. Gene expression signatures as a guide to treatment strategies for in-transit metastatic melanoma. Mol Cancer Ther. 2010;9:779–90.
141. Sigalotti L, Covre A, Fratta E, et al. Epigenetics of human cutaneous melanoma: setting the stage for new therapeutic strategies. J Transl Med. 2010;8:56.
142. Howell Jr PM, Liu S, Ren S, et al. Epigenetics in human melanoma. Cancer Control. 2009;16:200–18.
143. Liu S, Ren S, Howell Jr PM, Riker AI. Epigenetic biomarkers. In: Murphy M, editor. Diagnostic and prognostic biomarkers and therapeutic targets in melanoma. New York: Springer; in press.
144. Mueller DW, Bosserhoff AK. The evolving concept of 'melano-miRs'- microRNAs in melanomagenesis. Pigment Cell Melanoma Res. 2010;23:620–6.
145. Mueller DW, Bosserhoff AK. Role of miRNAs in the progression of malignant melanoma. Br J Cancer. 2009;101:551–6.

146. Takata M, Lin J, Takayanagi S, et al. Genetic and epigenetic alterations in the differential diagnosis of malignant melanoma and spitzoid lesion. Br J Dermatol. 2007;156:1287–94.
147. Lahtz C, Stranzenbach R, Fiedler E, et al. Methylation of PTEN as a prognostic factor in malignant melanoma of the skin. J Invest Dermatol. 2010;130:620–2.
148. Tanemura A, Terando AM, Sim MS, et al. CpG island methylator phenotype predicts progression of malignant melanoma. Clin Cancer Res. 2009;15:1801–7.
149. Mori T, Martinez SR, O'Day SJ, et al. Estrogen receptor-alpha methylation predicts melanoma progression. Cancer Res. 2006;66:6692–8.
150. Mori T, O'Day SJ, Umetani N, et al. Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. J Clin Oncol. 2005;23:9351–8.
151. Shen L, Kondo Y, Ahmed S, et al. Drug sensitivity prediction by CpG island methylation profile in the NCI-60 cancer cell line panel. Cancer Res. 2007;67:11335–43.
152. Rietschel P, Wolchok JD, Krown S, et al. Phase II study of extended-dose temozolomide in patients with melanoma. J Clin Oncol. 2008;26:2299–304.
153. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834–8.
154. Rosenfeld N, Aharonov R, Meiri E, et al. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol. 2008;26:462–9.
155. Satzger I, Mattern A, Kuettler U, et al. MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. Int J Cancer. 2010;126:2553–62.
156. Jukic DM, Rao UN, Kelly L, et al. MicroRNA profiling analysis of differences between the melanoma of young adults and older adults. J Transl Med. 2010;8:27.
157. Schultz J, Lorenz P, Gross G, et al. MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. Cell Res. 2008;18:549–57.
158. Segura MF, Belitskaya-Lévy I, Rose AE, et al. Melanoma MicroRNA signature predicts post-recurrence survival. Clin Cancer Res. 2010;16:1577–86.
159. Caramuta S, Egyházi S, Rodolfo M, et al. MicroRNA expression profiles associated with mutational status and survival in malignant melanoma. J Invest Dermatol. 2010;130:2062–70.
160. Leidinger P, Keller A, Borries A, et al. High-throughput miRNA profiling of human melanoma blood samples. BMC Cancer. 2010;10:262.
161. Heneghan HM, Miller N, Kelly R, et al. Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. Oncologist. 2010;15:673–82.
162. Healy E, Belgaid CE, Takata M, et al. Allelotypes of primary cutaneous melanoma and benign melanocytic nevi. Cancer Res. 1996;56:589–93.
163. van Dijk MC, Rombout PD, Mooi WJ, et al. Allelic imbalance in the diagnosis of benign, atypical and malignant Spitz tumours. J Pathol. 2002;197:170–8.
164. Takata M, Suzuki T, Ansai S, et al. Genome profiling of melanocytic tumors using multiplex ligation-dependent probe amplification (MLPA): its usefulness as an adjunctive diagnostic tool for melanocytic tumors. J Dermatol Sci. 2005;40:51–7.
165. van Dijk MC, Rombout PD, Boots-Sprenger SH, et al. Multiplex ligation-dependent probe amplification for the detection of chromosomal gains and losses in formalin-fixed tissue. Diagn Mol Pathol. 2005;14:9–16.
166. Kauffman L, Palma J, Wang Y, et al. Gene expression markers to facilitate melanoma diagnosis in skin biopsies [abstract]. J Cutan Pathol. 2009;36:110.
167. Lewis TB, Robison JE, Bastien R, et al. Molecular classification of melanoma using real-time quantitative reverse transcriptase-polymerase chain reaction. Cancer. 2005;104:1678–86.
168. Dadzie OE, Yang S, Emley A, et al. RAS and RAF mutations in banal melanocytic aggregates contiguous with primary cutaneous melanoma: clues to melanomagenesis. Br J Dermatol. 2009;160:368–75.
169. Rezaul K, Murphy M, Lundgren DH, et al. Combined mass spectrometry- and immunohistochemistry-based approach to determine protein expression in archival melanoma. Pigment Cell Melanoma Res. 2010; 23:849–52.

Chapter 6
Melanocytic Neoplasms II: Molecular Staging

Michael J. Murphy and J. Andrew Carlson

The TNM staging categories and groupings of the updated 2009 American Joint Committee on Cancer (AJCC) Melanoma Staging System are outlined in Tables 6.1 and 6.2 [1]. "*T*" parameters are defined by primary tumor thickness, ulceration, and mitotic status; "*N*" parameters by the number of lymph nodes with metastatic disease and extent of metastatic burden; and "*M*" parameters by the site(s) of the metastases and serum lactate dehydrogenase (LDH) levels [1]. The 5-year survival rate is ~90% for AJCC stage I melanoma and ~70% for AJCC stage II melanoma, but decreases significantly to 25–50% for AJCC stage III melanoma (depending on the number of lymph nodes involved), and ~10% for stage IV disease [2]. Because the identification of metastatic disease is a major prognostic factor for melanoma recurrence and outcome, accurate staging of this disease is important for optimal management of these patients. The clinical and histopathological features cannot accurately predict the behavior of melanoma in all cases [3]. Therefore, a need exists for biomarkers which would help to identify patients at risk for disease progression, in addition to those individuals whose disease has already progressed subclinically [3]. Numerous molecular biomarkers, which highlight the mechanisms of melanoma pathogenesis and progression, have been identified [3]. The clinical utility of a number of these molecular biomarkers, for improving upon routine histopathological methods in the staging and prognostication of melanoma patients, has also been investigated [3]. The role of molecular diagnostic techniques in the detection of (a) occult sentinel lymph node melanoma cells (SLNMCs) and (b) occult circulating melanoma cells (CMCs) is an area of active investigation and warrants further discussion. Although molecular analysis of melanocyte-related markers has also been undertaken in bone marrow specimens [4] and biological fluids (effusions and cerebrospinal fluid) [5, 6], the most commonly performed assays in melanoma patients have been on sentinel lymph nodes (SLNs) and peripheral blood (PB) specimens [7–10]. Metastatic melanoma cells are not found in either tissue sections or PB of normal individuals. Therefore, the detection of these cells in non-cutaneous specimens from melanoma patients with early-stage disease could identify those at high risk for metastasis. The amplification of tumor-related DNA or messenger RNA (mRNA) sequences, by such techniques as standard reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time polymerase chain reaction (qRT), has been undertaken in SLNs and PB of melanoma patients in an effort to detect the presence of occult tumor cells [7–10]. A list of specific and nonspecific molecular tumor markers used for the detection of SLNMCs and CMCs in melanoma patients is provided in Table 6.3. Of note, amplification of melanocyte-specific transcripts by standard RT-PCR allows for the detection of one melanoma cell among 10^6–10^7 non-tumor

M.J. Murphy (✉)
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, 21 South Rd., Farmington, CT 06030, USA
e-mail: drmichaelmurphy@netscape.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_6,

Table 6.1 TNM staging categories for cutaneous melanoma (Adapted from Balch et al. [1])

T	Thickness (mm)	Ulceration status/mitoses
Tis	NA	NA
T1	≤1.00	a. Without ulceration and mitoses <1/mm^2
		b. With ulceration or mitoses ≥1/mm^2
T2	1.01–2.00	a. Without ulceration
		b. With ulceration
T3	2.01–4.00	a. Without ulceration
		b. With ulceration
T4	>4.00	a. Without ulceration
		b. With ulceration
N	No. of metastatic nodes	Nodal metastatic burden
N0	0	NA
N1	1	a. Micrometastasis[a]
		b. Macrometastasis[b]
N2	2–3	a. Micrometastasis[a]
		b. Macrometastasis[b]
		c. In-transit metastases/satellites without metastatic nodes
N3	≥4 metastatic nodes, or matted nodes, or in-transit metastases/satellites with metastatic nodes	
M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

T tumor, *N* node, *M* metastasis, *NA* not applicable, *LDH* lactate dehydrogenase
[a]Micrometastases are diagnosed after sentinel lymph node biopsy and completion lymphadenectomy
[b]Macrometastases are defined as clinically detectable nodal metastases confirmed histopathologically

cells [11, 12]. The use of qRT may increase this sensitivity of detection to one melanoma cell per 10^7–10^8 background cells [9]. This is in contrast with the lower detection sensitivities for laboratory techniques more routinely employed in SLN analysis. Immunohistochemistry (IHC) can detect 1 melanoma cell in a background of 10^5–10^6 non-tumor cells [11, 12]. This sensitivity further decreases to 1 tumor cell per 10^4–10^5 non-tumor cells by light microscopic review alone (i.e., hematoxylin and eosin [H+E]-stained sections) of SLNs [11, 12]. Routine molecular testing could have diagnostic utility in the detection of subclinical and/or submicroscopic metastases in SLNs and/or PB of patients with melanoma. This chapter will also discuss the applicability of molecular techniques to microstage primary melanoma, in addition to the differentiation of second primary tumors from cutaneous metastases.

Microstaging of Primary Melanoma

Increasingly, molecular technologies have been evaluated as tools to more accurately microstage the primary tumor (i.e., T component of AJCC staging system) [13]. Based upon known significant differences in genetic and epigenetic alterations among different types of melanocytic tumors, most of these molecular-based ancillary approaches have been developed with a view to more accurately classify atypical melanocytic lesions [13]. Techniques investigated for this

Table 6.2 Anatomic staging groupings for cutaneous melanoma (Adapted from Balch et al. [1])

	Clinical staging[a]				Pathologic staging[b]		
	T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Any T	N>N0	M0	IIIA	T1-4a	N1a	M0
					T1-4a	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4a	N1b	M0
					T1-4a	N2b	M0
					T1-4a	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0
					T1-4b	N2c	M0
					Any T	N3	M0
IV	Any T	Any N	M1	IV	Any T	Any N	M1

[a] Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases

[b] Pathologic staging includes microstaging of the primary melanoma and histopathologic information about the regional lymph nodes after partial (i.e., sentinel node biopsy) or complete lymphadenectomy. Pathologic stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes

purpose have included: (a) allelic imbalance (AI) analysis; (b) comparative genomic hybridization (CGH); (c) multiplex ligation-dependent probe amplification (MLPA) (Fig. 3.6); (d) fluorescence in situ hybridization (FISH) (Fig. 3.6); (e) gene expression analysis using DNA microarray technology (Fig. 3.13) and RT-PCR; (f) analysis of BRAF, NRAS, and HRAS gene mutations; (g) evaluation of epigenetic biomarkers; and (h) a combination of mutational analysis and methylation-specific MLPA [13]. Importantly, these methodologies are applicable to formalin-fixed paraffin-embedded (FFPE) tissue, thereby facilitating the detection of genetic/epigenetic aberrations in the diagnostic setting. In the future, molecular testing may be standard-of-care in the routine work-up of morphologically ambiguous cutaneous melanocytic tumors. Molecular analysis could be employed as an adjunctive tool to histopathology, in order to: (a) assess the biological behavior of a melanocytic lesion (i.e., benign *vs.* malignant) and/or (b) discriminate between benign and malignant components of the same melanocytic lesion (i.e., precursor/associated melanocytic nevus *vs.* melanoma), thereby accurately determining Breslow thickness of the melanoma. Since Breslow thickness is the most significant prognostic factor for primary melanoma, its precise determination is essential for accurate staging (Tables 6.1 and 6.2). It provides relevant clinical information and influences management decisions, including excision margins and performance of sentinel lymph node biopsy (SLNB) [1]. The use of molecular technologies in primary melanoma and other melanocytic tumors is described in more detail in Chap. 5, and reviewed elsewhere [13].

Table 6.3 Specific and nonspecific molecular tumor markers used for the detection of SLNMCs and CMCs

Melanocyte-specific mRNA markers	Melanocyte-associated mRNA markers	General tumor-associated mRNA markers	Immunomodulatory markers	Non-coding RNA tumor markers	DNA tumor markers
TYR	Melan-A	MAGE-A3	IL-13	MicroRNAs	BRAF mutations
TYRP-1	p97	MAGE-A1	Leptin		Methylated DNA
TYRP-2/Dct	MUC-18	MAGE-A3/A6	LTbR		LOH of microsatellites
gp100	MIA	uMAGE-A	MIP1b		
PAX3	GalNAc-T	NY-ESO-1	IL-11Ra		
MITF	HMW-MAA	SSX 1–5	OX40		
PRAME		S-100β			
		YKL-40			
		CYT-MAA			
		Survivin			
		Bcl-2			
		Bcl-x			
		Bax			
		$VEGF_{121}$			
		PAI-1			
		SPP1			

SLNMCs sentinel lymph node melanoma cells, *CMCs* circulating melanoma cells, *TYR* tyrosinase, *TYRP-1* tyrosinase-related protein-1, *TYRP-2/Dct* tyrosinase-related protein-2/dopachrome tautomerase, *gp100* melanosomal protein/HMB-45, *PAX3* paired box transcription factor, *MITF* microphthalmia-associated transcription factor, *PRAME* preferentially expressed antigen in melanoma; recognized by cytotoxic lymphocytes, *Melan-A* melanoma antigen recognized by T-cells-1/MART-1, *p97* melanotransferrin, *MUC-18* melanoma cell adhesion molecule/MCAM, *MIA* melanoma inhibitory activity, *GalNAc-T* B(1→4)-N-acetylgalactosaminyl-transferase, *HMW-MAA* high-molecular weight melanoma-associated antigen, *MAGE-A3* melanoma antigen gene A3, *uMAGE-A* universal MAGE-A, *NY-ESO-1* and *SSX 1-5* cancer/testis antigens, *S-100β* acidic calcium-binding molecule, *YKL-40* mammalian chitinase-like protein, *CYT-MAA* cytoplasmic melanoma-associated antigen, *Survivin*, *bcl-2, bcl-x, bax* apoptosis-related genes, $VEGF_{121}$ vascular endothelial growth factor 121, *PAI-1* plasminogen activator inhibitor-1, *SPP1* osteopontin, *IL-13* interleukin 13; *LTbR* lymphotoxin β receptor, *MIP1b* macrophage inflammatory protein 1b, *IL-11Ra* interleukin 11Ra, *OX40* CD134/member of tumor necrosis factor receptor family, *LOH* loss of heterozygosity

Molecular Analysis of Sentinel Lymph Nodes (SLNs)

For cutaneous melanoma AJCC stages I and II, the most important prognostic indicators are (a) Breslow thickness and (b) the presence or absence of ulceration [1, 2, 14]. However, once patients develop regional lymph node metastases (AJCC stage III), the histopathological parameters of the primary melanoma (except for ulceration) are of limited value in predicting survival, and it is the number and extent of lymph node involvement that are strongly associated with prognosis [1, 2, 14]. In fact, the presence or absence of regional lymph node metastasis is the single most important prognostic factor for patients diagnosed with melanoma [15].

Since its introduction by Morton et al. in 1992 [16], SLNB has become a standard procedure in the staging and treatment of patients with primary melanoma [17, 18]. Approximately 50% of all cutaneous melanoma patients with tumor progression develop regional lymph node metastases first [19]. Accordingly, the AJCC Melanoma Staging Committee recommends that "*SLNB be performed as a staging procedure in patients for whom the information will be useful in planning subsequent treatments and follow-up regimens*" [1]. The SLN is defined as the lymph node nearest to the site of the primary melanoma on the direct lymphatic drainage pathway, and therefore, the first lymph node to receive drainage and cancer cells from a primary tumor site. The primary aims of SLNB are (a) to reduce morbidity associated with elective lymph node dissection and (b) to select patients with micrometastatic SLN disease for subsequent lymphadenectomy and/or adjuvant therapy [17, 20]. Many studies have confirmed that the presence or absence of metastatic melanoma in the SLN accurately predicts (in ~98% of cases) the presence or absence of disease in that lymph node basin [14, 21]. Approximately, 2% of patients with primary melanomas <1 mm in Breslow thickness have positive SLNs (i.e., with metastatic tumor deposits), in contrast to a significant minority of patients with primary melanomas ≥1 mm thickness who have positive SLNs (i.e., 7% for ≥1–1.99 mm, 13% for ≥2–2.99 mm, and 31% for ≥3 mm) [20]. Although, up to 6% of patients with primary tumors between 0.76 and 1 mm in thickness will have positive SLNs, SLNB has been generally recommended only for those patients with primary melanomas of ≥1 mm in Breslow thickness and clinically negative regional lymph nodes [17, 20].

Patients with SLN involvement by melanoma (identified on H+E and/or IHC stains) have a significantly higher risk of disease recurrence and death [22]. Fifty to 67% of patients with histopathologically positive SLN disease will develop recurrent disease within 5 years, which is more than double the recurrence rate for patients with histopathologically negative SLNs [22]. In addition, the 5-year survival rate for patients with SLN-positive disease is approximately half that of patients with SLN-negative disease (40% *vs.* 84%) [22]. Many studies suggest that SLNB increases disease-free survival in melanoma patients, but it may also function to alter the patterns of disease recurrence (i.e., reducing the rate of nodal recurrence, principally at the expense of an increased rate of distant metastases) [23–25]. The effect of SLNB on overall survival in melanoma patients remains controversial [18, 23–25]. Nonetheless, SLNB continues to be employed not only for disease staging and treatment planning, but also in the design of clinical trials in melanoma patients [18].

Standard procedures for laboratory analysis of the SLN include H+E staining and IHC. These facilitate the evaluation of lymph node architecture, cytologic features and cell surface markers, and allow differentiation of melanoma metastases from other malignant or benign cellular populations in lymph nodes, most importantly benign melanocytic nevi (Fig. 6.1) [18]. In fact, the updated 2009 AJCC Melanoma Staging System now accepts IHC-based detection alone of nodal metastases [if the diagnosis is based on at least one melanoma-associated marker (i.e., Melan-A, HMB-45) and the cells have malignant morphologic features that can be determined in IHC-stained tissue] [1]. By light microscopic examination of H+E stains alone, ~16% of clinical stages I and II melanoma patients will show occult SLN metastases [26]. Serial sectioning, with the addition of IHC (using antibodies such as S-100, HMB-45 and/or Melan-A), improves the sensitivity of metastases detection by as much as 10–45%, resulting in the detection of occult SLN metastases in an average of 20% of clinical

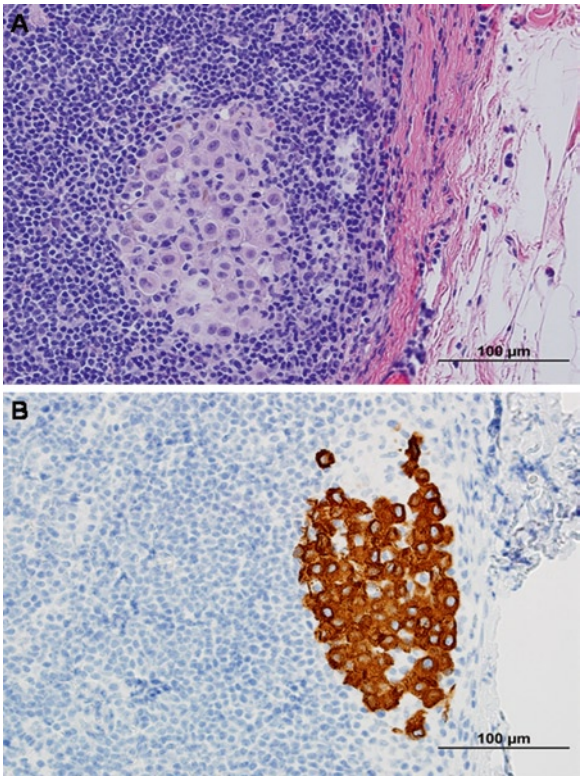

Fig. 6.1 Sentinel lymph node with metastatic melanoma deposit, identified on hematoxylin and eosin stain (**a**) and confirmed by Melan-A immunohistochemical stain (**b**) (Courtesy of Dr. Poornima Hegde, University of Connecticut Health Center, Farmington, CT, USA)

stages I and II patients [26]. However, despite the use of such a protocol, locoregional or distant recurrences are seen in 10–24% of patients with reportedly "negative" SLNs [7, 27]. Recurrent disease may result from occult melanoma metastases that are undetected by histopathological or combined histopathological-IHC analysis. Of note, typically <5% of the SLN is examined by routine testing, so sampling errors could account for false-negative results in many cases [15].

Molecular diagnostic techniques have been proposed as more sensitive means of detecting submicroscopic tumor cells in both SLNs [15, 28] and lymphatic fluid following lymph node dissection [29] in patients with melanoma. Hypothetically, these molecular tests could be potentially better predictors of those individuals at risk of developing regional or distant metastases. The diagnostic and prognostic utilities of RT-PCR and qRT to detect melanoma metastases in SLNs continue to be studied (Table 6.4). With one exception [30], all studies to date [7, 27, 31–37] have shown increased detection rates with the addition of RT-PCR (12–81%) over histopathological and/or IHC analysis alone (11–48%). RT-PCR of SLNs also identifies a significantly higher number of patients with micrometastatic disease in non-SLNs (i.e., adjacent lymph node basin) [38]. Many studies have utilized tyrosinase (TYR), as a single melanocyte-specific mRNA marker [7, 35, 36]. However, in an effort to improve assay specificity and sensitivity, a multimarker protocol is now more commonly employed (Tables 6.3 and 6.4). It is believed that the latter approach addresses some of the different biochemical and pathologic changes that are characteristic of melanoma cells, such as tumor cell heterogeneity with respect to types, numbers and expression levels of marker mRNA transcripts, mRNA half-life, and variations in mRNA quantity and quality [7, 10, 27, 31–34, 37].

More specific melanoma gene products are now being discovered and may become new targets in diagnostic protocols. For instance, using genome-wide microarray analyses, Soikkeli et al. [39]

Table 6.4 Selected studies using molecular methods to detect SLNMCs in melanoma patients

References	Method	Markers	Patients, *n*	PCR positivity (%)	Prognostic value
Hochberg [31]	RT-PCR	TYR, Melan-A, MIA	17	75	Yes
Kuo [32]	RT-PCR	TYR, Melan-A, TYRP-1, TYRP-2	77	74	Yes
Rimoldi [33]	RT-PCR	TYR, Melan-A	71	57.7	Yes
Takeuchi [34]	qRT	Melan-A, MAGE-A3, GalNAc-T, PAX3	215	46	Yes
Kammula [35]	RT-PCR	TYR	112	65.2	No
Mangas [36]	RT-PCR	TYR	180	68.9	No
Scoggins [37]	RT-PCR	TYR, Melan-A, MAGE-A3, gp100	1,446	42.9	No
Hilari [27]	qRT	TYR, Melan-A, SSX 2, MAGE-A3, PAX3, GalNAc-T	195	NR	No

SLNMCs sentinel lymph node melanoma cells
NR not reported

identified 27 genes (22 upregulated and 5 downregulated) that differentiated tumor-positive SLNs from tumor-negative SLNs (Fig. 6.2). A subsequent prospective study of Melan-A and TYR expression by RT-PCR confirmed their possible prognostic significance, stratifying patients with regard to tumor burden into distinct groups for tumor recurrence [39]. In addition, SPP1 (osteopontin) and PRAME (preferentially expressed antigen in melanoma, recognized by cytotoxic lymphocytes) were identified as melanoma-specific markers that could differentiate melanoma cells from benign melanocytic nevus cells in SLNs [39]. More recently, Ma et al. [40] have shown that it is feasible to profile microRNAs in FFPE SLN samples by cloning and sequencing techniques, with confirmatory Northern analysis and qRT (Fig. 6.3). MiRNAs are small noncoding RNAs (~21–25 nucleotides in length). They regulate the expression of mRNAs and may function as oncogenes or tumor suppressor genes, depending on the cellular context and/or their target genes [40]. Interestingly, let-7 microRNA family members were found to be differentially expressed in tumor-positive and tumor-negative SLNs in a melanoma patient (Fig. 6.3) [40]. Therefore, analysis of microRNA expression in SLNs could potentially be used for diagnostic and/or prognostic purposes in melanoma patients. In addition, the immune system is known to be suppressed locally and/or systemically before melanoma metastases develop [41, 42]. It is believed that activation or downregulation of genes for pro- and anti-inflammatory cytokines and chemokines, and their receptors, may control the immunosuppressive microenvironment that facilitates metastasis [41, 42]. Therefore, assessment of the SLN microenvironment vis-à-vis immune surveillance is emerging as another possible diagnostic avenue. Using cDNA microarray analysis and qRT validation, Torisu-Itakura et al. [41] found that expression levels of interleukin-13 (IL-13), leptin, lymphotoxin β receptor (LTbR), and macrophage inflammatory protein 1b (MIP1b) were significantly higher, and expression level of IL-11Ra was lower, in melanoma-positive compared with melanoma-negative SLNs. Similarly, John et al. [43] identified differentially expressed transcripts (including immunologic signaling genes), that were capable of distinguishing clinical outcomes in melanoma patients with stage III (lymph node) disease, using gene expression profiling by oligonucleotide microarrays and qRT. Other studies, which also included analyses of stage III tumors, have confirmed that results of gene expression profiling correlate with clinical outcome in melanoma patients [44, 45]. In another investigation, Sarff et al. [42] demonstrated that the expression of OX40 (CD134, a member of the tumor necrosis factor receptor superfamily) on CD4+ T-cells draining primary cutaneous melanomas decreased with (a) more advanced primary tumor features (i.e., increasing tumor thickness, ulceration) and (b) SLN involvement by tumor. Therefore, immune-mediator gene expression analysis could aid in the assessment of SLNs in melanoma patients. According to Torisu-Itakura et al. [41],

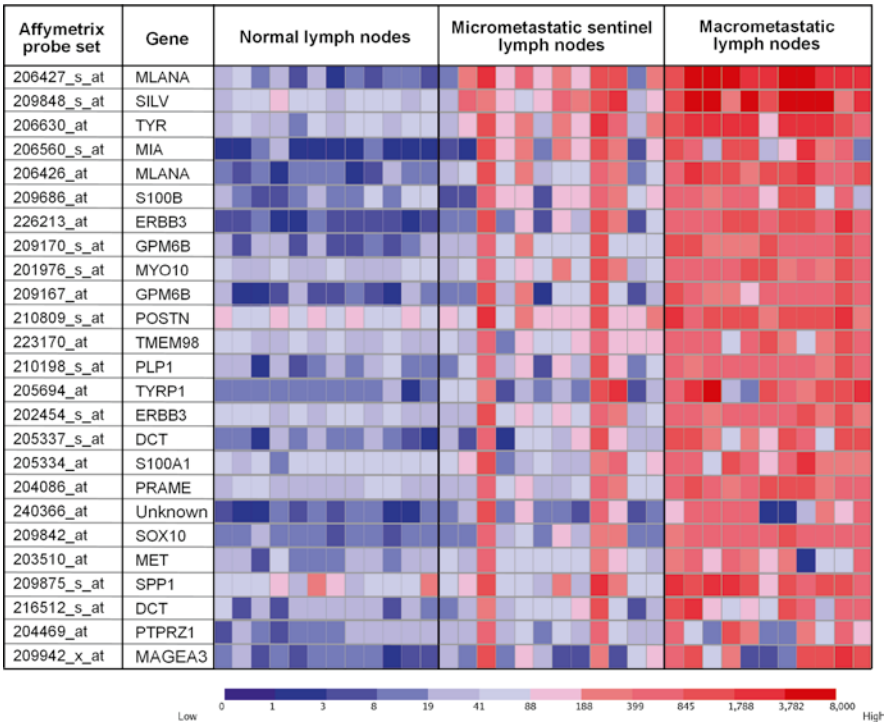

Fig. 6.2 Identification of marker genes for melanoma micrometastasis detection in sentinel lymph nodes (SLNs) using genome-wide gene expression analyses. The heat map depicts Affymetrix microarray (HG-U133 Set) data, calculated by significance analysis of microarrays (*SAM*). Genes exhibiting ≥4-fold overexpression in micrometastatic SLNs as compared to normal lymph nodes (and their respective gene expression levels in clinically detected macrometastatic lymph nodes) are shown. Rows represent genes (probe sets) and columns represent expression levels in different samples. A *blue–red* color scale displays the signal intensities (from low to high). Microarray results were validated by subsequent RT-PCR analyses of MLANA, TYR, MIA, S100B, ERBB3, and PRAME in a larger series of samples (160 patients, 516 SLN samples). Of note, SPP1 and PRAME proved to be melanoma-specific markers, capable of potentially differentiating melanoma cells from benign melanocytic nevi in SLNs (Courtesy of Dr. Erkki Hölttä, University of Helsinki, Helsinki, Finland)

markers of the tumor microenvironment should be found throughout the SLN, unlike direct (melanocyte-related) markers of SLN metastasis. The latter are subject to sampling error, unless the entire SLN is sectioned for analysis. Theoretically, the presence of SLN melanoma micrometastasis could be determined by examining a single section from any part of the SLN with an immune mediator-directed protocol [41]. The expression of other general tumor-associated transcripts has also been investigated [46, 47]. For example, the expression of apoptosis-related genes (i.e., survivin, bcl-2, bcl-x, and bax) has been determined by RT-PCR and IHC in SLNs from melanoma patients, with survivin expression found to correlate with outcome [46]. The utility of qRT detection of angiogenesis- and invasion-related markers in SLNs of melanoma patients has also been evaluated, with $VEGF_{121}$ and PAI-1 expression significantly associated with the presence of micrometastases [47]. In addition, loss of heterozygosity (LOH) on the APAF-1 locus (12q22-23) in lymph nodes with metastatic melanoma deposits correlated with poor disease outcome [48]. APAF-1 is a key factor in the mitochondrial apoptotic pathway downstream of p53 [48].

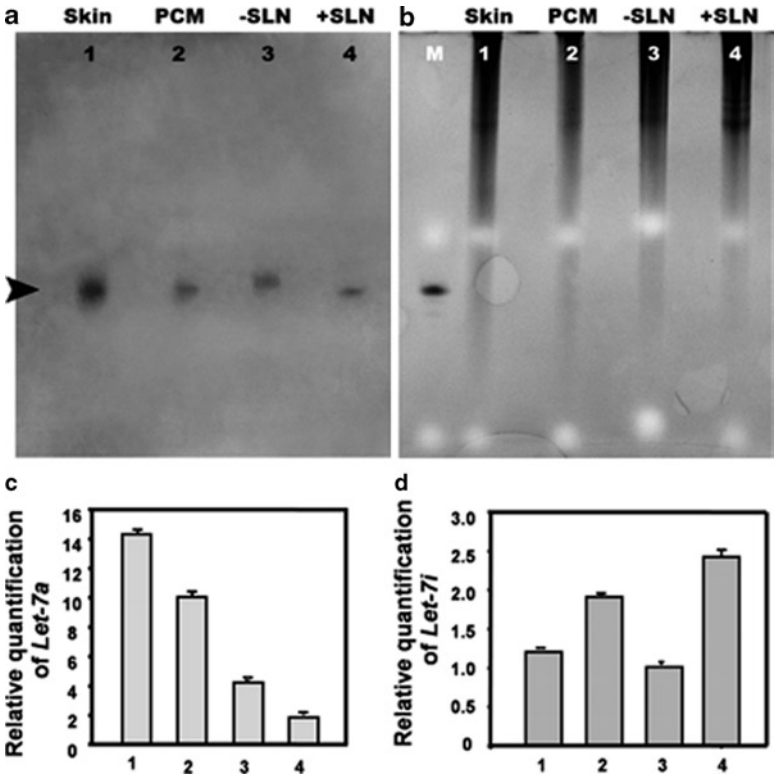

Fig. 6.3 Profiling of microRNAs in formalin-fixed paraffin-embedded specimens from a patient with melanoma. (**a**) Northern blot analysis (denaturing 15% polyacrylamide gel) showing the expression of the human microRNA let-7a in tissue samples from a 52-year-old man with scalp melanoma, including residual primary cutaneous melanoma (PCM); uninvolved skin >2.0 cm away from the primary tumor in a wide local excision (Skin); sentinel lymph node negative for metastasis (−SLN); and SLN positive for metastasis (+SLN). A 21/22-bp band is identified (*arrowhead*) corresponding to the size of lin-4 in (**b**). (**b**) For loading control, the 15% polyacrylamide gel for Northern blot was stained by ethidium bromide (EtBr) before transferring to membrane. Lane M represents synthetic *Caenorhabditis elegans* microRNA lin-4 loaded for size comparison. (**c**) Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) demonstrated downregulation of let-7a expression in PCM (lane 2) and +SLN (lane 4), when compared with uninvolved skin (lane 1) and −SLN (lane 3). (**d**) In contrast, let-7i expression was upregulated in PCM (lane 2) and +SLN (lane 4), when compared with normal skin (lane 1) and −SLN (lane 3). Each reaction was performed in triplicate; *error bars* show standard deviations (From Ma et al. [40]. Reprinted with permission from the American Society for Investigative Pathology and the Association for Molecular Pathology, copyright © 2009)

More recently, Dalton et al. [49] have proposed a FISH-based approach as an adjunctive tool to histopathological evaluation of SLNs from melanoma patients. Probes targeting chromosomes 6p25, 6 centromere, 6q23, and 11q13 are used to determine the presence of genomic aberrations in SLNs with small foci of melanocytes (i.e., differential diagnosis of metastatic melanoma *vs.* nodal melanocytic nevus) (Fig. 6.4). Of 24 SLNs with melanoma deposits in the original study, 20 (83%) showed aberrations by FISH, which was similar to the sensitivity of the technique in primary melanomas (85%) [49]. In addition, the specific aberrations present in the primary tumor could also be detected in its corresponding SLN metastasis [49]. Therefore, FISH-positive results in a SLN have high specificity for the diagnosis of metastatic melanoma. However, in the absence of diagnostic microscopic features of malignant disease in a SLN, a definitive interpretation of FISH-negative results in a SLN would require genomic analysis of the corresponding primary tumor.

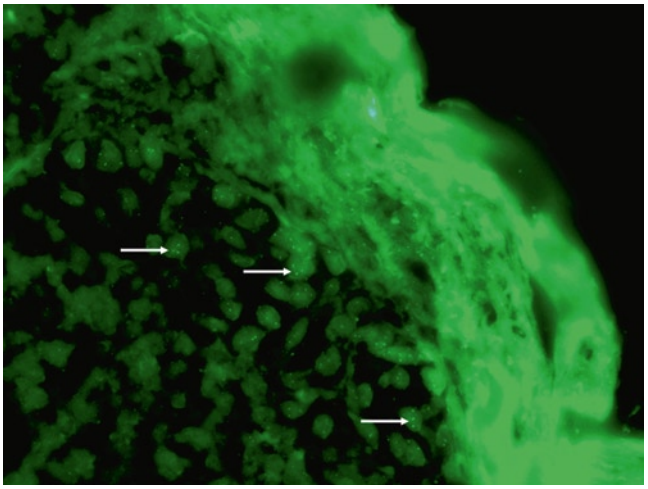

Fig. 6.4 Fluorescence in situ hybridization (*FISH*) as an adjunctive tool to histopathological evaluation of sentinel lymph nodes (*SLNs*) in patients with melanoma. Subcapsular cellular infiltrate demonstrating increased copies of CCND1 (*arrows*), supporting the diagnosis of a melanoma metastasis (Courtesy of Dr. Pedram Gerami, Northwestern University, Chicago, IL, USA)

FISH-negative results in a SLN could be used to support a histopathological diagnosis of benign nodal melanocytic nevus, in the presence of discordant FISH-positive results in the accompanying primary sample. Of note, 17–22% of primary melanomas and their corresponding SLN metastases are FISH-negative using these four probes, precluding a definitive evaluation of any clonal relationship between such paired tumor sets by this method [49].

Conceptually, patients with molecular-negative SLNs may be at low risk for progression and/or poor outcome, with little benefit from adjuvant therapies. Patients with molecular-positive SLNs may be at high risk for aggressive disease and benefit from rigorous clinical follow-up and further therapeutic intervention (Fig. 6.5). However, despite numerous studies, the clinical relevance of RT-PCR-based testing of SLNs from melanoma patients remains unclear [7]. The molecular "upstaging" of SLNs correlates with clinical outcome parameters in many studies (Table 6.4) [7, 31–34]. Studies have shown that: (a) the more mRNA markers used, the greater the sensitivity of occult metastasis detection [27, 31–34, 37]; (b) the expression of two or more markers is associated with significantly decreased disease-free survival in patients with histopathologically negative SLNs [32–34]; and (c) decreased overall survival is associated with both Melan-A RT-PCR-positivity [31] and the presence of melanocyte-related mRNA transcripts (more specifically, increasing number of markers) identified by qRT [34]. A recent systematic review and meta-analysis of 22 studies, including more than 4,000 melanoma patients who underwent SLNB, demonstrated that RT-PCR status correlated with: (a) TNM stage (stage I to II *vs.* III; RT-PCR positivity, 46.6% *vs.* 95.1%; $P < .0001$); (b) disease recurrence (RT-PCR positive *vs.* negative; relapse rate, 16.8% *vs.* 8.7%; $P < .0001$); (c) disease-free survival (hazard ratio [HR], 3.41; 95% CI, 1.86–6.24; $P < .0001$); and (d) worse overall survival (HR, 5.08; 95% CI, 1.83–14.08; $P = .002$) [7]. Therefore, molecular analysis of SLNs in melanoma patients may have clinical utility as (a) the recurrence rate in patients upstaged by RT-PCR appears in many studies to be significantly higher than for patients with RT-PCR-negative SLNs and (b) the determination of individuals with both histopathologically and RT-PCR-negative SLNs potentially identifies a group of melanoma patients who may not benefit from further surgical or adjuvant therapy. However, there are a number of studies which have failed

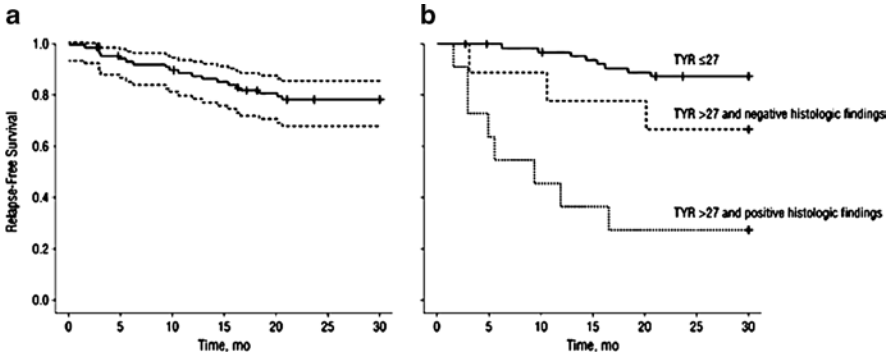

Fig. 6.5 Quantitative reverse transcription-polymerase chain reaction (*RT-PCR*) analysis of melanocytic differentiation antigens in sentinel lymph nodes (*SLNs*) with melanoma micrometastases. Relapse-free survival (*RFS*) of the overall cohort (**a**) and by tyrosinase (*TYR*) expression and histologic group (**b**). The use of both 27 copies/copy of TBP control gene as a TYR expression cutoff for RT-PCR and histologic examination distinguishes the following three groups of patients after 30 months of follow-up: (1) a group with both positive histologic findings and TYR expression of >27 copies/copy of TBP, which has a high rate of relapse (27% RFS); (2) a second group of patients with TYR expression of >27 copies/copy of TBP and negative histologic findings (67% RFS); and (3) a third group of patients with TYR expression of ≤27 copies/copy of TBP (all with negative histologic findings, except for 2 patients), which has an even better prognosis (87% RFS). The use of 27 copies/copy of TBP as a TYR cutoff for RT-PCR may distinguish true micrometastasis from illegitimate transcription of melanocytic differentiation antigens and isolated melanoma cells. *Dashed lines* in (**a**) represent the 95% confidence intervals; *tick marks* in (**a**) and (**b**) represent censored observations. TBP = TATA box-binding protein (From Vitoux et al. [47]. Reprinted with permission from the American Medical Association, copyright © 2009)

to confirm the prognostic value of molecular analysis in otherwise histopathologically negative SLNs (Table 6.4) [27, 35–37]. Hilari et al. [27] noted that multimarker qRT analysis of SLNs did not correlate with melanoma recurrence. Scoggins et al. [37] reported no difference in disease-free survival or overall survival between melanoma patients with molecular-positive or molecular-negative SLNs. In another study, Kammula et al. [35] noted that differences in recurrence rates between the RT-PCR-positive and RT-PCR-negative groups were statistically significant at 42 months, but no longer statistically significant at 67 months. This latter finding suggested that the RT-PCR-negative group had a defined long-term failure rate that was not different from the RT-PCR-positive group, with the exception of their time to recurrence [35]. According to the authors, this finding only became apparent after extended follow-up was performed, reiterating the risks of false-negativity, and arguing against the hypothesis that the main benefits of molecular analysis are (a) its negative predictive value and (b) its ability to stratify patients with histopathologically negative SLNs into a low risk group based on negative RT-PCR results [35]. In fact, there may be a lag time to metastasis/systemic disease associated with a small amount of tumor, as more tumor cell divisions/time would be required to produce clinically recognizable disease [50]. The conflicting data between all these studies may have resulted from significant differences in length of clinical follow-up, in addition to different methodologies employed, such as SLN sampling techniques, use of different molecular markers, and varying RT-PCR protocols (including standard gel-based RT-PCR, nested RT-PCR, RT-PCR with Southern blot detection, and qRT) (Table 6.4). Future studies evaluating molecular staging techniques may require longer follow-up periods (>5 years) to accurately define clinical outcome in patients with histopathologically negative SLNs.

As stated previously, standard RT-PCR studies are optimized to detect as little as 1 melanoma cell in 10^6–10^7 non-tumor cells, and typically report the presence or absence of a particular marker with limited quantification [11, 13]. In addition to a multimarker protocol, a number of studies have also employed a qRT approach (Table 6.4). qRT is capable of accurately and reproducibly detecting and

quantifying individual marker copy numbers, and has been shown to be at least as sensitive as standard RT-PCR for the detection of molecular marker transcripts in SLNs of melanoma patients [27, 34]. The use of qRT also significantly increases the analytical power of the assay, as absolute numbers of tumor cells may be determined by comparing levels of transcript expression between tumor and control samples [10]. In addition, qRT is particularly suited to routine laboratory use, as reactions can be performed within ~30 min, in a single-step procedure. The detection of amplified fragments is accomplished by melting curve analysis, without the requirement for subsequent amplicon manipulation (i.e., gel-based), as in standard RT-PCR-based studies [27, 34]. qRT is employed with the assumption that some of the causes of false-positivity with standard RT-PCR methods (i.e., such as the presence of nodal benign melanocytic nevus cells) would produce lower signals with this method (i.e., than true melanoma metastases). In this respect, significant differences in median melanocyte-derived mRNA levels have been found by qRT between: (a) SLNs with histopathologically verified micrometastases (high); (b) SLNs with benign nevus inclusions (intermediate); and (c) SLNs without evidence of either benign or malignant intranodal melanocytes (low), suggesting that this quantitative molecular protocol may increase assay precision and be more useful than standard RT-PCR for the clinical evaluation of SLNs in melanoma patients [51, 52]. However, there is a "gray-zone" where substantial overlap in mRNA levels exists between the three groups [51]. In addition, multimarker qRT assays do not improve distinction between these three groups when compared with single marker analyses [51].

The Breslow thickness of a primary melanoma is an accurate predictor of SLN metastasis [53]. Accordingly, studies have investigated if there is a correlation between transcript detection rates and/or levels in SLNs and primary tumor prognostic markers (i.e., primary tumor thickness and/or ulceration). In many of these studies, patients with RT-PCR-positive and qRT-positive SLNs had significantly greater primary tumor thickness [32, 33, 38, 51, 54] and risk of tumor ulceration [54] than transcript-negative patients. However, other studies have not found a correlation between primary tumor thickness [35, 55] or ulceration [35] and transcript detection in SLNs by either RT-PCR or qRT.

In addition to determining the presence or absence of melanoma within a SLN, the tumor burden (i.e., the area of tumor in the SLN relative to total nodal area) has also been suggested as a predictor of metastases in non-SLNs, disease recurrence and melanoma-specific death [17]. The numbers and/or levels of mRNA transcripts detected by molecular methods may reflect this tumor burden. Abrahamsen et al. [51] identified that both TYR and Melan-A mRNA quantity (highest expressive SLN per patient), as detected by qRT, correlated with tumor burden (defined as the number of levels with tumor relative to the total number of levels examined) in SLNs with melanoma metastases. Takeuchi et al. [34] found that: (a) the mRNA copy number of a particular marker in histopathologically negative SLNs was significantly higher for patients who recurred; (b) disease-free survival and overall survival were significantly shorter when histopathologically negative SLNs expressed one or more markers; and (c) increasing numbers of detected markers correlated significantly with progressive decreases in disease-free survival and overall survival. The recurrence rate was 41% for patients with SLNs expressing one marker, 69% for patients with two markers, and 100% for patients with three markers [34]. In another study, Starz et al. [30] also noted that the detection rate of TYR mRNA by RT-PCR correlated with tumor burden in SLNs. RT-PCR was sufficiently sensitive to detect all "*extended peripheral*" (S2) and "*deeply invasive*" (S3) micrometastases, but only a minority (46%) of cases with "*few localized melanoma cells in the subcapsular zone*" (S1) [30]. In addition, over a median follow-up period of 50.5 months, melanoma patients with low tumor burden in SLNs (S1) did not show any significant differences in local recurrence or distant metastasis when compared to patients free of SLN disease (S0) [30]. A similar correlation between "occult" tumor burden in regional lymph nodes and prognosis has been reported in other human tumors [56]. This suggests that the failure of RT-PCR to detect low tumor burden melanoma

micrometastases in SLNs may be irrelevant to prognosis and further therapeutic decisions, and tolerable for clinical purposes in melanoma patients [30]. The goal is to define a clinically relevant cut-off value for tumor burden by either (a) light microscopy (i.e., micrometer to evaluate diameter of metastasis) or (b) number of molecular markers detected and/or mRNA copy number (i.e., gene expression levels) by qRT, in an effort to stratify melanoma patients into a group with a high risk of recurrence (high tumor burden in SLN) and a group with a more favorable prognosis and low risk of relapse (low tumor burden in SLN). Giese et al. [55] have suggested that an increase in the specificity of the molecular assay could be achieved by decreasing its sensitivity. However, Hilari et al. [27] counter that any attempt to set a quantitative threshold as a cut-off and/or combining multiple markers to improve sensitivity or specificity may result in false interpretations of prognostic utility. The updated 2009 AJCC Melanoma Staging System does not recommend a lower threshold for staging lymph node-positive disease ("*isolated tumor cells or tumor deposits <0.1 mm, which meet the criteria for histologic or IHC detection of melanoma should be scored lymph node-positive*") [1]. At this time, the prognostic significance of isolated tumor cells in the SLNs of melanoma patients remains unclear. A number of studies have proposed that micrometastases (up to 0.2 mm) are clinically irrelevant, with patients showing a clinical course similar to individuals with negative SLNs [57–59]. In contrast, other researchers have demonstrated that a minority of patients (~12%) with micrometastases have additional metastatic disease on follow-up lymphadenectomy, and show a significantly reduced melanoma-specific survival rate compared to patients with negative SLNs (89% *vs.* 94%; $p = .02$) [60].

This chapter highlights the potential diagnostic and prognostic impact of adjunct molecular studies in individuals with melanoma. Molecular tests may be used to identify a population of patients at risk for melanoma recurrence who are not readily determined by light microscopy [3, 7, 20, 61, 62]. For example, the percentage of those melanoma patients with histopathologically negative, but RT-PCR-positive SLNs who will experience disease recurrence (12–25%) is elevated compared to patients with both histopathologically and RT-PCR-negative SLNs (up to 9%), but significantly less than those patients with both histopathologically and RT-PCR-positive SLNs (31–67%) (Fig. 6.5) [26]. However, despite some apparent clinical utility for molecular analysis of SLNs in melanoma patients, the problem of false-positive and false-negative results with these assays deserves further discussion. With the addition of molecular tests, the detection rate of suspected occult metastases (i.e., positive results) may be as high as 70–75% (with an average of ~50%) [3, 26, 31]. This "over-detection" by molecular methods (i.e., clinically insignificant results in many patients) may represent false-positives due to: (a) carry-over/amplicon contamination; (b) amplification of residual DNA in the RNA extract; (c) illegitimate transcription; (d) detection of unwanted transcripts from SLN benign melanocytic nevi (4–22% of cases), melanophages (that have ingested melanoma cells/organelles) and/or Schwann cells; (e) the detection of melanoma cells incapable of tumor progression, that are dormant, or that are limited to the SLN by immune surveillance; (f) the SLNB may have surgically cured some SLN-positive patients; or (g) the possibility that clinical recurrences may not have occurred during the defined follow-up periods in these studies [3, 21, 33, 61, 62]. For example, Li et al. [54], using TYR as a sole marker, demonstrated that 63% of patients were positive by RT-PCR. However, the vast majority (90%) of these individuals did not show clinical evidence of disease recurrence, suggesting that TYR analysis alone may be too sensitive for routine clinical use [54]. High false-positive rates have been documented when TYR was employed as a single marker in other studies [28]. In addition, molecular analysis of SLNs can potentially give rise to false-negative results secondary to a number of factors [3, 21, 33, 61, 62], including: (a) RNA degradation; (b) RT-PCR inefficiency; (c) sampling error (i.e., low copy number of amplifiable mRNA targets); (d) all cells within a tumor may not necessarily express a particular marker; (e) some RT-PCR assays may be stratifying SLNs by burden of disease, and not by the absolute presence or absence of disease; or (f) some aggressive tumors may bypass the draining lymph node

basin (in one study, 7 of 24 patients had TYR-positive RT-PCR in non-SLNs, despite a negative result in the SLN [63]).

Nonetheless, the short turnaround time and cost-effectiveness of molecular testing may promote its wider use in the staging of melanoma patients [61]. The possibility that RT-PCR and qRT-based techniques can detect metastatic melanoma cells in SLNs, which show no histopathological or immunohistochemical evidence of tumor, is exciting. Sampling (i.e., all or part of the lymph node), mRNA degradation, and logistical issues in the preparation and storage of specimens all complicate the optimization and standardization of molecular tests on frozen SLNs. The use of RNA extracted from FFPE material for multimarker analysis may be an alternative for evaluating patients who have histopathologically negative SLNs [21, 30, 32]. FFPE specimens are abundantly available in pathology archives worldwide, resist mRNA degradation during storage, and are linked to a wealth of patient data, including clinical outcomes related to disease course and/or response to treatment regimens. However, there is some evidence that the sensitivity of RT-PCR analysis of FFPE tissue may be lower than that of fresh/frozen tissue [8]. In addition, it would not be feasible to completely abandon light microscopy and analyze SLNs by RT-PCR or qRT only (or at least as the initial test). The processing techniques for RT-PCR and qRT are different from those of routine histology, and would preclude histopathological corroboration of molecular results, should all of the sample have been submitted for molecular analysis. The use of large portions of SLNs for these studies would not be acceptable, since micrometastases or melanocytic nevi may be focally distributed. Combined histopathological and IHC analysis remains the gold standard for identification of melanoma cells in SLNs. Adjunct molecular analyses, using adjacent tissue sections, may further improve the diagnostic accuracy. For example, the detection of marker mRNA, without a morphologic correlate on initial sections, would encourage the pathologist to investigate the significance of the molecular results by performing additional H + E and/or IHC stains ("extended protocol"). Of note, it has been shown that previously IHC-stained archival slides (stored for up to 90 days) of lymph nodes with metastatic melanoma can be used for subsequent mRNA analysis [64]. In this way, combined histopathological, IHC and molecular testing should minimize both false-negative and false-positive results. Furthermore, recent gene expression profiling studies have reported the identification of melanoma-specific genes (i.e., PLAB and L1CAM) that allow for a more accurate differentiation of melanoma from benign melanocytic nevi, making them better candidates than conventional markers (i.e., TYR and Melan-A) for metastatic melanoma diagnosis in SLNs [65, 66].

In view of the conflicting results from numerous studies, the current poor specificity of molecular tests for SLN staging/prognostication in melanoma patients is an obvious drawback. This is a particular concern becuase of the morbidity associated with proposed treatment options (i.e., lymphadenectomy and/or adjuvant interferon alfa-2b) for patients with molecular-positive SLNs [27]. The utility of molecular diagnostic testing of SLNs in patients with melanoma has not yet been clearly established. Currently, there is no consensus on whether RT-PCR-based detection of SLN micrometastatic disease has demonstrable diagnostic and/or prognostic value to warrant its inclusion in routine clinical practice. Although molecular evidence of SLN metastasis has been identified in patients who have early-stage melanoma, its clinical relevance remains to be determined, as current molecular markers and/or techniques lack sufficient sensitivity and specificity. A definitive role for molecular markers can only be determined with large, multicenter, randomized controlled trials, which could investigate the efficacy and clinical impact of molecular analysis of SLNs in melanoma patients, and validate panels of molecular markers as a basis for risk stratification. In this regard, the second multicenter selective lymphadenectomy trial (MSLT-II), a phase III international trial that will determine the benefit of multimarker qRT assays in SLN specimens, is underway [15].

Molecular Analysis of Peripheral Blood (PB) Specimens

While melanoma usually first metastasizes via local lymphatics to the regional lymph node basin, distant metastases are often secondary to hematogenous spread. In addition, ~30% of primary melanomas metastasize directly to distant organs (stage IV) without intermediate lymph node/stage III involvement [19]. Therefore, the detection of circulating melanoma cells (CMCs) in the PB could have utility in: (a) identifying disease progression and/or recurrence; (b) stratifying patients into risk groups in order to predict prognosis; (c) selecting patients who would benefit from adjuvant therapy; and (d) monitoring therapeutic response [8–10]. Similar to studies on SLNs, molecular analysis of PB samples in melanoma patients has involved the use of RT-PCR-based techniques to detect single, or a combination of, specific and nonspecific mRNA markers of CMCs (Tables 6.3 and 6.5). Other PB-based assays employed to identify metastasis and disease progression in patients with melanoma include: (a) detection of circulating microRNAs; (b) direct isolation of melanoma cells; (c) detection of tumor-specific circulating DNA; and (d) evaluation of serological protein markers (i.e., LDH, S-100β, MIA). The latter are beyond the scope of this chapter and have been extensively reviewed elsewhere [67–69].

Detection of Circulating Tumor-Related mRNAs

Molecular tests to identify circulating tumor cells have been used to predict patient outcome for several malignancies, including leukemia and prostate cancer [10]. The detection of CMCs in melanoma patients who are "clinically" disease-free is a promising avenue to determine those individuals with occult micrometastases, and therefore at risk for disease progression. Similar to studies on SLNs, combined multimarker and/or qRT assays have been employed in an effort to improve test sensitivity and specificity (Table 6.5) [70–78]. Both the number and expression levels of melanoma-associated mRNA transcripts detected in PB are found to be significantly associated with disease stage and progression in melanoma patients [8–10, 71, 72, 74]. A lack of detectable markers in PB correlates with a low risk of disease recurrence in melanoma patients who show no clinical or radiological evidence of residual or metastatic disease [72]. Of note, chemotherapy has been found to

Table 6.5 Selected studies using molecular methods to detect CMCs in melanoma patients

Reference	Method	Markers	Patients, *n*	Disease stages	Prognostic value
Mellado [70]	RT-PCR	TYR	120	II–IV	Yes
Reynolds [71]	RT-PCR	TYR, Melan-A, MAGE-A3, gp100	118	II–IV	Yes
Wascher [72]	RT-PCR	TYR, Melan-A, uMAGE-A	30	III	Yes
Keilholz [73]	qRT	TYR, Melan-A, gp100	122	III, IV	Yes
Koyanagi [74]	qRT	Melan-A, MAGE-A3, GalNAc-T, PAX3	94	I–IV	Yes
Koyanagi [75]	qRT	MITF	90	I–IV	Yes
Strohal [76]	RT-PCR	TYR, Melan-A	76	I–IV	No
Brownbridge [77]	RT-PCR	TYR, Melan-A	299	I–IV	No
Palmieri [78]	RT-PCR	TYR, Melan-A, p97	200	I–IV	No
Scoggins [37]	RT-PCR	TYR, Melan-A, MAGE-A3, gp100	1,446	I–IV	No

CMCs circulating melanoma cells

induce an early clearance of CMCs [70, 79]. A number of studies have investigated whether the detection of CMCs in melanoma patients is of prognostic value, with respect to such parameters as disease-free survival, relapse/recurrence-free survival, progression-free survival, and overall survival. As for SLN investigations, results of CMC-based studies are conflicting (Table 6.5). Overall, a slight majority of studies have reported a prognostic role for such a molecular test [70–75]. However, up to 40% of studies have not found the molecular detection of CMCs in melanoma patients to be predictive of clinical outcome [37, 76–78]. Melanocyte-related transcript determination in PB appears to be either a surrogate marker for clinical staging (in negative detections) or a predictive factor for disease-specific survival (in positive detections). The conflicting data among studies with respect to prognostic utility may be explained by differences in length of clinical follow-up, time interval between and methods of specimen collection, mRNA extraction techniques, mRNA integrity, selection of molecular markers (related to marker expression heterogeneity by tumor cells), RT-PCR protocols, and data interpretation and statistical analyses [9]. Other issues could include illegitimate transcription, processed pseudogenes, and the complex biology that is associated with metastasis development (such as intermittent shedding of tumor cells and tumor dormancy) [9]. While the mobilization of tumor cells from the primary tumor site is necessary for metastasis formation, it is not sufficient. Animal models have shown that only 0.01–0.001% of circulating cancer cells (derived from solid tumors) are capable of developing a metastatic colony [80]. Therefore, it is likely that a significant proportion of CMCs, which are detectable by RT-PCR, are not capable of contributing to the formation of metastases in melanoma patients. Patients with negative molecular results at initial diagnosis may have subsequent positive results (with or without systemic therapy), and show disease progression [81]. In contrast, up to 25% of patients with a positive molecular result can show one or more negative results during follow-up, and not develop recurrent disease [82]. This latter phenomenon could be related to effective subsequent systemic therapy, metastatic inefficiency of tumor cells, or a combination of these or other factors [82]. Therefore, a single molecular analysis performed during diagnostic work-up may merely reflect the stage of disease (i.e., tumor burden) at that time, and not provide additional prognostic information in individual melanoma patients. It has been proposed that the prolonged presence of CMCs may be an important factor in the selection of viable tumor cells with greater capacity for distant site colonization [8]. Therefore, the molecular persistence or (re-)appearance of CMCs during follow-up could be more strongly associated with disease progression and poor outcome in melanoma patients. Accordingly, serial evaluations for the presence of CMCs during the postoperative period or following adjuvant therapy may be more useful than a single test (Fig. 6.6). For example, patients with consistently negative RT-PCR results demonstrate significantly higher disease-free survival and overall survival compared to those patients with positive results in more than one test during prolonged follow-up [83, 84]. In addition, patients who demonstrate a decrease in CMCs with neoadjuvant chemotherapy have significantly lower rates of disease recurrence than patients who show no change in CMC levels [15]. Furthermore, for melanoma patients treated with interferon for advanced-stage disease (stages II-IV), it has been found that those with persistence or development of CMC-positive results show a significantly lower disease-free survival compared with patients who become CMC-negative [70]. However, another study has reported that RT-PCR assessment is not predictive of outcome following adjuvant interferon treatment [85]. Sequential assays to detect CMCs may provide (a) an early marker of the effectiveness of therapy, particularly in patients with no clinical evidence of disease and (b) an early indicator of disease recurrence, with the re-appearance of markers in the PB. The detection of mRNA transcripts in the PB, both at baseline and during follow-up, could be considered a reliable prognostic parameter associated with response to treatment, development of new metastatic sites, time to progression and survival. However, the role of CMC-based assays in the management of melanoma patients remains controversial. In general, these tests have been limited to clinical trials, and have not yet become a standard part of routine clinical practice.

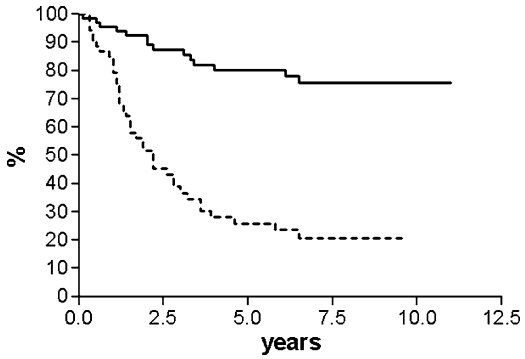

Fig. 6.6 Serial peripheral blood tyrosinase (*TYR*) mRNA evaluation by reverse transcription-polymerase chain reaction (RT-PCR) in patients with melanoma. Overall survival of 125 melanoma patients following radical lymph node dissection for nodal metastases is presented. Serial peripheral blood TYR mRNA determinations were performed on all patients during follow-up. Fifty-five patients showed one or more positive determinations, while samples of the remaining 70 patients remained negative in all cases. The survival of patients with one or more positive samples (- - - - -) was significantly lower (5-year: 26%) than that of negative (———) patients (5-year: 78%; $p < 0.001$) (Courtesy of Dr. Pietro Quaglino, University of Turin, Turin, Italy)

Future research in this field will focus on the identification of novel markers of CMCs that can more accurately predict disease progression. Several clinical studies, such as the phase III multicenter malignant melanoma active immunotherapy trial (MMAIT), are currently validating CMC-based assays.

Detection of Circulating Tumor-Related MicroRNAs

A recent study provides strong evidence that microRNA (miRNA) expression signatures in PB may be useful diagnostic biomarkers for melanoma [86]. MiRNAs are endogeneous small (~22 nucleotide) noncoding RNAs that regulate transcription and translation. Using a microarray-based approach, 51 differentially regulated miRNAs, including 21 downregulated miRNAs and 30 upregulated miRNAs, were identified in the PB of melanoma patients as compared with healthy controls [86]. A subset of 16 significantly deregulated miRNAs distinguished melanoma patients from healthy individuals, with an accuracy of 97.4% [86]. In another study, circulating levels of five cancer-associated miRNAs (let-7a, miR-10b, miR-145, miR-155, and miR-21) were deregulated in the presence of several cancers (including melanoma), with no specific one of these five markers denoting a particular malignancy [87]. The usefulness of miRNA signatures as prognostic, predictive, or early detection biomarkers requires further study.

Isolation of Melanoma Cells

A number of research groups have been successful in isolating CMCs directly from PB, using techniques such as cell culture and immunomagnetic-bead cell sorting [88–91]. The DNA and mRNA extracted from CMCs can be analyzed by qRT, in an effort to characterize the genomic and transcriptomic expression of these cells [91]. While the sensitivity of these isolation assays is

within the range of data published for RT-PCR studies, these techniques are labor-intensive and time-consuming, and to date have not been found to be clinically useful in melanoma patients [88–91]. More recently, platforms based on microfluidic technology and automated immunomagnetic enrichment and staining (CellSearch™) have been shown to be both highly selective and sensitive for the detection of circulating tumor cells in PB samples of cancer patients [85]. However, the identification of CMCs in individuals with melanoma by these latter methods remains to be investigated.

Detection of Circulating Tumor-Related DNA

Intact circulating tumor-related DNA can be found in the PB of melanoma patients, as a result of tumor cell turnover, physically disrupted tumor cells, and/or tumor necrosis or apoptosis [15, 91–101]. This may be detectable as loss of heterozygosity (LOH) of DNA microsatellites, methylated DNA, and mutations of either mitochondrial DNA (mtDNA) or nuclear DNA (including mutant BRAF) [15, 91–101]. Circulating tumor-related DNA has genetic changes similar to primary tumors, and therefore may have clinical utility as a marker of disease stage, therapeutic response, and/or disease recurrence [15, 92, 93]. However, DNA from "normal" (nonmalignant) cells is also a common finding in PB (probably as a result of normal cellular apoptosis) [92]. Therefore, the challenge has been to find DNA markers specific for melanoma cells [92]. A number of studies have reported an association between detectable circulating microsatellite loss, methylated DNA, mtDNA alterations, and mutant BRAF with disease stage and progression [94–96, 100], response to therapy [93, 97–100], and overall survival [99, 100] in melanoma patients. Prospective studies, combining serial PB analyses with long-term follow-up, are needed to fully evaluate the clinical utility of these assays.

Differentiation of Second Primary Tumor from Cutaneous Metastasis

The ability to differentiate between a second primary tumor and a cutaneous metastasis (i.e., AJCC stage I/II *vs.* III/IV) has obvious significance for prognosis, therapy and survival outcome in individual melanoma patients, as well as implications for broader epidemiological studies (Fig. 6.7) [1].

Patients with a history of melanoma have an increased risk (1–8%) of developing a second primary tumor, but are also at varying risk (2–20%) of developing cutaneous metastases from any prior lesion [102–105]. Cutaneous metastases of melanoma can occur in both the early and late phases of disease progression, with locoregional recurrence (AJCC stage IIIB) representing ~80% of cases, and distant skin metastases (stage IV) accounting for the remaining 20% [105]. Interestingly, patients with locoregional metastases have a lower disease-free survival, but a longer time to progression to visceral involvement, compared with patients with distant skin metastases [105]. Currently, a distinction between a second primary tumor and a recurrent/metastatic lesion is based on clinical history, changes on physical examination (i.e., proximity of lesions), and microscopic findings (i.e., similarity of cytomorphological and immunohistochemical features, presence or absence of a precursor lesion, and/or epidermotropism). However, it can be difficult to definitively categorize a "second" neoplasm on the basis of comparative histopathologic and immunophenotypic features [106]. As a consequence, a number of studies have investigated if molecular technologies could be used to delineate either a clonal relationship or independent origin among

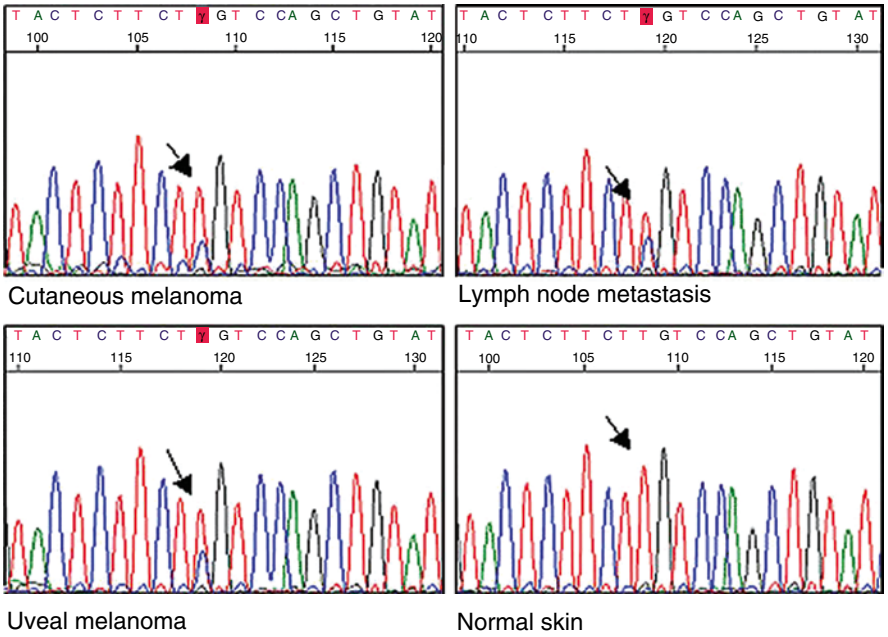

Fig. 6.7 Molecular confirmation of melanoma metastasis versus secondary primary tumor. Sequencing analysis demonstrates an identical NRAS exon 3 codon 61 mutation (c.182A>G, p.Gln61Arg) in primary cutaneous melanoma and subsequent lymph node and uveal metastases, but not in normal control tissue of the patient (excluding a germline mutation) (From Küsters-Vandevelde et al. [115]. Reprinted with permission from Springer, copyright © 2007)

pairs of cutaneous melanoma samples, and therefore have potential diagnostic utility in differentiating skin-tropic metastatic melanomas from second primary lesions [102–104, 107–109]. In those instances where paired tumors show cytologically and immunohistochemically divergent features, molecular studies may have a role in distinguishing a metastatic melanoma from a tumor of different origin/differentiation [108]. Of note, differences between the transcriptional profiles of melanoma metastases and other solid tumors have been described [110]. Molecular analysis could also be used to distinguish melanoma from clear cell sarcoma, a tumor which shows similar morphologic (i.e., presence of melanin), histochemical, immunohistochemical (i.e., S100+/HMB-45+), and ultrastructural (i.e., presence of pre-melanosomes) features (see Chap. 8).

Paired skin-derived melanoma samples have been evaluated by such techniques as X-chromosome inactivation analysis [103, 104] and LOH testing of multiple microsatellite markers [102, 103, 107], in addition to BRAF gene [102], p16 gene [108], and CDKN2A locus [109] mutational assays. Other studies on paired (cutaneous and non-cutaneous) tumor samples have focused more on an investigation of the genetic basis of melanoma pathogenesis and metastatic progression (in addition to possible diagnostic and prognostic utilities), employing such methods as karyotyping, LOH, FISH, CGH, oligonucleotide and cDNA microarrays, and CDKN2A, NRAS, and KIT gene mutational analyses [44, 49, 111–117]. The detection of identical mutations in: (a) p16 genes [108]; (b) CDKN2A loci [109]; and (c) NRAS genes and CDKN2A loci [115], in paired tumor samples, has been used to establish the diagnosis of melanoma metastases to the skin and other sites (i.e., lymph node, eye) (Fig. 6.7). Identical KIT mutations in both primary melanomas and their metastases have also been reported [including the same exon 11, V559A activating mutation in a primary acral lentiginous melanoma (ALM) and its corresponding skin metastasis] [116]. It should be stressed that such gene mutational analyses must be interpreted with caution. Firm conclusions that the tumor pair are related (i.e., primary and corresponding metastasis) can only be reached if an identical mutation is found in both tumors, and not present in the germline [108, 109]. In cases of discordant findings, no definitive conclusions can be drawn, as mutations could

be either acquired or lost with tumor progression [109]. The majority of melanoma metastases do appear to share a common clonal origin with their matched primary tumors [104]. However, multiple coexisting, genetically independent metastatic tumors can be found in an individual patient, and putatively arise from several distinct subpopulations/clones in the original melanoma [104]. It has been argued that the presence of mutational heterogeneity both (a) within an individual melanoma and (b) between a recurrent/metastatic melanoma and its primary tumor may be a pitfall for diagnostic molecular testing in this setting [102, 114]. Divergence of metastatic subclones could occur early in the evolution of the primary tumor, and clonal evolution of melanoma may not necessarily follow the linear growth-dominance concept of the metastatic cancer cell, as proposed for other human tumors [114]. In contrast to the transformation from a benign melanocyte to melanoma, the ability of a fully formed melanoma cell to give rise to a metastasis may require the presence of genomic stability [118]. Several studies have shown more similarities in gene expression among autologous samples taken at different stages from one individual than between patients with similar stage disease [43]. A recent oligonucleotide microarray study found no statistically significant differences in gene expression between primary melanomas and their corresponding metastases ($n = 9$, including 5 cutaneous metastases) [44]. In contrast, cDNA microarrays readily distinguished a patient's primary and corresponding metastasis in another study [117]. A four-probe FISH-based study found that SLN metastatic melanoma deposits can show (a) less, more, or equal numbers of genetic aberrations and (b) identical or different types of genetic aberrations, compared with their corresponding primary melanomas [49]. Karyotyping studies, on paired primary and metastatic melanomas, have shown common alterations (interpreted as supporting disease clonality), as well as additional changes in the metastases [111]. In recent CGH- and FISH-based studies on five patients, none of the metastatic tumors (including two cutaneous metastases) was found to be genetically identical to its primary melanoma [112, 113]. Shared chromosomal alterations between paired tumor sets were noted [112, 113]. However, in all cases, the metastases had acquired new chromosomal alterations that were not identified in the primary tumors [112, 113]. The higher genomic instability at metastatic sites suggests that melanoma progression is based on the accumulation of multiple genetic alterations. Therefore, comparative studies could be used to explore the chromosomal alterations associated with aggressive behavior and to identify metastasis-related genetic aberrations (in addition to tumor initiation factors), but may have limited value in determining the nature/origin of a second tumor. A degree of LOH discordance can be present between a tumor pair, even if they share a common clonal origin [107]. Of course, LOH concordance (i.e., loss of the same allele at the same locus) could occur by chance in two distinct tumors [107]. In addition, it has been postulated that there exists a population of melanoma stem cells with core genomic instability that have not yet acquired tumorigenic mutations, analogous to the melanoma field cell concept in ALM (see Chap. 9) [102, 119]. Theoretically, multiple tumor cell populations with different genotypic and phenotypic characteristics could arise in an individual tumor from such stem cells through different molecular pathways [102]. These concepts have important implications both for (a) the current view of melanoma biology and metastases (i.e., a single transformed cell produces a clonal population of malignant cells, with subsequent development of genetically divergent subpopulations that may acquire the ability to metastasize) [103] and (b) the evolving field of targeted therapy for patients with melanoma [120, 121]. Recently, Begg et al. [122] proposed a statistical test that could compare patterns of allelic losses at candidate genetic loci, and be used to determine if a diagnosed second tumor shares a common clonal origin or is biologically independent of the original primary lesion. Clonality would be favored if the number of concordant mutations between tumors exceeds the number expected on the basis of chance [107]. Using this statistical testing strategy, their results suggest that the majority of melanomas, which are diagnosed as second primaries on the basis of their clinical and histopathological features, do indeed represent independent occurrences of the disease (Fig. 6.8) [107]. These findings support the validity of current histopathological criteria used by experienced pathologists to distinguish metastases from second primary tumors [107]. In the future, clinical and histopathological evaluation may be supplemented by molecular diagnostic testing in an effort to definitively identify the origin of second malignancies in patients with melanoma.

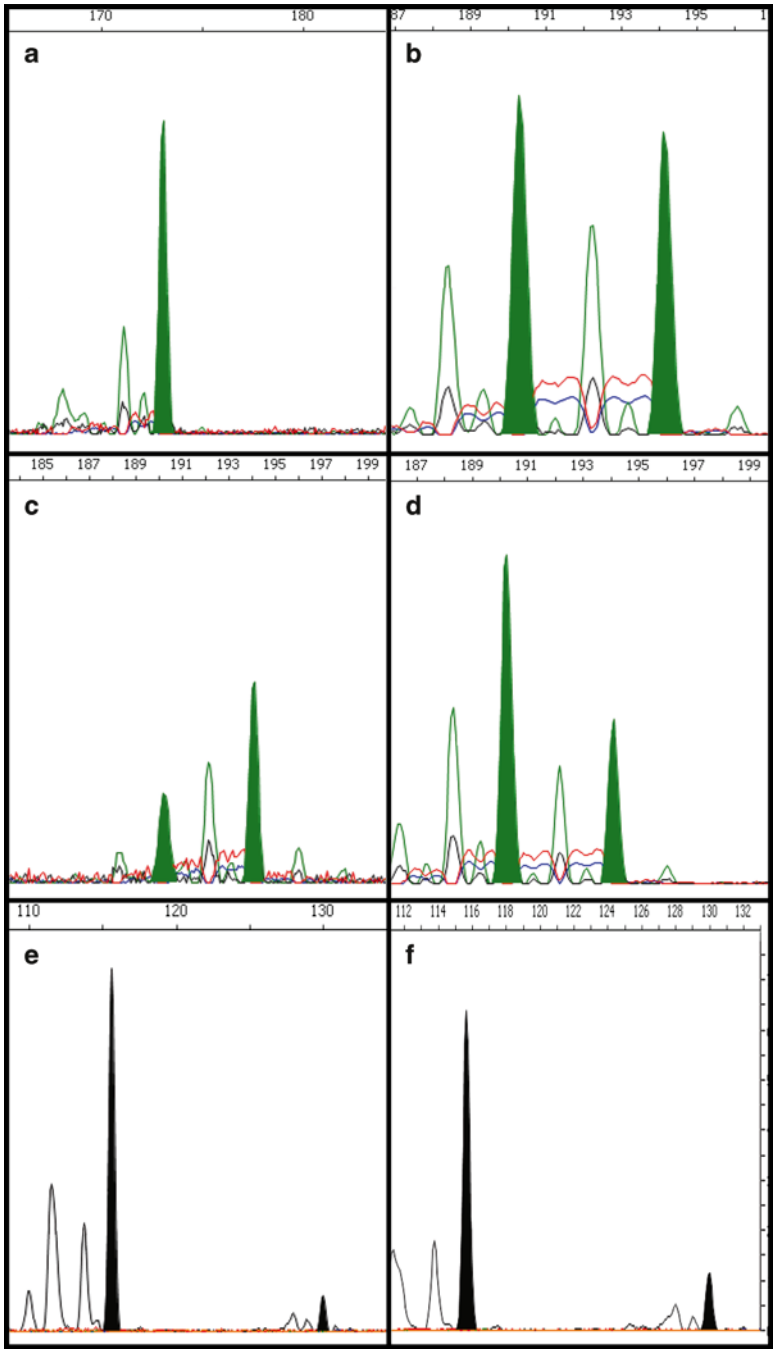

Fig. 6.8 Comparison of the somatic mutational profiles of a pair of melanomas in an individual patient: determination of allelic gains and losses by fragment-size analysis. The electropherograms depict results obtained for the dinucleotide (CA) markers D2S139 (**a**–**d**) and D1S214 (**e**–**f**). Figures (**a**) and (**b**) exemplify noninformative and informative normal DNA samples, respectively. The tumor pair of a melanoma patient display discordant allelic loss for D2S139: loss of the short allele in tumor #1 (**c**) and loss of the long allele in tumor #2 (**d**). For the marker D1S214, both of the patient's tumors show concordant allelic loss, or loss of the same allele (**e**–**f**). *C* cytosine, *A* adenine (Courtesy of Drs. Irene Orlow and Colin Begg, Memorial Sloan-Kettering Cancer Center, New York)

References

1. Balch CM, Gershenwald JE, Soong SJ, et al. Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol. 2009;27:6199–206.
2. Balch CM, Buzaid AC, Soong SJ, et al. Final version of the American Joint Committee on cancer staging system for cutaneous melanoma. J Clin Oncol. 2001;19:3635–48.
3. Carlson JA, Slominski A, Linette GP, et al. Biomarkers in melanoma: staging, prognosis and detection of early metastases. Expert Rev Mol Diagn. 2003;3:303–30.
4. Waldmann V, Wacker J, Deichmann M, et al. Prognosis of metastatic melanoma: no correlation of tyrosinase mRNA in bone marrow and survival time. Recent Results Cancer Res. 2001;158:118–25.
5. Savoia P, Quaglino P, Osella-Abate S, et al. Tyrosinase mRNA RT-PCR analysis as an additional diagnostic tool for the identification of melanoma cells in biological fluid samples other than blood: a preliminary report. Int J Biol Markers. 2005;20:11–7.
6. Hoon DS, Kuo CT, Wascher RA, et al. Molecular detection of metastatic melanoma cells in cerebrospinal fluid in melanoma patients. J Invest Dermatol. 2001;117:375–8.
7. Mocellin S, Hoon DS, Pilati P, et al. Sentinel lymph node molecular ultrastaging in patients with melanoma: a systematic review and meta-analysis of prognosis. J Clin Oncol. 2007;25:1588–95.
8. Palmieri G, Casula M, Sini MC, et al. Issues affecting molecular staging in the management of patients with melanoma. J Cell Mol Med. 2007;11:1052–68.
9. Nezos A, Lembessis P, Sourla A, et al. Molecular markers detecting circulating melanoma cells by reverse transcription polymerase chain reaction: methodological pitfalls and clinical relevance. Clin Chem Lab Med. 2009;47:1–11.
10. Medic S, Pearce RL, Heenan PJ, et al. Molecular markers of circulating melanoma cells. Pigment Cell Res. 2007;20:80–91.
11. Wang X, Heller R, VanVoorhis N, et al. Detection of submicroscopic lymph node metastases with polymerase chain reaction in patients with malignant melanoma. Ann Surg. 1994;220:768–74.
12. Shivers SC, Li W, Lin J, et al. The clinical relevance of molecular staging for melanoma. Recent Results Cancer Res. 2001;158:187–99.
13. Takata M. Genetic/epigenetic markers to distinguish melanomas from other melanocytic neoplasms. In: Murphy M, editor. Diagnostic and prognostic biomarkers and therapeutic targets in Melanoma. New York: Humana Press; 2011 (in press).
14. Carlson JA, Slominski A, Linette GP, et al. Malignant melanoma 2003: predisposition, diagnosis, prognosis, and staging. Am J Clin Pathol. 2003;120(Suppl):S101–27.
15. Martinez SR, Hoon DS. Molecular markers in malignant cutaneous melanoma: gift horse or one-trick pony? J Cell Biochem. 2005;96:473–83.
16. Morton DL, Wen DR, Wong JH, et al. Technical details of intraoperative lymphatic mapping for early stage melanoma. Arch Surg. 1992;127:392–9.
17. Cochran AJ, Roberts A, Wen DR, et al. Update on lymphatic mapping and sentinel node biopsy in the management of patients with melanocytic tumours. Pathology. 2004;36:478–84.
18. Phan GQ, Messina JL, Sondak VK, et al. Sentinel lymph node biopsy for melanoma: indications and rationale. Cancer Control. 2009;16:234–9.
19. Leiter U, Meier F, Schittek B, et al. The natural course of cutaneous melanoma. J Surg Oncol. 2004;86:172–8.
20. Perrott RE, Glass LF, Reintgen DS, et al. Reassessing the role of lymphatic mapping and sentinel lymphadenectomy in the management of cutaneous malignant melanoma. J Am Acad Dermatol. 2003;49:567–88.
21. Morton DL, Hoon DS, Cochran AJ, et al. Lymphatic mapping and sentinel lymphadenectomy for early-stage melanoma: therapeutic utility and implications of nodal microanatomy and molecular staging for improving the accuracy of detection of nodal micrometastases. Ann Surg. 2003;238:538–49.
22. Lewis TB, Robison JE, Bastien R, et al. Molecular classification of melanoma using real-time quantitative reverse transcriptase-polymerase chain reaction. Cancer. 2005;104:1678–86.
23. Russell-Jones R. Sentinel node and survival in melanoma. Br J Dermatol. 2005;153:1093–5.
24. Gutzmer R, Al Ghazal M, Geerlings H, et al. Sentinel node biopsy in melanoma delays recurrence but does not change melanoma-related survival: a retrospective analysis of 673 patients. Br J Dermatol. 2005;153:1137–41.
25. Roka F, Kittler H, Cauzig P, et al. Sentinel node status in melanoma patients is not predictive for overall survival upon multivariate analysis. Br J Cancer. 2005;92:662–7.
26. Carlson JA, Ross JS, Slominski AJ. New techniques in dermatopathology that help to diagnose and prognosticate melanoma. Clin Dermatol. 2009;27:75–102.
27. Hilari JM, Mangas C, Xi L, et al. Molecular staging of pathologically negative sentinel lymph nodes from melanoma patients using multimarker, quantitative real-time rt-PCR. Ann Surg Oncol. 2009;16:177–85.

28. Prichard RS, Dijkstra B, McDermott EW, et al. The role of molecular staging in malignant melanoma. Eur J Surg Oncol. 2003;29:306–14.
29. Nowecki ZI, Rutkowski P, Kulik J, et al. Molecular and biochemical testing in stage III melanoma: multimarker reverse transcriptase-polymerase chain reaction assay of lymph fluid after lymph node dissection and preoperative serum lactate dehydrogenase level. Br J Dermatol. 2008;159:597–605.
30. Starz H, Haas CJ, Schulz GM, et al. Tyrosinase RT-PCR as a supplement to histology for detecting melanoma and nevus cells in paraffin sections of sentinel lymph nodes. Mod Pathol. 2003;16:920–9.
31. Hochberg M, Lotem M, Gimon Z, et al. Expression of tyrosinase, MIA and MART-1 in sentinel lymph nodes of patients with malignant melanoma. Br J Dermatol. 2002;146:244–9.
32. Kuo CT, Hoon DS, Takeuchi H, et al. Prediction of disease outcome in melanoma patients by molecular analysis of paraffin-embedded sentinel lymph nodes. J Clin Oncol. 2003;21:3566–72.
33. Rimoldi D, Lemoine R, Kurt AM, et al. Detection of micrometastases in sentinel lymph nodes from melanoma patients: direct comparison of multimarker molecular and immunopathological methods. Melanoma Res. 2003;13:511–20.
34. Takeuchi H, Morton DL, Kuo C, et al. Prognostic significance of molecular upstaging of paraffin-embedded sentinel lymph nodes in melanoma patients. J Clin Oncol. 2004;22:2671–80.
35. Kammula US, Ghossein R, Bhattacharya S, et al. Serial follow-up and the prognostic significance of reverse transcriptase-polymerase chain reaction–staged sentinel lymph nodes from melanoma patients. J Clin Oncol. 2004;22:3989–96.
36. Mangas C, Hilari JM, Paradelo C, et al. Prognostic significance of molecular staging study of sentinel lymph nodes by reverse transcriptase-polymerase chain reaction for tyrosinase in melanoma patients. Ann Surg Oncol. 2006;13:910–8.
37. Scoggins CR, Ross MI, Reintgen DS, et al. Prospective multi-institutional study of reverse transcriptase polymerase chain reaction for molecular staging of melanoma. J Clin Oncol. 2006;24:2849–57.
38. Blaheta HJ, Schittek B, Breuninger H, et al. Detection of melanoma micrometastasis in sentinel nodes by reverse transcription-polymerase chain reaction correlates with tumor thickness and is predictive of micrometastatic disease in the lymph node basin. Am J Surg Pathol. 1999;23:822–8.
39. Soikkeli J, Lukk M, Nummela P, et al. Systematic search for the best gene expression markers for melanoma micrometastasis detection. J Pathol. 2007;213:180–9.
40. Ma Z, Lui WO, Fire A, et al. Profiling and discovery of novel miRNAs from formalin-fixed, paraffin-embedded melanoma and nodal specimens. J Mol Diagn. 2009;11:420–9.
41. Torisu-Itakura H, Lee JH, Scheri RP, et al. Molecular characterization of inflammatory genes in sentinel and nonsentinel nodes in melanoma. Clin Cancer Res. 2007;13:3125–32.
42. Sarff M, Edwards D, Dhungel B, et al. OX40 (CD134) expression in sentinel lymph nodes correlates with prognostic features of primary melanomas. Am J Surg. 2008;195:621–5.
43. John T, Black MA, Toro TT, et al. Predicting clinical outcome through molecular profiling in stage III melanoma. Clin Cancer Res. 2008;14:5173–80.
44. Winnepenninckx V, Lazar V, Michiels S, et al. Melanoma Group of the European Organization for research and treatment of cancer. Gene expression profiling of primary cutaneous melanoma and clinical outcome. J Natl Cancer Inst. 2006;98:472–82.
45. Mandruzzato S, Callegaro A, Turcatel G, et al. A gene expression signature associated with survival in metastatic melanoma. J Transl Med. 2006;4:50.
46. Gradilone A, Gazzaniga P, Ribuffo D, et al. Survivin, bcl-2, bax, and bcl-X gene expression in sentinel lymph nodes from melanoma patients. J Clin Oncol. 2003;21:306–12.
47. Vitoux D, Mourah S, Kerob D, et al. Highly sensitive multivariable assay detection of melanocytic differentiation antigens and angiogenesis biomarkers in sentinel lymph nodes with melanoma micrometastases. Arch Dermatol. 2009;145:1105–13.
48. Fujimoto A, Takeuchi H, Taback B, et al. Allelic imbalance of 12q22-23 associated with APAF-1 locus correlates with poor disease outcome in cutaneous melanoma. Cancer Res. 2004;64:2245–50.
49. Dalton SR, Gerami P, Kolaitis NA, et al. Use of fluorescence in-situ hybridization (FISH) to distinguish intranodal nevus from metastatic melanoma. Am J Surg Pathol. 2010;34:231–7.
50. Carlson JA. Tumor doubling time of cutaneous melanoma and its metastasis. Am J Dermatopathol. 2003;25:291–9.
51. Abrahamsen HN, Sorensen BS, Nexo E, et al. Pathologic assessment of melanoma sentinel nodes: a role for molecular analysis using quantitative real-time reverse transcription-PCR for MART-1 and tyrosinase messenger RNA. Clin Cancer Res. 2005;11:1425–33.
52. Abrahamsen HN, Nexo E, Steiniche T, et al. Quantification of melanoma mRNA markers in sentinel nodes: pre-clinical evaluation of a single-step real-time reverse transcriptase-polymerase chain reaction assay. J Mol Diagn. 2004;6:253–9.

53. Lens MB, Dawes M, Newton-Bishop JA, et al. Tumour thickness as a predictor of occult lymph node metastases in patients with stage I and II melanoma undergoing sentinel lymph node biopsy. Br J Surg. 2002;89:1223–7.
54. Li W, Stall A, Shivers SC, et al. Clinical relevance of molecular staging for melanoma: comparison of RT-PCR and immunohistochemistry staining in sentinel lymph nodes of patients with melanoma. Ann Surg. 2000;231:795–803.
55. Giese T, Engstner M, Mansmann U, et al. Quantification of melanoma micrometastases in sentinel lymph nodes using real-time RT-PCR. J Invest Dermatol. 2005;124:633–7.
56. Reed W, Bohler PJ, Sandstad B, et al. Occult metastases in axillary lymph nodes as a predictor of survival in node-negative breast carcinoma with long-term follow-up. Breast J. 2004;10:174–80.
57. Thomas JM. Caution with sentinel node biopsy in cutaneous melanoma. Br J Surg. 2006;93:129–30.
58. van Akkooi AC, de Wilt JH, Verhoef C, et al. Clinical relevance of melanoma micrometastases (<0.1 mm) in sentinel nodes: are these nodes to be considered negative? Ann Oncol. 2006;17:1578–85.
59. Govindarajan A, Ghazarian DM, McCready DR, et al. Histological features of melanoma sentinel lymph node metastases associated with status of the completion lymphadenectomy and rate of subsequent relapse. Ann Surg Oncol. 2007;14:906–12.
60. Scheri RP, Essner R, Turner RR, et al. Isolated tumor cells in the sentinel node affect long-term prognosis of patients with melanoma. Ann Surg Oncol. 2007;14:2861–6.
61. Davids V, Kidson SH, Hanekom GS. Melanoma patient staging: histopathological versus molecular evaluation of the sentinel node. Melanoma Res. 2003;13:313–24.
62. Davids V, Kidson SH, Hanekom GS. Accurate molecular detection of melanoma nodal metastases: an assessment of multimarker assay specificity, sensitivity, and detection rate. Mol Pathol. 2003;56:43–51.
63. Lukowsky A, Bellmann B, Ringk A, et al. Detection of melanoma micrometastases in the sentinel lymph node and in nonsentinel nodes by tyrosinase polymerase chain reaction. J Invest Dermatol. 1999;113:554–9.
64. Gjerdrum LM, Abrahamsen HN, Villegas B, et al. The influence of immunohistochemistry on mRNA recovery from microdissected frozen and formalin-fixed, paraffin-embedded sections. Diagn Mol Pathol. 2004;13:224–33.
65. Talantov D, Mazumder A, Yu JX, et al. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res. 2005;11:7234–42.
66. de Wit NJ, Rijntjes J, Diepstra JH, et al. Analysis of differential gene expression in human melanocytic tumour lesions by custom made oligonucleotide arrays. Br J Cancer. 2005;92:2249–61.
67. Solassol J, Mangé A. Identification of serum melanoma progression biomarkers through proteomic-based approaches. Expert Rev Proteomics. 2009;6:341–3.
68. Ugurel S, Utikal J, Becker JC. Tumor biomarkers in melanoma. Cancer Control. 2009;16:219–24.
69. Findeisen P, Peccerella T, Neumaier M, et al. Proteomics for biomarker discovery in malignant melanoma. Expert Rev Dermatol. 2008;3:209–20.
70. Mellado B, Del Carmen MV, Colomer D, et al. Tyrosinase mRNA in blood of patients with melanoma treated with adjuvant interferon. J Clin Oncol. 2002;20:4032–9.
71. Reynolds SR, Albrecht J, Shapiro RL, et al. Changes in the presence of multiple markers of circulating melanoma cells correlate with clinical outcome in patients with melanoma. Clin Cancer Res. 2003;9:1497–502.
72. Wascher RA, Morton DL, Kuo C, et al. Molecular tumor markers in the blood: early prediction of disease outcome in melanoma patients treated with a melanoma vaccine. J Clin Oncol. 2003;21:2558–63.
73. Keilholz U, Goldin-Lang P, Bechrakis NE, et al. Quantitative detection of circulating tumor cells in cutaneous and ocular melanoma and quality assessment by real-time reverse transcriptase-polymerase chain reaction. Clin Cancer Res. 2004;10:1605–12.
74. Koyanagi K, Kuo C, Nakagawa T, et al. Multimarker quantitative real-time PCR detection of circulating melanoma cells in peripheral blood: relation to disease stage in melanoma patients. Clin Chem. 2005;51:981–8.
75. Koyanagi K, O'Day SJ, Gonzalez R, et al. Microphthalmia transcription factor as a molecular marker for circulating tumor cell detection in blood of melanoma patients. Clin Cancer Res. 2006;12:1137–43.
76. Strohal R, Mosser R, Kittler H, et al. MART-1/Melan-A and tyrosinase transcripts in peripheral blood of melanoma patients: PCR analyses and follow-up testing in relation to clinical stage and disease progression. Melanoma Res. 2001;11:543–8.
77. Brownbridge GG, Gold J, Edward M, et al. Evaluation of the use of tyrosinase-specific and melanA/MART-1-specific reverse transcriptase-coupled–polymerase chain reaction to detect melanoma cells in peripheral blood samples from 299 patients with malignant melanoma. Br J Dermatol. 2001;144:279–87.
78. Palmieri G, Ascierto PA, Perrone F, et al. Prognostic value of circulating melanoma cells detected by reverse transcriptase-polymerase chain reaction. J Clin Oncol. 2003;21:767–73.
79. Koyanagi K, O'Day SJ, Gonzalez R, et al. Serial monitoring of circulating melanoma cells during neoadjuvant biochemotherapy for stage III melanoma: outcome prediction in a multicenter trial. J Clin Oncol. 2005;23:8057–64.
80. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes Memorial Award Lecture. Cancer Res. 1990;50:6130–8.

81. Osella-Abate S, Savoia P, Quaglino P, et al. Tyrosinase expression in the peripheral blood of stage III melanoma patients is associated with a poor prognosis: a clinical follow-up study of 110 patients. Br J Cancer. 2003;89:1457–62.
82. Quaglino P, Savoia P, Osella-Abate S, et al. RT-PCR tyrosinase expression in the peripheral blood of melanoma patients. Expert Rev Mol Diagn. 2004;4:727–41.
83. Voit C, Kron M, Rademaker J, et al. Molecular staging in stage II and III melanoma patients and its effect on long-term survival. J Clin Oncol. 2005;23:1218–27.
84. Palmieri G, Satriano SM, Budroni M, et al. Serial detection of circulating tumour cells by reverse transcriptase-polymerase chain reaction assays is a marker for poor outcome in patients with malignant melanoma. BMC Cancer. 2006;6:266.
85. Fusi A, Collette S, Busse A, et al. Circulating melanoma cells and distant metastasis-free survival in stage III melanoma patients with or without adjuvant interferon treatment (EORTC 18991 side study). Eur J Cancer. 2009;45:3189–97.
86. Leidinger P, Keller A, Borries A, et al. High-throughput miRNA profiling of human melanoma blood samples. BMC Cancer. 2010;10:262.
87. Heneghan HM, Miller N, Kelly R, et al. Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. Oncologist. 2010;15:673–82.
88. Pretlow TP, Bailey JM, Herrera GA, et al. Culture in soft agar of melanoma cells separated from human peripheral blood. Br J Cancer. 1986;53:411–4.
89. Benez A, Schiebel U, Fierlbeck G. Morphologically intact melanoma cells may be detected in peripheral blood of melanoma patients. Recent Results Cancer Res. 2001;158:113–7.
90. Ulmer A, Schmidt-Kittler O, Fischer J, et al. Immunomagnetic enrichment, genomic characterization, and prognostic impact of circulating melanoma cells. Clin Cancer Res. 2004;10:531–7.
91. Kitago M, Koyanagi K, Nakamura T, et al. mRNA expression and BRAF mutation in circulating melanoma cells isolated from peripheral blood with high molecular weight melanoma-associated antigen-specific monoclonal antibody beads. Clin Chem. 2009;55:757–64.
92. Ziegler A, Zangemeister-Wittke U, Stahel RA. Circulating DNA: a new diagnostic gold mine? Cancer Treat Rev. 2002;28:255–71.
93. Shinozaki M, O'Day SJ, Kitago M, et al. Utility of circulating B-RAF DNA mutation in serum for monitoring melanoma patients receiving biochemotherapy. Clin Cancer Res. 2007;13:2068–74.
94. Takeuchi H, Fujimoto A, Hoon DS. Detection of mitochondrial DNA alterations in plasma of malignant melanoma patients. Ann NY Acad Sci. 2004;1022:50–4.
95. Fujiwara Y, Chi DD, Wang H, et al. Plasma DNA microsatellites as tumor-specific markers and indicators of tumor progression in melanoma patients. Cancer Res. 1999;59:1567–71.
96. Taback B, Fujiwara Y, Wang HJ, et al. Prognostic significance of circulating microsatellite markers in the plasma of melanoma patients. Cancer Res. 2001;61:5723–6.
97. Taback B, O'Day SJ, Boasberg PD, et al. Circulating DNA microsatellites: molecular determinants of response to biochemotherapy in patients with metastatic melanoma. J Natl Cancer Inst. 2004;96:152–6.
98. Fujimoto A, O'Day SJ, Taback B, et al. Allelic imbalance on 12q22-23 in serum circulating DNA of melanoma patients predicts disease outcome. Cancer Res. 2004;64:4085–8.
99. Mori T, O'Day SJ, Umetani N, et al. Predictive utility of circulating methylated DNA in serum of melanoma patients receiving biochemotherapy. J Clin Oncol. 2005;23:9351–8.
100. Mori T, Martinez SR, O'Day SJ, et al. Estrogen receptor-alpha methylation predicts melanoma progression. Cancer Res. 2006;66:6692–8.
101. Marini A, Mirmohammadsadegh A, Nambiar S, et al. Epigenetic inactivation of tumor suppressor genes in serum of patients with cutaneous melanoma. J Invest Dermatol. 2006;126:422–31.
102. Edinger JT, Radfar A, Jukic DM. Two cutaneous malignant melanomas at the same anatomic site: a case report with molecular evaluation. J Cutan Pathol. 2009;36(Suppl 1):74–9.
103. Bahrami S, Cheng L, Wang M, et al. Clonal relationships between epidermotropic metastatic melanomas and their primary lesions: a loss of heterozygosity and X-chromosome inactivation-based analysis. Mod Pathol. 2007;20:821–7.
104. Katona TM, Jones TD, Wang M, et al. Genetically heterogeneous and clonally unrelated metastases may arise in patients with cutaneous melanoma. Am J Surg Pathol. 2007;31:1029–37.
105. Savoia P, Fava P, Nardò T, et al. Skin metastases of malignant melanoma: a clinical and prognostic survey. Melanoma Res. 2009;19:321–6.
106. Guerriere-Kovach PM, Hunt EL, Patterson JW, et al. Primary melanoma of the skin and cutaneous melanomatous metastases: comparative histologic features and immunophenotypes. Am J Clin Pathol. 2004;122:70–7.
107. Orlow I, Tommasi DV, Bloom B, et al. Evaluation of the clonal origin of multiple primary melanomas using molecular profiling. J Invest Dermatol. 2009;129:1972–82.

108. Quezado MM, Abati AD, Albuquerque AV, et al. Morphologic diversity in malignant melanoma: the potential use of microdissection and the polymerase chain reaction for diagnosis. Mod Pathol. 1998;11:1010–5.
109. Blokx WA, Lesterhuis WJ, Andriessen MP, et al. CDKN2A (INK4A-ARF) mutation analysis to distinguish cutaneous melanoma metastasis from a second primary melanoma. Am J Surg Pathol. 2007;31:637–41. Erratum in: Am J Surg Pathol. 2007;31:1137. Lesterhuis, Joost J [corrected to Lesterhuis, W Joost]; Punt, Kees J A [corrected to Punt, Cornelis J A].
110. Wang E, Panelli MC, Zavaglia K, et al. Melanoma-restricted genes. J Transl Med. 2004;2:34.
111. Balaban GB, Herlyn M, Clark Jr WH, et al. Karyotypic evolution in human malignant melanoma. Cancer Genet Cytogenet. 1986;19:113–22.
112. Balázs M, Adám Z, Treszl A, et al. Chromosomal imbalances in primary and metastatic melanomas revealed by comparative genomic hybridization. Cytometry. 2001;46:222–32.
113. Balázs M, Adám Z, Bégány A, et al. Involvement of chromosome losses in the progression and metastasis formation of a human malignant melanoma. Cancer Genet Cytogenet. 1999;109:114–8.
114. Morita R, Fujimoto A, Hatta N, et al. Comparison of genetic profiles between primary melanomas and their metastases reveals genetic alterations and clonal evolution during progression. J Invest Dermatol. 1998;111:919–24.
115. Küsters-Vandevelde HV, Keunen JE, Wesseling P, et al. Occurrence of ocular melanoma thirteen years after skin melanoma: two separate primaries or metastatic disease? A case solved with NRAS and CDKN2A (INK4A-ARF) mutational analysis. Virchows Arch. 2008;452:331–6.
116. Torres-Cabala CA, Wang WL, Trent J, et al. Correlation between KIT expression and KIT mutation in melanoma: a study of 173 cases with emphasis on the acral-lentiginous/mucosal type. Mod Pathol. 2009;22:1446–56.
117. Haqq C, Nosrati M, Sudilovsky D, et al. The gene expression signatures of melanoma progression. Proc Natl Acad Sci USA. 2005;102:6092–7.
118. Kauffmann A, Rosselli F, Lazar V, et al. High expression of DNA repair pathways is associated with metastasis in melanoma patients. Oncogene. 2008;27:565–73.
119. North JP, Kageshita T, Pinkel D, et al. Distribution and significance of occult intraepidermal tumor cells surrounding primary melanoma. J Invest Dermatol. 2008;128:2024–30.
120. Russo AE, Torrisi E, Bevelacqua Y, et al. Melanoma: molecular pathogenesis and emerging target therapies (review). Int J Oncol. 2009;34:1481–9.
121. Flaherty KT, Hodi FS, Bastian BC. Mutation-driven drug development in melanoma. Curr Opin Oncol. 2010;22:178–83.
122. Begg CB, Eng KH, Hummer AJ. Statistical tests for clonality. Biometrics. 2007;63:522–30.

Chapter 7
Non-Melanoma Skin Cancers and Hereditary Cancer Syndromes

Zhenyu Ji, Victor Neel, and Hensin Tsao

Non-melanoma skin cancers (NMSC), which include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are among the most common human neoplasms, accounting for nearly as many human tumors as all other cancers combined. More than one million new cases of NMSC are diagnosed each year in the USA [1]. Although their growth is often slow when compared to other cancers, NMSC can be locally destructive and many have the capacity to metastasize to distant organs. Therefore, early diagnosis is critical for successful treatment of these tumors [2]. The current gold standard for diagnosis of NMSC is tissue biopsy for histopathological analysis [3]. However, a definitive diagnosis is sometimes not possible on the basis of morphological features [4, 5]. In this chapter, we will provide an update on recent advances in molecular technologies which may help resolve diagnostic ambiguities and potentially offer insights into patient outcomes and treatment response of NMSC.

Both BCC and SCC are keratinocyte-derived skin tumors that predominantly occur at chronically sun-exposed sites [6]. BCC is the most common form of NMSC, accounting for more than 75% of cases. Although they are locally invasive and destroy surrounding tissue, BCC rarely metastasize. The metastatic potential of SCC is higher than that of BCC, especially those tumors found at mucosal sites, in areas of chronic wound healing and/or in immunocompromised patients [7]. In addition to BCC and SCC, there exist several keratinocytic dysplasias associated with ultraviolet radiation (UVR)-damaged skin, including actinic keratosis (AK), SCC in situ (Bowen's disease), and keratoacanthoma (KA) [8]. Both AK and SCC in situ can evolve to overt SCC, whereas KA rarely progress and can resolve spontaneously [9].

Basal Cell Carcinoma

Basal cell carcinomas (BCC) are slow-growing epithelial tumors, composed of nests and cords of basaloid cells with hyperchromatic nuclei, scant cytoplasm, and peripheral palisading, in addition to stromal retraction and mucin deposition [10]. Karyotyping and fluorescence in situ hybridization (FISH) analyses have identified trisomy of chromosome 6 as a cytogenetic aberration in tumor cells of aggressive and metastatic BCC [11, 12]. In fact, acquisition of trisomy 6 in BCC may be associated

H. Tsao (✉)
Wellman Center for Photomedicine, Department of Dermatology and MGH Cancer Center,
Massachusetts General Hospital, Harvard Medical School, Bartlett 622,
48 Blossom Street, Boston, MA 02114, USA
e-mail: htsao@partners.org

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_7,

with metastatic potential. Karyotying and comparative genomic hybridization (CGH) studies have also revealed regions of recurrent copy number (CN) gain in BCC, including 6p, 7, 9p, and X, and CN loss on 9q [13–15]. This loss has been confirmed in 30–50% of BCC samples by loss of heterozygosity (LOH) analysis, which further refined this region to 9q22.3, the site of the tumor suppressor gene *PTCH1* [13, 16–18]. PTCH1 acts as a transmembrane receptor and its loss results in upregulation of the sonic hedgehog (SHH) signaling pathway [19].

Dysregulation of the SHH pathway is a critical event in BCC development. PTCH1 negatively regulates SHH signaling through inhibition of a transmembrane protein Smoothened (SMO) (Fig. 7.1). SHH binds to PTCH1, relieving the pathway inhibition induced by unbound PTCH1. SMO is then disinhibited and transmits signals through a series of interacting proteins, including suppressor of fused (SUFU), ultimately resulting in activation of the GLI family of transcription factors, GLI1, GLI2, and GLI3 [10]. GLIs transactivate genes such as *GLI1*, *PTCH1*, *HIP*, and *PDGFRα* [20]. The SHH signaling pathway is highly conserved across different species, including humans, and is involved in cell fate determination and organogenesis [20]. Besides deletion, *PTCH1* point mutations and rearrangements have also been detected in sporadic BCC [21, 22]. Activating *SMO* mutations have been described in 10% of sporadic BCC [23]. In addition, both *HIP* and *PTCH1* mRNA overexpression have been found in all samples of BCC when compared with normal skin [24].

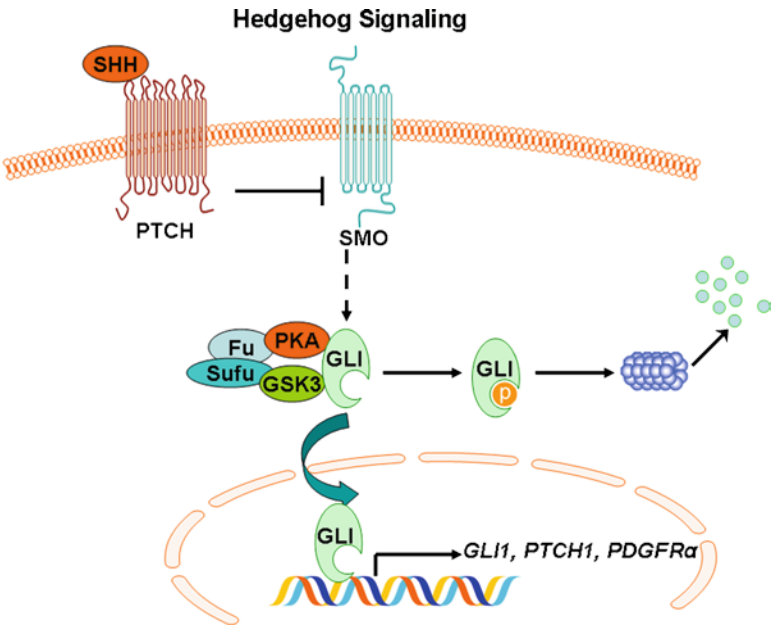

Fig. 7.1 Hedgehog signaling pathway. Hedgehog signaling is initiated by the binding of Hedgehog ligand to Patched (PTCH), a 12-transmembrane protein receptor. Sonic hedgehog (*SHH*) is one of the hedgehogs in mammalian cells. PTCH acts as an inhibitor of Smoothened (SMO), a 7-transmembrane G protein-coupled receptor. Downstream of SMO is a multiprotein complex, which comprises the transcription factor GLI, the serine/threonine kinase Fused (*Fu*), Suppressor of fused (*Sufu*), protein kinase A (*PKA*), and glycogen synthase kinase 3 (*GSK3*). In the absence of SHH, PTCH inhibits the activity of SMO. This inhibition is relieved by SHH binding. SMO activation leads to dissociation of GLI from the cytoplasmic protein complex and nuclear translocation, followed by enhanced transcription of its target genes *GLI1*, *PTCH1*, *HIP*, and *PDGFRα*. Activation of the hedgehog signaling pathway has been described in several human cancers, including BCC, gastrointestinal cancers, and brain cancers

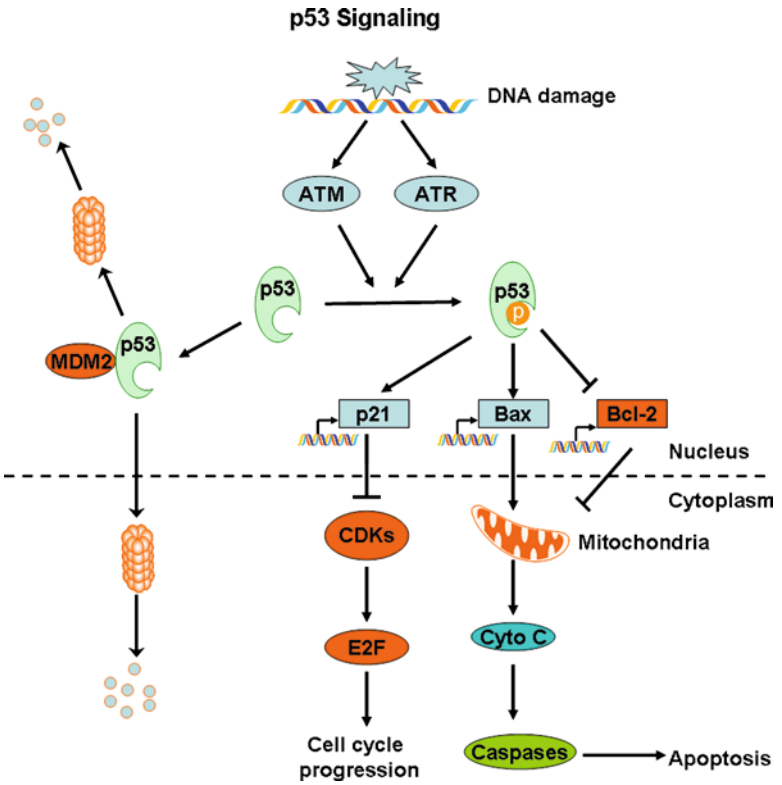

Fig. 7.2 p53 signaling pathway. The tumor suppressor protein p53 exhibits sequence-specific DNA-binding, directly interacts with various cellular and viral proteins, and induces cell cycle arrest in response to DNA damage. In response to signals generated by a variety of genotoxic stresses, such as ultraviolet radiation or DNA damage, p53 is expressed and undergoes post-translational modification that results in its accumulation within the nucleus. The p53-dependent pathways help to maintain genomic stability by eliminating damaged cells either by arresting them permanently or by inducing apoptosis. p53 promotes the transcription of p21, which, in turn, binds to and inhibits cyclin-dependent kinases (*CDKs*) and E2F, thus blocking the G1-S transition. p53 also induces transcription of the pro-apoptosis gene Bax, which leads to cytochrome c (*Cyto C*) release from mitochondria, caspase activation, and apoptosis. p53 activity is controlled through an autoregulatory loop involving the E3 ligase MDM2. Binding of MDM2 to p53 targets the latter for degradation, and inhibits p53-induced cell cycle arrest and apoptosis. Disruption of p53 protein itself or other components of the p53 pathway are major events in many human cancers, including skin tumors

The tumor suppressor gene *TP53* (Fig. 7.2), which is involved in DNA damage response and genome surveillance, is mutated and inactivated in about 50% of BCC [25, 26]. It has been suggested that *TP53* mutation is a crucial, but late event in BCC progression [26]. Most *TP53* mutations in skin cancers are of the "UVR signature" type (i.e., C → T and/or CC → TT transitions at dipyrimidine sequences) [27, 28]. Recently, it has been proposed that mutational hot spots in codon 177 of the *TP53* gene are specific for BCC, whereas mutations in codon 278 are associated with primary cutaneous SCC [29]. The functional basis of this codon selection is unknown. Mutational analyses of *PTCH1*, *SMO*, and *TP53* have been accomplished using single-strand conformation polymorphism (SSCP) [30]. It has been suggested that measurement of SHH pathway target gene expression by quantitative reverse transcription-polymerase chain reaction (RT-PCR) may be of help in diagnosing BCC and guiding future therapies [31]. The use of molecular technologies to study BCC is also discussed in Chaps. 4, 9, and 21.

Squamous Cell Carcinoma

Squamous cell carcinoma (SCC) is another major subtype of NMSC. SCC exhibit more genetic aberrations compared to BCC, which may explain their greater metastatic potential [16]. Frequent regions of LOH have been identified at 3p, 9p, 13q, 17p, and 17q [16]. It is plausible that these distinct regions contain putative tumor suppressor genes involved in SCC development and progression.

For example, loss of 17p is associated with dysfunction of the tumor suppressor gene *TP53* [16]. LOH analysis of SCC in situ has revealed recurrent loss of the *TP53* gene in ~27% of cases [32]. Brash et al. [33] have found that approximately 90% of SCC have *TP53* gene mutations. LOH analysis has also revealed recurrent loss of 9p in some SCC samples [34]. The 9p21 locus contains a well-known tumor suppressor gene *CDKN2A*, which plays a critical role in SCC development. Mutational analysis and transcript expression studies further suggest that inactivation of *CDKN2A* is also important for SCC progression [35–37]. Loss at 3p in SCC is associated with inactivation of the tumor suppressor gene *FHIT* (3p14.2), whose involvement in oral SCC is already established [38]. Deletion of 3p12-p14, 3p24-pter, and 13q13 has also been commonly observed in cutaneous SCC [39]. Future higher-resolution mapping will help clarify the role of these putative tumor suppressor gene containing sites in NMSC tumorigenesis. Many other CN alterations have been reported in SCC, including losses at 4p, 5q, 8p, and 10p, and gains at 3q, 5p, 7p, 8q, 11q, 17q, 18q, 19p, and 20q [40–42]. Among all these changes, a number of loci (3p21, 17p13, and 9p21) are commonly deleted in SCC, BCC, and KA.

LOH among different types and stages of SCC has also been studied. All poorly differentiated SCC and 60% of SCC in situ show genetic loss at 3p21 [43]. Primary tumors demonstrated gain at 12q13, which was not detected in the metastases, while metastatic lesions showed unique gains of 8q, 9q, and Xp21, and losses of 8p and 10p12-pter [41]. Evaluation of differences in LOH between primary and metastatic SCC may provide a method to identify metastasis-prone tumors. In addition, significantly higher numbers of chromosomal aberrations are detected in SCC compared to AK, with 18q deletion appearing to be more specific for SCC [44].

Gain of 8q24, which contains the oncogene *MYC*, is a recurrent cytogenetic aberration in SCC [45]. *MYC* amplification, rearrangement, and/or overexpression may be important for keratinocyte dedifferentiation and tumor progression [45]. *MYC* gain can be determined by FISH analysis, results of which show a significant correlation with MYC protein overexpression in tissue samples, as determined by immunohistochemistry [45]. The level of MYC expression is associated with the stage of disease and may be considered a marker of tumor aggressiveness.

Protein markers may serve as reliable prognostic predictors of SCC. Strong expression of phosphorylated STAT3 (p-STAT3) [46] and decreased expression of E-cadherin [47] are associated with a poorly differentiated phenotype, a propensity for tissue invasion, and increased risk of metastasis to regional lymph nodes. Immunohistochemical staining reveals an increase in FoxP3 expression in SCC with perineural invasion [48]. Methylation of *FOXE1*, a candidate tumor suppressor gene, occurs frequently in SCC and is associated with significantly downregulated gene expression [49].

Although there are currently no definitive molecular markers for SCC, further studies using genomic and transcriptomic technologies may identify such markers with both diagnostic and prognostic value in this tumor. SCC is also discussed in Chaps. 4 and 9.

Actinic Keratosis

Actinic keratosis (AK) is considered a pre-cancerous lesion, with up to 10% of AK transforming into SCC [50]. Microscopically, AK is characterized by atypical keratinocytes usually restricted to the lower portion of the epidermis; although, these changes may extend up to the mid-part of

the stratum Malpighi [51]. Molecular analyses of AK indicate that these lesions are genetically unstable, with a high number of recurrent aberrations. Interestingly, LOH studies demonstrate that AK harbor a higher rate of LOH (43%) compared to SCC (25%), with almost 20% of AK showing loss of chromosome 8 [52]. A high frequency of LOH has been observed at 3p, 9p, 9q, 13q, 17p, and 17q for AK (sites of LOH observed in SCC). This supports the observation that SCC may evolve from AK (i.e., AK is a precursor of SCC) [37, 52–54]. AK show a lower frequency of LOH at 9p21 (the *CDKN2A* tumor suppressor gene region) compared to SCC (21% *vs.* 46%), suggesting that progression of AK to SCC may involve the inactivation of *CDKN2A*.

Mutational inactivation of *TP53* may be a crucial step in the pathogenesis of AK. *TP53* mutations with UVR signature changes can be easily detected in AK [27]. Ziegler et al. [27] showed that inactivating p53 in mouse skin reduced the appearance of sunburn cells (apoptotic keratinocytes generated by overexposure to UVR). Therefore, keratinocytes appear to possess a p53-dependent "suicide" response to DNA damage that aborts precancerous cells. If this response is abrogated by a prior *TP53* mutation, UVR can select for clonal expansion of p53-mutated cells (Fig. 9.3) with subsequent development of AK. Thus, UVR can act twice: both as tumor initiator and tumor promoter. Immunohistochemistry identifies p53 protein expression in skin biopsies of AK [55]. The precursor status of AK has also been corroborated by cytogenetic studies [56]. Karyotypic similarities between SCC and AK are reported, including structural rearrangements involving chromosomal band 3p13 and the centromeric region of chromosome 3 [56]. These changes may be early genetic events associated with malignant transformation in the skin.

Keratoacanthoma

Keratoacanthoma (KA) is a well-differentiated, rapidly growing, and self-regressing keratinocytic neoplasm of unknown etiology. KA does not exhibit distinct histopathological features nor specific protein biomarkers that allow a definitive discrimination from SCC [57].

LOH analysis of KA demonstrates only isolated losses at 9p, 9q, and 10q [58]. CGH studies have also confirmed a lower degree of chromosomal instability in KA compared to SCC [42]. KA shows recurrent gain at 9q, and losses from 3p and 9p [42]. Only loss of 3p is seen at approximately the same frequency in KA and SCC [42]. This low frequency of genomic derangement in KA not only supports a less malignant phenotype, but also provides a potential approach to genetically differentiate KA from SCC. In contrast, *HRAS* mutation appears to be more frequent in KA than in SCC, and is associated with a gain of chromosome 11p [59]. Further genetic analysis of KA may provide detailed information on KA-related genes.

Apoptosis-related protein expression analysis demonstrates reduced Bcl-2, in conjunction with elevated Bak, in regressing KA [60]. In contrast, a steady level of Bcl-2 expression and reduced Bak expression is found in proliferating SCC, possibly explaining the aggressiveness of this tumor [60]. In addition, p16 appears to be lost in SCC, but not in cases of KA [61].

Non-Melanoma Skin Cancer Syndromes

Basal Cell Nevus Syndrome (BCNS, Gorlin Syndrome)

BCNS is an autosomal dominant disease characterized by the rapid development of multiple BCC early in life [62]. BCNS is linked to chromosome 9q22, which harbors the *PTCH1* tumor suppressor gene. Genetic aberrations of the *PTCH1* gene in this syndrome include nonsense, frameshift,

in-frame, splice-site, interstitial, and missense mutations [63]. About 70% of germline *PTCH1* mutations are rearrangements, resulting in truncated proteins, although no hot spots have been identified [64, 65]. In accordance with the Knudson two-hit hypothesis, loss of the wild-type allele has been demonstrated in BCNS patients [22]. The *PTCH1* gene consists of 23 exons, and encodes a 1447-amino-acid integral membrane protein, including a 12-transmembrane region. As discussed in the previous section, inactivating mutations of *PTCH1* lead to upregulation of SHH signaling pathway activity in BCC [66, 67].

High-resolution chromosomal banding of peripheral blood lymphocytes of these patients reveals interstitial deletions of the long arm of chromosome 9 [63]. Both array-CGH and FISH have been used to determine the extent of chromosomal deletion [63, 68]. In addition, sequence analysis of the *PTCH1* gene is shown to be useful in definitively diagnosing BCNS [69]. Few recurrent mutations and no genotype–phenotype correlations have been found for BCNS [65]. Analysis of polymorphic DNA markers on chromosome 9 can be used for prenatal diagnosis of this syndrome and identification of the parental origin of gene deletion [70]. Immunohistochemistry with non-mutated site-targeting anti-PTCH1 antibody does not differentiate BCNS-associated from sporadic forms of BCC, because both display similar patterns and intensity of staining [71].

Bazex–Dupre–Christol Syndrome (BDCS)

BDCS is characterized by follicular atrophoderma, hypotrichosis, and early development of BCC [72]. An X-linked dominant mode of inheritance has been suggested, with genes responsible for this syndrome mapped to Xq24-q27 [73]. The *UBE2A* gene, whose yeast homolog is involved in DNA repair following UVR-induced damage, has been proposed as a candidate gene for BDCS, although a clear link has not yet been established [73]. The main differential diagnosis of BDCS is BCNS. A distinction can be made by genomic analysis, such as *PTCH1* gene sequencing and mutation studies. Identification of the gene(s) responsible for BDCS may provide a tool to definitively diagnose this syndrome.

Rombo Syndrome

Rombo syndrome is characterized by atrophoderma vermiculatum of the face, multiple milia, telangiectases, acral erythema, and a propensity to develop BCC [74]. An autosomal dominant mode of transmission was suggested by the presence of male-to-male transmission in the originally described family with this syndrome [74]. A sporadic case was recently reported by Van Steensel et al. [75]. The gene responsible for this syndrome is still unknown.

Multiple Self-healing Squamous Epithelioma

Multiple self-healing squamous epithelioma (MSSE) is an autosomal dominant disorder characterized by multiple self-resolving cutaneous epithelial tumors that occur as early as the first decade and as late as the fifth decade of life [76]. The affected locus has been mapped to an 800-kb region on chromosome 9q22.1-q22.3 [77]. LOH analysis suggests that a tumor suppressor gene might be located at this site. The locus contains genes such as *PTCH1*, *ZNF169*, *PHF2*, and *FANCC*. However, mutations of these genes have not been identified by sequencing studies [78]. Though MSSE

involves sun-exposed sites, mutations of the DNA repair gene *XPA* have also been excluded [79]. The disease-causing gene(s) in this syndrome await identification.

Muir–Torre Syndrome

Muir–Torre syndrome (MTS) is a rare autosomal dominant condition characterized by sebaceous gland tumors and/or KA, in addition to visceral malignancies that include gastrointestinal and genitourinary cancers [80–83]. MTS usually results from germline mutation(s) in one or more of the DNA mismatch repair (MMR) genes. MMR genes commonly implicated include *MSH-2* (>90%), *MLH-1* (<10%), and, more recently, *MSH-6* [80–83]. Immunohistochemical testing of skin biopsies for the MMR proteins, MSH-2, MLH-1, and MSH-6, is reported to be very useful in detecting these defects in cutaneous tumors in MTS, especially in cases where the diagnosis is uncertain or a silent phenotype is present [81–83]. A high concordance (~90%) between MMR protein expression by immunohistochemistry and microsatellite status is seen; however, maintenance of MMR protein expression does not exclude the possibility of an underlying DNA repair defect. Studies in MTS patients indicate that lack of expression of either MLH-1 or MSH-2 is associated with microsatellite instability (MSI) in 100% of cases, while maintenance of expression of both proteins is predictive of microsatellite stability in 93% of cases [81–83]. MSI refers to the appearance of abnormally long or short microsatellites (repeated sequences of DNA of 1–6 base pairs in length) in an individual's DNA, as a result of defects in the normal DNA repair process [84]. Mutations in DNA repair genes result in the accumulation of errors in microsatellite sequences, so that they become either longer or shorter. Based on current evidence, a lack of expression of any one of the MMR proteins warrants additional studies, such as MSI testing and/or germline mutational analysis, guided by clinical suspicion and the patient's family history. Documentation of MSI indicates that the proband and his/her family members require strict cancer surveillance and mutational analysis. However, it is important to bear in mind that both sebaceous and/or visceral neoplasms associated with MTS may be microsatellite stable, and also that sporadic cutaneous sebaceous neoplasms can demonstrate MSI [81–83].

Xeroderma Pigmentosum

Xeroderma pigmentosum (XP) represents a group of autosomal recessive disorders characterized by intense photosensitivity and early onset of skin tumors, such as AK, BCC, SCC, and melanoma [76]. Germline mutations of the XP genes (*XPA* to *XPG*) are responsible for this disease. The XP genes are involved in nucleotide excision repair (NER) following UVR-induced DNA damage (Fig. 7.3). The elevated risk of skin cancer development in patients with XP is not due solely to a deficient DNA repair system [85]. Alterations of other oncogenes and tumor suppressor genes have also been reported [76].

XP has been diagnosed by measuring post-UVR unscheduled DNA synthesis (UDS) in fibroblasts cultured from skin biopsies, a technique developed by Cleaver et al. [86]. Although this assay is reliable, it is time-consuming and labor-intensive. PCR-based methods, such as PCR-restriction fragment length polymorphism (PCR-RFLP), are now more commonly employed [87]. PCR-RFLP is also very useful for early prenatal diagnosis, with fetal amniotic fluid chorionic villi as a source of DNA template [87]. The PCR-SSCP method is useful in identifying new mutations in XP patients [88, 89]. Host cell reactivation (HCR) is another rapid and sensitive assay for the diagnosis of XP. HCR utilizes a UVR-treated plasmid containing the sequence of a reporter gene,

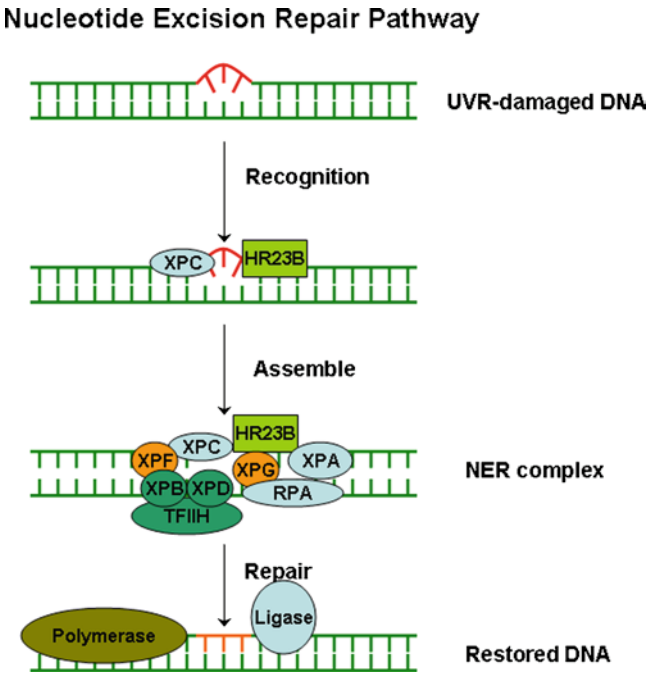

Fig. 7.3 Nucleotide excision repair pathway. Normally, ultraviolet radiation (*UVR*)-induced DNA damage is recognized by the XPC and HR23 proteins, promoting protein complex formation. A complex including TFIIH is formed at the lesion site, and the two helicases XPB and XPD unwind the DNA molecule surrounding the lesion. This is followed by recruitment of RPA, a major eukaryotic single-stranded DNA-binding protein, which protects both of the separated strands in the complex. The endonucleases XPF and XPG then cleave the damaged region. The gap is subsequently filled by DNA polymerases, followed by ligation through the action of DNA ligases. The disruption of this nucleotide excision repair (*NER*) pathway is involved in several disorders, including xeroderma pigmentosum (*XP*). Different defects in the NER pathway lead to the variant forms of XP

such as chloramphenicol acetyltransferase (CAT). This is transfected into XP cell lines from which repair and restoration of CAT activity is measured [90, 91]. Other reporter genes, such as luciferase or β-galactosidase, can also be utilized for HCR.

Other Less Common Non-Melanoma Skin Tumors

Merkel Cell Carcinoma

Merkel cell carcinoma (MCC), or trabecular carcinoma of the skin, is a rare, aggressive cutaneous neuroendocrine malignancy [76]. UVR-induced damage has been implicated in the pathogenesis of this tumor, although more recent evidence points to a previously uncharacterized polyomavirus (MCPyV) as playing a central role [92, 93]. Gene expression studies of cultured MCC cell lines have suggested the possibility of two subtypes of this disease with distinct transcriptomic profiles (i.e., signal transduction pattern *vs.* cell cycle control and proliferation pattern). LOH studies have identified possible tumor suppressor loci for MCC at 1p32-p36 and 10q23, chromosomal regions which have been implicated in the development of other cancers [76, 94]. Using high resolution array-CGH analysis, MCC tissue samples frequently show loss of

regions on chromosomes 3p, 4, 5q, 7, 10 and 13, with gain of regions on chromosomes 1, 3q, 5p, and 6 [95]. Loss of the *pRb1* gene region and amplification of the *L-Myc* gene region are found in 26% and 31% of tumors, respectively [95]. Importantly, a higher frequency of genomic aberrations is reported to be associated with reduced survival in patients with MCC [95]. In contrast, recent evidence suggests that MCC tumors with higher MCPyV abundance demonstrate better clinical outcomes [96]. Immunohistochemistry is commonly employed in the differential diagnosis of MCC from other small round cell tumors. For example, cytokeratin 20 (CK20) is the most widely used immunohistochemical marker and characteristically shows a perinuclear dot-like expression pattern in MCC [97]. Other protein markers that appear to be reliable in distinguishing MCC from small cell carcinoma of the lung have also been described, and include neurofilaments, TTF-1 and MASH1 [98].

MCC is heterogeneous clinically, morphologically, in its genomic aberrations, MCPyV abundance, and also in the expression of a limited number of proteins investigated to date. The identification of molecular and protein biomarkers (applicable to clinical tissue and biological fluids), that would help to refine the information gained by knowledge of routine clinical and histopathological prognostic factors, could be used to develop more standardized criteria for defining and reporting MCC, facilitate the stratification of MCC patients based on the risk for metastasis and poor outcome, and aid in the development of novel targeted therapies. It has been envisioned that the results of research-based genomic and transcriptomic analyses of skin tumors, such as MCC, will provide avenues for the future clinical-based use of molecular diagnostic and prognostic tests. In fact, defined genetic-morphological classification systems have already been proposed for other skin tumors. However, a distinct molecular pathway for the development and progression of MCC has yet to be identified [92]. Several major genomic aberrations, such as those found in melanoma (i.e., MAPK pathway), have revealed little or no involvement in MCC, although research is on-going [92].

Pilomatricoma

Pilomatricoma (or calcifying epithelioma of Malherbe) is a slow-growing, benign skin tumor that shows similar histomorphological features to the hair follicle [99]. Activating mutations of the beta-catenin gene have been linked to the development of this tumor [100]. Dysregulation of beta-catenin signaling has been reported in Gardner syndrome, whose presentation includes multiple pilomatricomas [76]. In addition, deletion or truncation of the nuclear receptor-binding SET domain containing protein (*NSD1*) gene has been reported in familial Sotos syndrome, in which multiple large pilomatricomas develop [101].

Cylindroma

Cylindroma is a rare cutaneous neoplasm characterized by intradermal cellular nodules surrounded by basement membrane-like material. Both sporadic and autosomal dominant familial cases have been reported. LOH analysis has identified a putative tumor suppressor gene on chromosome 16q12 [102]. Refined mapping and positional cloning of this region led to the discovery of germline and somatic mutations of the cylindromatosis gene (*CYLD*), in both familial cylindromatosis and sporadic cylindroma cases [103]. Although its function is as yet unknown, detection of *CYLD* gene mutation is expected to provide a valuable method for diagnosis.

Conclusions

Molecular techniques may play a decisive role in the differential diagnosis and prognostication of histopathologically ambiguous NMSC. Traditional cytogenetic analysis has enabled the identification of a number of chromosomal abnormalities associated with these tumors. Newer technologies are expected to further refine the role of such genomic aberrations. With the development of recent methodologies, such as array-CGH, gene expression profiling, and protein microarrays, novel biomarkers of diagnostic and prognostic value in NMSC are expected to emerge rapidly [104–106].

References

1. Housman TS, Williford PM, Feldman SR, et al. Nonmelanoma skin cancer: an episode of care management approach. Dermatol Surg. 2003;29:700–11.
2. Mogensen M, Jemec GB. Diagnosis of nonmelanoma skin cancer/keratinocyte carcinoma: a review of diagnostic accuracy of nonmelanoma skin cancer diagnostic tests and technologies. Dermatol Surg. 2007;33:1158–74.
3. Bastian BC, LeBoit PE, Pinkel D. Genomic approaches to skin cancer diagnosis. Arch Dermatol. 2001;137:1507–11.
4. Corona R, Mele A, Amini M, et al. Interobserver variability on the histopathologic diagnosis of cutaneous melanoma and other pigmented skin lesions. J Clin Oncol. 1996;14:1218–23.
5. Wakely SL, Baxendine-Jones JA, Gallagher PJ, et al. Aberrant diagnoses by individual surgical pathologists. Am J Surg Pathol. 1998;22:77–82.
6. Albert MR, Weinstock MA. Keratinocyte carcinoma. CA Cancer J Clin. 2003;53:292–302.
7. Nguyen TH. Mechanisms of metastasis. Clin Dermatol. 2004;22:209–16.
8. Kane CL, Keehn CA, Smithberger E, et al. Histopathology of cutaneous squamous cell carcinoma and its variants. Semin Cutan Med Surg. 2004;23:54–61.
9. Schwartz RA. Keratoacanthoma: a clinico-pathologic enigma. Dermatol Surg. 2004;30(2 Pt 2):326–33. discussion 333.
10. Epstein EH. Basal cell carcinomas: attack of the hedgehog. Nat Rev Cancer. 2008;8:743–54.
11. Casalone R, Mazzola D, Righi R, et al. Cytogenetic and interphase FISH analyses of 73 basal cell and three squamous cell carcinomas: different findings in direct preparations and short-term cell cultures. Cancer Genet Cytogenet. 2000;118:136–43.
12. Nangia R, Sait SN, Block AW, et al. Trisomy 6 in basal cell carcinomas correlates with metastatic potential: a dual color fluorescence in situ hybridization study on paraffin sections. Cancer. 2001;91:1927–32.
13. Ashton KJ, Weinstein SR, Maguire DJ, et al. Molecular cytogenetic analysis of basal cell carcinoma DNA using comparative genomic hybridization. J Invest Dermatol. 2001;117:683–6.
14. Jin Y, Mertens F, Persson B, et al. Nonrandom numerical chromosome abnormalities in basal cell carcinomas. Cancer Genet Cytogenet. 1998;103:35–42.
15. Jin Y, Martins C, Salemark L, et al. Nonrandom karyotypic features in basal cell carcinomas of the skin. Cancer Genet Cytogenet. 2001;131:109–19.
16. Quinn AG, Sikkink S, Rees JL. Basal cell carcinomas and squamous cell carcinomas of human skin show distinct patterns of chromosome loss. Cancer Res. 1994;54:4756–9.
17. Shanley SM, Dawkins H, Wainwright BJ, et al. Fine deletion mapping on the long arm of chromosome 9 in sporadic and familial basal cell carcinomas. Hum Mol Genet. 1995;4:129–33.
18. Shen T, Park WS, Boni R, et al. Detection of loss of heterozygosity on chromosome 9q22.3 in microdissected sporadic basal cell carcinoma. Hum Pathol. 1999;30:284–7.
19. Saldanha G, Fletcher A, Slater DN. Basal cell carcinoma: a dermatopathological and molecular biological update. Br J Dermatol. 2003;148:195–202.
20. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 2001;15:3059–87.
21. Aszterbaum M, Rothman A, Johnson RL, et al. Identification of mutations in the human PATCHED gene in sporadic basal cell carcinomas and in patients with the basal cell nevus syndrome. J Invest Dermatol. 1998;110:885–8.
22. Gailani MR, Stahle-Backdahl M, Leffell DJ, et al. The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. Nat Genet. 1996;14:78–81.

23. Xie J, Murone M, Luoh SM, et al. Activating smoothened mutations in sporadic basal-cell carcinoma. Nature. 1998;391:90–2.
24. Tojo M, Kiyosawa H, Iwatsuki K, et al. Expression of a sonic hedgehog signal transducer, hedgehog-interacting protein, by human basal cell carcinoma. Br J Dermatol. 2002;146:69–73.
25. Rady P, Scinicariello F, Wagner Jr RF, et al. p53 mutations in basal cell carcinomas. Cancer Res. 1992;52:3804–6.
26. Ziegler A, Leffell DJ, Kunala S, et al. Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers. Proc Natl Acad Sci USA. 1993;90:4216–20.
27. Ziegler A, Jonason AS, Leffell DJ, et al. Sunburn and p53 in the onset of skin cancer. Nature. 1994;372:773–6.
28. Tornaletti S, Pfeifer GP. Slow repair of pyrimidine dimers at p53 mutation hotspots in skin cancer. Science. 1994;263:1436–8.
29. Boukamp P. Non-melanoma skin cancer: what drives tumor development and progression? Carcinogenesis. 2005;26:1657–67.
30. Reifenberger J, Wolter M, Knobbe CB, et al. Somatic mutations in the PTCH, SMOH, SUFUH and TP53 genes in sporadic basal cell carcinomas. Br J Dermatol. 2005;152:43–51.
31. Hatta N, Hirano T, Kimura T, et al. Molecular diagnosis of basal cell carcinoma and other basaloid cell neoplasms of the skin by the quantification of Gli1 transcript levels. J Cutan Pathol. 2005;32:131–6.
32. Lee HJ, Kim JS, Ha SJ, et al. p53 gene mutations in Bowen's disease in Koreans: clustering in exon 5 and multiple mutations. Cancer Lett. 2000;158:27–33.
33. Brash DE, Ziegler A, Jonason AS, et al. Sunlight and sunburn in human skin cancer: p53, apoptosis, and tumor promotion. J Investig Dermatol Symp Proc. 1996;1:136–42.
34. Saridaki Z, Koumantaki E, Liloglou T, et al. High frequency of loss of heterozygosity on chromosome region 9p21-p22 but lack of p16INK4a/p19ARF mutations in Greek patients with basal cell carcinoma of the skin. J Invest Dermatol. 2000;115:719–25.
35. Chang TG, Wang J, Chen LW, et al. Loss of expression of the p16 gene is frequent in malignant skin tumors. Biochem Biophys Res Commun. 1997;230:85–8.
36. Soufir N, Moles JP, Vilmer C, et al. P16 UV mutations in human skin epithelial tumors. Oncogene. 1999;18:5477–81.
37. Mortier L, Marchetti P, Delaporte E, et al. Progression of actinic keratosis to squamous cell carcinoma of the skin correlates with deletion of the 9p21 region encoding the p16(INK4a) tumor suppressor. Cancer Lett. 2002;176:205–14.
38. Tanimoto K, Hayashi S, Tsuchiya E, et al. Abnormalities of the FHIT gene in human oral carcinogenesis. Br J Cancer. 2000;82:838–43.
39. Sikkink SK, Rehman I, Rees JL. Deletion mapping of chromosome 3p and 13q and preliminary analysis of the FHIT gene in human nonmelanoma skin cancer. J Invest Dermatol. 1997;109:801–5.
40. Popp S, Waltering S, Holtgreve-Grez H, et al. Genetic characterization of a human skin carcinoma progression model: from primary tumor to metastasis. J Invest Dermatol. 2000;115:1095–103.
41. Shimizu T, Izumi H, Oga A, et al. Epidermal growth factor receptor overexpression and genetic aberrations in metastatic squamous-cell carcinoma of the skin. Dermatology. 2001;202:203–6.
42. Clausen OP, Aass HC, Beigi M, et al. Are keratoacanthomas variants of squamous cell carcinomas? A comparison of chromosomal aberrations by comparative genomic hybridization. J Invest Dermatol. 2006;126:2308–15.
43. Ashton KJ, Carless MA, Griffiths LR. Cytogenetic alterations in nonmelanoma skin cancer: a review. Genes Chromosom Cancer. 2005;43:239–48.
44. Ashton KJ, Weinstein SR, Maguire DJ, et al. Chromosomal aberrations in squamous cell carcinoma and solar keratoses revealed by comparative genomic hybridization. Arch Dermatol. 2003;139:876–82.
45. Toll A, Salgado R, Yebenes M, et al. MYC gene numerical aberrations in actinic keratosis and cutaneous squamous cell carcinoma. Br J Dermatol. 2009;161:1112–8.
46. Suiqing C, Min Z, Lirong C. Overexpression of phosphorylated-STAT3 correlated with the invasion and metastasis of cutaneous squamous cell carcinoma. J Dermatol. 2005;32:354–60.
47. Koseki S, Aoki T, Ansai S, et al. An immunohistochemical study of E-cadherin expression in human squamous cell carcinoma of the skin: relationship between decreased expression of E-cadherin in the primary lesion and regional lymph node metastasis. J Dermatol. 1999;26:416–22.
48. Tallon B, Bhawan J. FoxP3 expression is increased in cutaneous squamous cell carcinoma with perineural invasion. J Cutan Pathol. 2010;37:1184–5.
49. Venza I, Visalli M, Tripodo B, et al. FOXE1 is a target for aberrant methylation in cutaneous squamous cell carcinoma. Br J Dermatol. 2010;162:1093–7.
50. Johnson TM, Rowe DE, Nelson BR, et al. Squamous cell carcinoma of the skin (excluding lip and oral mucosa). J Am Acad Dermatol. 1992;26:467–84.

51. Quatresooz P, Pierard-Franchimont C, Paquet P, et al. Crossroads between actinic keratosis and squamous cell carcinoma, and novel pharmacological issues. Eur J Dermatol. 2008;18:6–10.
52. Rehman I, Takata M, Wu YY, et al. Genetic change in actinic keratoses. Oncogene. 1996;12:2483–90.
53. Ortonne JP. From actinic keratosis to squamous cell carcinoma. Br J Dermatol. 2002;146 Suppl 61:20–3.
54. Anwar J, Wrone DA, Kimyai-Asadi A, et al. The development of actinic keratosis into invasive squamous cell carcinoma: evidence and evolving classification schemes. Clin Dermatol. 2004;22:189–96.
55. Kanjilal S, Strom SS, Clayman GL, et al. p53 mutations in nonmelanoma skin cancer of the head and neck: molecular evidence for field cancerization. Cancer Res. 1995;55:3604–9.
56. Jin Y, Jin C, Salemark L, et al. Clonal chromosome abnormalities in premalignant lesions of the skin. Cancer Genet Cytogenet. 2002;136:48–52.
57. Sanchez EY, Simon P, Requena L, et al. Solitary keratoacanthoma: a self-healing proliferation that frequently becomes malignant. Am J Dermatopathol. 2000;22:305–10.
58. Waring AJ, Takata M, Rehman I, et al. Loss of heterozygosity analysis of keratoacanthoma reveals multiple differences from cutaneous squamous cell carcinoma. Br J Cancer. 1996;73:649–53.
59. Leon J, Kamino H, Steinberg JJ, et al. H-ras activation in benign and self-regressing skin tumors (keratoacanthomas) in both humans and an animal model system. Mol Cell Biol. 1988;8:786–93.
60. Batinac T, Zamolo G, Coklo M, et al. Expression of cell cycle and apoptosis regulatory proteins in keratoacanthoma and squamous cell carcinoma. Pathol Res Pract. 2006;202:599–607.
61. Burnworth B, Popp S, Stark HJ, et al. Gain of 11q/cyclin D1 overexpression is an essential early step in skin cancer development and causes abnormal tissue organization and differentiation. Oncogene. 2006;25:4399–412.
62. Kimonis VE, Goldstein AM, Pastakia B, et al. Clinical manifestations in 105 persons with nevoid basal cell carcinoma syndrome. Am J Med Genet. 1997;69:299–308.
63. Yamamoto K, Yoshihashi H, Furuya N, et al. Further delineation of 9q22 deletion syndrome associated with basal cell nevus (Gorlin) syndrome: report of two cases and review of the literature. Congenit Anom (Kyoto). 2009;49:8–14.
64. Boutet N, Bignon YJ, Drouin-Garraud V, et al. Spectrum of PTCH1 mutations in French patients with Gorlin syndrome. J Invest Dermatol. 2003;121:478–81.
65. Wicking C, Shanley S, Smyth I, et al. Most germ-line mutations in the nevoid basal cell carcinoma syndrome lead to a premature termination of the PATCHED protein, and no genotype-phenotype correlations are evident. Am J Hum Genet. 1997;60:21–6.
66. Hahn H, Wicking C, Zaphiropoulous PG, et al. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell. 1996;85:841–51.
67. Johnson RL, Rothman AL, Xie J, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science. 1996;272:1668–71.
68. Chen CP, Lin SP, Wang TH, et al. Perinatal findings and molecular cytogenetic analyses of de novo interstitial deletion of 9q (9q22.3-q31.3) associated with Gorlin syndrome. Prenat Diagn. 2006;26:725–9.
69. Veenstra-Knol HE, Scheewe JH, van der Vlist GJ, et al. Early recognition of basal cell naevus syndrome. Eur J Pediatr. 2005;164:126–30.
70. Bialer MG, Gailani MR, McLaughlin JA, et al. Prenatal diagnosis of Gorlin syndrome. Lancet. 1994;344:477.
71. Abe S, Kabashima K, Sakabe J, et al. Coincident two mutations and one single nucleotide polymorphism of the PTCH1 gene in a family with naevoid basal cell carcinoma syndrome. Acta Derm Venereol. 2008;88:635–6.
72. Yung A, Newton-Bishop JA. A case of Bazex-Dupre-Christol syndrome associated with multiple genital trichoepitheliomas. Br J Dermatol. 2005;153:682–4.
73. Vabres P, Lacombe D, Rabinowitz LG, et al. The gene for Bazex-Dupre-Christol syndrome maps to chromosome Xq. J Invest Dermatol. 1995;105:87–91.
74. Michaelsson G, Olsson E, Westermark P. The Rombo syndrome: a familial disorder with vermiculate atrophoderma, milia, hypotrichosis, trichoepitheliomas, basal cell carcinomas and peripheral vasodilation with cyanosis. Acta Derm Venereol. 1981;61:497–503.
75. van Steensel MA, Jaspers NG, Steijlen PM. A case of Rombo syndrome. Br J Dermatol. 2001;144:1215–8.
76. Tsao H. Genetics of nonmelanoma skin cancer. Arch Dermatol. 2001;137:1486–92.
77. Bose S, Morgan LJ, Booth DR, et al. The elusive multiple self-healing squamous epithelioma (MSSE) gene: further mapping, analysis of candidates, and loss of heterozygosity. Oncogene. 2006;25:806–12.
78. D'Alessandro M, Coats SE, Morley SM, et al. Multiple self-healing squamous epithelioma in different ethnic groups: more than a founder mutation disorder? J Invest Dermatol. 2007;127:2336–44.
79. Wright AL, Gawkrodger DJ, Branford WA, et al. Self-healing epitheliomata of Ferguson-Smith: cytogenetic and histological studies, and the therapeutic effect of etretinate. Dermatologica. 1988;176:22–8.
80. Muir EG, Bell AJ, Barlow KA. Multiple primary carcinomata of the colon, duodenum, and larynx associated with kerato-acanthomata of the face. Br J Surg. 1967;54:191–5.

81. Abbas O, Mahalingam M. Cutaneous sebaceous neoplasms as markers of Muir-Torre syndrome: a diagnostic algorithm. J Cutan Pathol. 2009;36:613–9.
82. Chhibber V, Dresser K, Mahalingam M. MSH-6: extending the reliability of immunohistochemistry as a screening tool in Muir-Torre syndrome. Mod Pathol. 2008;21:159–64.
83. Cesinaro AM, Ubiali A, Sighinolfi P, et al. Mismatch repair proteins expression and microsatellite instability in skin lesions with sebaceous differentiation: a study in different clinical subgroups with and without extracutaneous cancer. Am J Dermatopathol. 2007;29:351–8.
84. Dietmaier W, Wallinger S, Bocker T, et al. Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. Cancer Res. 1997;57:4749–56.
85. Norgauer J, Idzko M, Panther E, et al. Xeroderma pigmentosum. Eur J Dermatol. 2003;13:4–9.
86. Cleaver JE. Defective repair replication of DNA in xeroderma pigmentosum. Nature. 1968;218:652–6.
87. Moriwaki S, Kraemer KH. Xeroderma pigmentosum-bridging a gap between clinic and laboratory. Photodermatol Photoimmunol Photomed. 2001;17:47–54.
88. Orita M, Iwahana H, Kanazawa H, et al. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA. 1989;86:2766–70.
89. Sato M, Nishigori C, Yagi T, et al. Aberrant splicing and truncated-protein expression due to a newly identified XPA gene mutation. Mutat Res. 1996;362:199–208.
90. Carreau M, Eveno E, Quilliet X, et al. Development of a new easy complementation assay for DNA repair deficient human syndromes using cloned repair genes. Carcinogenesis. 1995;16:1003–9.
91. Khan SG, Levy HL, Legerski R, et al. Xeroderma pigmentosum group C splice mutation associated with autism and hypoglycinemia. J Invest Dermatol. 1998;111:791–6.
92. Lemos B, Nghiem P. Merkel cell carcinoma: more deaths but still no pathway to blame. J Invest Dermatol. 2007;127:2100–3.
93. Feng H, Shuda M, Chang Y, et al. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;319:1096–100.
94. Van Gele M, Leonard JH, Van Roy N, et al. Frequent allelic loss at 10q23 but low incidence of PTEN mutations in Merkel cell carcinoma. Int J Cancer. 2001;92:409–13.
95. Paulson KG, Lemos BD, Feng B, et al. Array-CGH reveals recurrent genomic changes in Merkel cell carcinoma including amplification of L-Myc. J Invest Dermatol. 2009;129:1547–55.
96. Bhatia K, Goedert JJ, Modali R, et al. Immunological detection of viral large T antigen identifies a subset of Merkel cell carcinoma tumors with higher viral abundance and better clinical outcome. Int J Cancer. 2010;127:1493–6.
97. Bobos M, Hytiroglou P, Kostopoulos I, et al. Immunohistochemical distinction between merkel cell carcinoma and small cell carcinoma of the lung. Am J Dermatopathol. 2006;28:99–104.
98. Ralston J, Chiriboga L, Nonaka D. MASH1: a useful marker in differentiating pulmonary small cell carcinoma from Merkel cell carcinoma. Mod Pathol. 2008;21:1357–62.
99. Turhan-Haktanir N, Demir Y, Sahin O, et al. Pilomatricoma: a review of six pediatric cases with nine lesions. Turk J Pediatr. 2009;51:44–8.
100. Chan EF, Gat U, McNiff JM, et al. A common human skin tumour is caused by activating mutations in beta-catenin. Nat Genet. 1999;21:410–3.
101. Gilaberte Y, Ferrer-Lozano M, Olivan MJ, et al. Multiple giant pilomatricoma in familial Sotos syndrome. Pediatr Dermatol. 2008;25:122–5.
102. Biggs PJ, Chapman P, Lakhani SR, et al. The cylindromatosis gene (cyld1) on chromosome 16q may be the only tumour suppressor gene involved in the development of cylindromas. Oncogene. 1996;12:1375–7.
103. Bignell GR, Warren W, Seal S, et al. Identification of the familial cylindromatosis tumour-suppressor gene. Nat Genet. 2000;25:160–5.
104. Cai WW, Mao JH, Chow CW, et al. Genome-wide detection of chromosomal imbalances in tumors using BAC microarrays. Nat Biotechnol. 2002;20:393–6.
105. Oostlander AE, Meijer GA, Ylstra B. Microarray-based comparative genomic hybridization and its applications in human genetics. Clin Genet. 2004;66:488–95.
106. Hartmann M, Roeraade J, Stoll D, et al. Protein microarrays for diagnostic assays. Anal Bioanal Chem. 2009;393:1407–16.

Chapter 8
Cutaneous Sarcomas and Soft Tissue Proliferations

Omar Jassim and John D. Pfeifer

Primary cutaneous sarcomas account for 14% of all sarcomas [1]. Partly owing to their relative rarity, diagnosis of these tumors is often challenging. All too often a pathologist is faced with a small biopsy, and has to differentiate a common benign mesenchymal tumor from its malignant counterpart, or classify a malignant tumor based on a limited tissue sample. Fortunately, basic science discoveries over the last several decades have demonstrated that many benign and malignant mesenchymal tumors of the skin have characteristic genetic changes. This chapter will discuss how adjunct molecular genetic testing can be used in the diagnosis and prognostication of these lesions, and in directing patient therapy.

For many sarcomas of the skin and subcutaneous tissue, a chromosomal translocation produces a fusion gene that is the key to tumor development. Therefore, molecular genetic testing of primary cutaneous and metastatic sarcomas is primarily focused on the detection of translocations, fusion genes, or fusion gene transcripts by: (a) conventional cytogenetic analysis (karyotyping); (b) fluorescence in situ hybridization (FISH), using either metaphase (dividing) or interphase (nondividing) cells; or (c) reverse transcription polymerase chain reaction (RT-PCR). However, for some neoplasms, it is gene deletions and/or gene inactivation by point mutations that are associated with tumorigenesis. While routine cytogenetic analysis or FISH can demonstrate gross structural abnormalities in these latter cases, accurate identification of the point mutation requires DNA sequence analysis. For still other tumor types, it is activating mutations, also only detectable by DNA sequence analysis, that are responsible for oncogenesis. More recently, gene expression profiling strategies have shown promise (a) for facilitating the classification of sarcomas of uncertain histogenesis, (b) as predictors of patient outcome, and (c) as methods to uncover novel therapeutic strategies [2]. The study of tumor RNA using cDNA/oligonucleotide microarrays, which simultaneously analyze the expression of thousands of genes, has resulted in the identification of novel tumor-specific biomarkers and altered biochemical pathways in subtypes of sarcoma [2].

J.D. Pfeifer (✉)
Lauren V. Ackerman Laboratory of Surgical Pathology,
Department of Pathology and Immunology, Washington University School of Medicine,
3rd Floor, Peters Building, 660 South Euclid Ave., St. Louis, MO 63110, USA
e-mail: pfeifer@path.wustl.edu

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_8,

Malignant Round Cell Tumors and Other Tumors of Uncertain Histogenesis

Ewing Sarcoma/Peripheral Neuroectodermal Tumor

Ewing sarcoma/peripheral neuroectodermal tumor (EWS/PNET) is a prototypic malignant round cell tumor of childhood and adolescence that classically arises in the soft tissue and bone (Fig. 8.1). Once thought to be an uncommon neoplasm, EWS/PNET now accounts for ~20% of all malignant soft tissue tumors in children. It has been described in patients of all ages [3, 4], and may involve a wide variety of sites besides soft tissue and bone, including the skin and subcutis [5–7]. While molecular characterization of the genetic features of EWS/PNET has led to recognition of a broader clinicopathologic spectrum of this tumor, the progenitor cell from which the sarcoma arises remains unknown.

Genetics. The genetic hallmark of EWS/PNET is a balanced translocation that results in a chimeric gene in which the *EWS* gene at 22q12 is fused with a member of the *ETS* family of transcription factors (Table 8.1 and Fig. 8.2). The most common translocation, present in over 90% of cases, is t(11;22)(q24;q12), which results in an *EWS-FLI1* fusion gene [8–10]. Between 5% and 10% of tumors result from a translocation that produces an *EWS-ERG* fusion gene [10, 11]. Fusion genes between *EWS* and other members of the *ETS* family, including *ETV1*, *E1AF*, *FEV*, and *ZSG*, each account for less than 1% of cases of EWS/PNET. A subset of EWS/PNET harbor translocations in which *TLS* is fused to *ERG* or *FEV* [12, 13], a finding that is not surprising given the extensive similarity between *TLS* and *EWS* [14, 15]. The recent demonstration that EWS/PNET-like tumors (i.e., lesions that have the same morphology as conventional EWS/PNET, but do not show diffuse

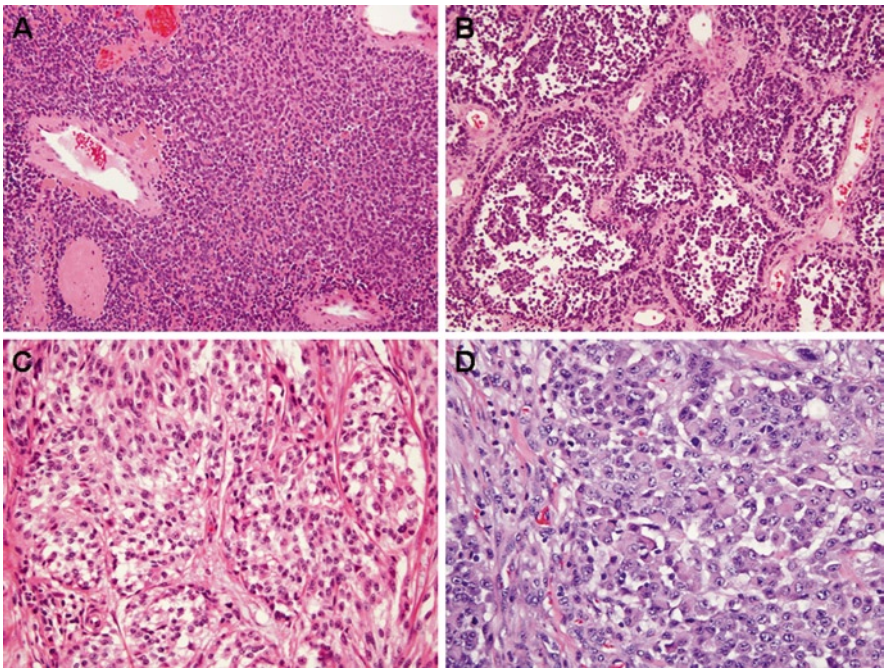

Fig. 8.1 (**a**) Ewing sarcoma/peripheral neuroectodermal tumor (*EWS/PNET*). (**b**) Alveolar rhabdomyosarcoma (*ARMS*). (**c**) Clear cell sarcoma (*CCS*). (**d**) Malignant rhabdoid tumor (*MRT*)

Table 8.1 Summary of recurring genetic aberrations in malignant round cell tumors and other tumors of uncertain histogenesis in the skin

Tumor	Aberration	Gene(s) involved	Estimated prevalence
Ewing sarcoma/peripheral neuroectodermal tumor	t(11;22)(q24;q12)	*EWS-FLI1*	85–95%
	t(21;22)(q22;q12)	*EWS-ERG*	5–10%
	t(7;22)(p22;q12)	*EWS-ETV1*	Rare
	t(17;22)(q21;q12)	*EWS-E1AF*	Rare
	t(2;22)(q33;q12)	*EWS-FEV*	Rare
	Inversion of 22q	*EWS-ZSG (EWS-PATZ1)*	Rare
	t(16;21)(p11;q22)	*TLS-ERG*	Rare
	t(2;16)(q33;p11)	*TLS-FEV*	Rare
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWS-WT1*	>80%
	t(21;22)(q22;q12)	*EWS-ERG*	Rare
Embryonal rhabdomyosarcoma	Gains of 2,7,8,12, and 13; losses of 1,6,9,14, and 17	*IGF2, GOK, PTCH, TP53;* many unknown	–
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX3-FKHR*	75%
	t(1;13)(p36;q14)	*PAX7-FKHR*	10%
Clear cell sarcoma	t(12;22)(q13;q12)	*EWS-ATF1*	>90%
	t(2;22)(q33;q12)	*EWS-CREB1*	<10%
Extrarenal malignant rhabdoid tumor	Bi-allelic inactivation of 22q11.2	*hSNF5/INI1*	75–100%
Proximal-type epithelioid sarcoma	Bi-allelic inactivation of 22q11.2	*hSNF5/INI1*	25–100%
Alveolar soft part sarcoma	Non-reciprocal der[17] t(X;17)(p11;q25)	*ASPL-TFE3*	100%
Undifferentiated soft tissue sarcoma	Changes characteristic of a specific sarcoma subtype can be detected in ~40% of cases	Fusion transcript characteristic of specific sarcoma subtype	–
Merkel cell carcinoma	No recurrent genomic abnormality	–	–
	Merkel Cell Polyomavirus (MCPyV)	–	80%

membranous CD99 immunoreactivity) harbor a variety of other translocations has further highlighted the increased genetic complexity of these neoplasms [16, 17].

Because the exact position of the genomic break can vary, structural heterogeneity is a prominent feature of the fusion genes (Fig. 8.2). For example, at least 18 different in-frame *EWS-FLI1* chimeric transcripts can be produced from breakpoints within the conventional exons of *EWS* and *FLI1* (most of which have been detected *in vivo*). Cryptic exons contribute even greater structural diversity. Since the exonic structure of *ERG* and *FLI1* is very similar, it is not surprising that several variants of the *EWS-ERG* fusion transcripts have also been described [10, 18, 19]. Although translocations that result in *EWS-ETS* fusion genes are the characteristic genetic abnormality in EWS/PNET, secondary chromosomal aberrations, including trisomy 8, trisomy 12, and gain of 1q, are present in >50% of cases, and have been associated with an unfavorable outcome [20, 21].

EWS-ETS fusion genes encode a chimeric protein in which a strong transactivation domain in the N-terminal region of *EWS* is fused to a DNA-binding domain in the C-terminal region of the *ETS* family member [22, 23]. This novel protein can initiate and maintain EWS/PNET tumorigenesis [22, 23]. Since *EWS* transcription is driven by a strongly and broadly active promoter, the chimeric protein is highly expressed [24].

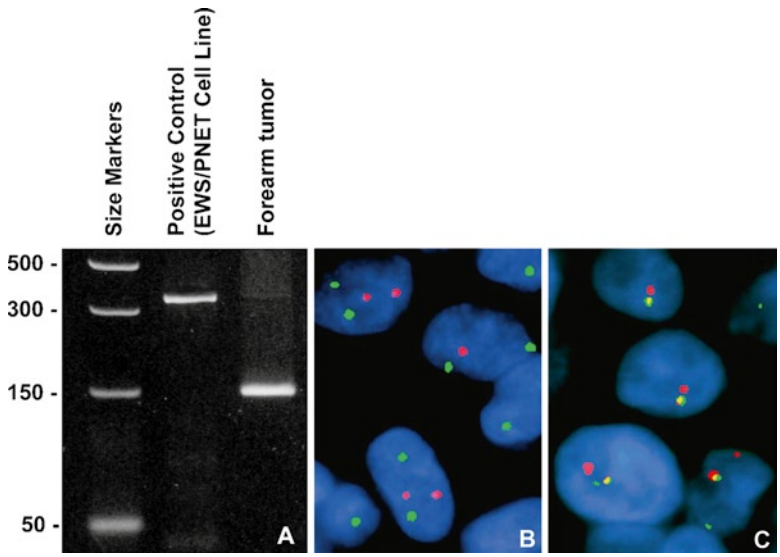

Fig. 8.2 Molecular analysis of Ewing sarcoma/peripheral neuroectodermal tumor (*EWS/PNET*). (**a**) Reverse transcription polymerase chain reaction (*RT-PCR*) demonstration of the *EWS-FLI1* fusion transcript that results from the t(11;22)(q24;q12) translocation, a characteristic of EWS/PNET. Variability in the location of the translocation breakpoint produces structural heterogeneity of the *EWS-FLI1* fusion gene, which is reflected in the different sizes of the RT-PCR products. The band size of 353 bp in the positive control cell line results from an *EWS* exon 7 to *FLI1* exon 5 fusion; the band size of 150 bp in the forearm tumor results from an *EWS* exon 7 to *FLI1* exon 8 fusion. (**b–c**) Probe fusion FISH for t(11;22). The probe for *EWS* at 22q12 is labeled *red*; the probe for *FLI1* at 11q24 is labeled *green*. (**b**) The split *red* and *green* signals provide no evidence of the translocation in the negative control. (**c**) In the forearm tumor, one *yellow* fusion signal in the majority of nuclei indicates the presence of the translocation

Although gene fusions between *EWS* and a member of the *ETS* family are characteristic of EWS/PNET, they have also been described in isolated cases of a variety of other tumors. These include biphenotypic sarcomas with myogenic and neural differentiation (also known as malignant ectomesenchymoma) and other polyphenotypic tumors [25–29], malignant rhabdoid tumor (MRT) [30], mesenchymal chondrosarcoma [31], mixed embryonal and alveolar rhabdomyosarcoma [27], and even synovial sarcoma (SS) [32]. However, the significance of these gene fusions in the latter tumors remains to be defined, and may merely reflect the chromosomal instability that is characteristic of malignant tumors rather than a genetic alteration responsible for tumorigenesis [33, 34]. Importantly, fusion genes that are an epiphenomenon of a tumor's clonal evolution may be present in only a very small subset of cells and still be detectable by PCR-based methods. Although uncommon, the presence *EWS-ETS* fusion genes in tumors other than EWS/PNET emphasizes that a diagnosis of EWS/PNET should be based primarily on the clinical and histopathological features of a case, rather than the molecular genetic test result alone [32, 35–37].

Molecular testing. RT-PCR has been shown to be an extremely useful method for demonstrating the fusion transcripts characteristic of EWS/PNET (Fig. 8.2). However, in view of (a) the number of different *ETS* family genes that can partner with *EWS*, and (b) the structural heterogeneity that results from the variable translocation breakpoint, the sensitivity of PCR-based approaches depends on the comprehensiveness of the testing protocols [38]. *EWS-FLI1* and related fusion transcripts can be detected by RT-PCR from both fresh and formalin-fixed, paraffin-embedded (FFPE) tissue. However, the testing of FFPE samples places significant constraints on the methodology, including the need to amplify shorter target sequences, often via a nested approach, both of which increase the risk of contamination and amplification of nonspecific sequences [35, 39]. In addition, analysis of FFPE

tissue has lower sensitivity than assays on fresh (or fresh frozen) tumor samples [32, 36, 40]. Because such a wide variety of fusion transcripts is possible, it is important to directly or indirectly confirm the identity of the RT-PCR product by either DNA sequence or melting curve analysis [36].

Interphase FISH, using a probe-splitting or break-apart approach (Fig. 8.2), can also be employed to detect the translocations characteristic of EWS/PNET [30, 41–43]. Using probes that bracket *EWS*, this approach yields results that are concordant with RT-PCR in 67–83% of cases [44, 45]. However, given the complexity of the chromosomal rearrangements seen in EWS/PNET and EWS/PNET-like tumors, a comprehensive set of probes is required for high test sensitivity.

Prognostic features of transcript type. Several studies have shown that patients whose tumors harbor an *EWS* exon 7 to *FLI1* exon 6 fusion (so-called type I fusion) have significantly better survival compared to those with other fusion types [46, 47]. Type I fusion genes encode a protein that has weaker transactivation activity and is associated with a lower proliferative rate [22, 23].

Detection of minimal disease. For cases of EWS/PNET arising in bone and soft tissue, RT-PCR detection of submicroscopic bone marrow (BM) involvement may be used for the purpose of tumor staging [48, 49]. Importantly, the presence of molecular-positive BM at the time of diagnosis is significantly associated with an adverse outcome by univariate analysis [50]. However, the significance of RT-PCR-detected submicroscopic disease in the peripheral blood (PB) compartment is unclear [50, 51]. One factor that may complicate such analysis of PB samples is tumor cell mobilization secondary to a recent surgical procedure [52].

Immunohistochemistry. Both monoclonal and polyclonal antibodies have demonstrated utility in the diagnosis of EWS/PNET [53]. Because FLI1 is also expressed by vascular tumors and Merkel cell carcinoma (MCC) [54–56], an immunopanel that includes stains for CD99, in addition to FLI1, has highest sensitivity and specificity for EWS/PNET, especially when combined with confirmatory FISH testing [53].

Alveolar Rhabdomyosarcoma

Alveolar rhabdomyosarcoma (ARMS) is a primitive malignant round cell tumor that shows partial skeletal muscle differentiation (Fig. 8.1). The tumor arises in all age groups, but most often in adolescents and young adults. Accounting for ~20% of all rhabdomyosarcomas, ARMS most commonly arises in the deep soft tissue of the extremities, although the tumor can also involve the paraspinal and perineal regions, the paranasal sinuses, and the skin [57–61]. Congenital ARMS may present as the so-called blueberry muffin baby [62, 63].

Genetics. Approximately 75% of ARMS harbor the t(2;13)(q35;q14) translocation, producing a fusion gene in which the 5′ end of the *PAX3* gene on chromosome 2 is fused with the 3′ end of the *FKHR* gene on chromosome 13 [64, 65]. Up to 10% of cases harbor the t(1;13)(p36;q14) translocation, in which the 5′ end of the *PAX7* gene on chromosome 1 is fused with the aforementioned *FKHR* gene [66]. The chromosomal breakpoints that produce *PAX3-FKHR* fusion genes are always located in intron 7 of *PAX3* and intron 1 of *FKHR*, and the breakpoints that produce *PAX7-FKHR* genes are always located in intron 7 of *PAX7* and intron 1 of *FKHR* [67]. Consequently, there is no structural heterogeneity of encoded chimeric proteins [67]. Approximately 15–20% of ARMS show: low expression of the standard *PAX3-FKHR* or *PAX7-FKHR* fusions; variant fusions of *PAX3* or *PAX7* with other genes; or no detectable rearrangements in *PAX3*, *PAX7*, or *FKHR* [68, 69].

The chimeric PAX-FKHR proteins consist of (a) an N-terminal DNA-binding domain (composed of paired-box and homeobox domains) encoded by *PAX*, and (b) a C-terminal transactivation domain encoded by *FKHR*. The proteins act as aberrant transcription factors that cause excessive activation of genes with *PAX* binding sites [70].

Prognosis for ARMS is generally poor compared with embryonal rhabdomyosarcoma (ERMS), justifying an effort to distinguish these two tumor types [2]. Because *PAX-FKHR* rearrangements are not hallmarks of any other tumor type, including ERMS, the demonstration of a *PAX-FKHR* fusion is thought to be diagnostic of ARMS [71, 72]. Molecular testing is most useful when the microscopic features are not classic (i.e., mixed alveolar and embryonal patterns). The rare cases of putative ERMS that do contain *PAX-FKHR* fusions probably represent: (a) ARMS with mixed embryonal and alveolar histology, in which only the embryonal pattern was present in the areas sampled for microscopic evaluation; (b) ARMS with solid alveolar histology that were misclassified as ERMS; or (c) true ERMS for which the rearrangement is an epiphenomenon [71].

Molecular testing. The lack of structural heterogeneity of the encoded chimeric proteins makes RT-PCR-based assays for *PAX-FKHR* fusion transcripts very straightforward. Primer pairs that are specific for *PAX3-FKHR* and *PAX7-FKHR* fusion transcripts have been described. In addition, the availability of consensus primers, which bind to the highly homologous 5′ regions of *PAX3* and *PAX7*, make it possible to amplify either fusion transcript in a single RT-PCR reaction [73]. Published data indicate that RT-PCR demonstrates *PAX-FKHR* fusion transcripts in slightly <75% of ARMS when fresh tissue is used for testing, but in only ~55% of cases when FFPE tissue is analyzed [36]. As noted previously, ~15–20% of cases do not harbor either the t(2;13) or t(1;13), limiting the utility of RT-PCR (and other molecular methodologies), regardless of technical modifications to the testing protocol.

FISH has also been used to detect the translocations characteristic of ARMS, by both probe-fusion and probe break-apart approaches on either metaphase chromosomes or interphase nuclei [74–76]. Comparison of FISH-based assays and RT-PCR analysis demonstrates excellent concordance between both testing methods [77].

Prognostic features. The clinical behavior of ARMS that harbor a *PAX3-FKHR* fusion gene is different from those that harbor a *PAX7-FKHR* fusion gene. Tumors with a *PAX7-FKHR* fusion gene occur in younger patients, are locally less invasive, and show a lower propensity for BM involvement [78–80]. The estimated 4-year overall survival rate is 75% for tumors that harbor *PAX7-FKHR* versus 8% for tumors with *PAX3-FKHR*. These differences suggest that, in addition to a role for molecular genetic testing in the diagnosis of ARMS, identification of the particular *PAX-FKHR* fusion-type provides important prognostic information.

Detection of minimal disease. An RT-PCR-based assay, capable of detecting one tumor cell in 10^5 normal cells, has been used to demonstrate molecular evidence of BM involvement in up to 15% of histopathologically-negative samples [81]. Patients with molecularly detected submicroscopic BM involvement tend to have a worse outcome [81]. At present, there is no role for RT-PCR-based testing for tumor cells in PB samples from patients with ARMS [81].

Clear Cell Sarcoma

Clear cell sarcoma (CCS) is a rare tumor (~1% of all soft tissue tumors) that predominantly arises in the deep soft tissues of young adults, although a wide range of anatomic sites, including the skin, may be involved (Fig. 8.1) [82–87]. Despite the characterization of the genetic basis of CCS, the identity of the progenitor cell from which the tumor arises remains unknown. Although malignant melanoma of soft parts is often used as a synonym for CCS, malignant melanoma differs from CCS in several important respects. For example, CCS rarely involves the epidermis, and harbors a translocation that has not been documented in cutaneous melanoma.

Genetics. The t(12;22)(q13;q12) translocation is the hallmark of CCS (Fig. 8.3) [88–91]. It is present in >90% of cases, and produces an *EWS-ATF1* fusion gene [92, 93]. Differences in the position

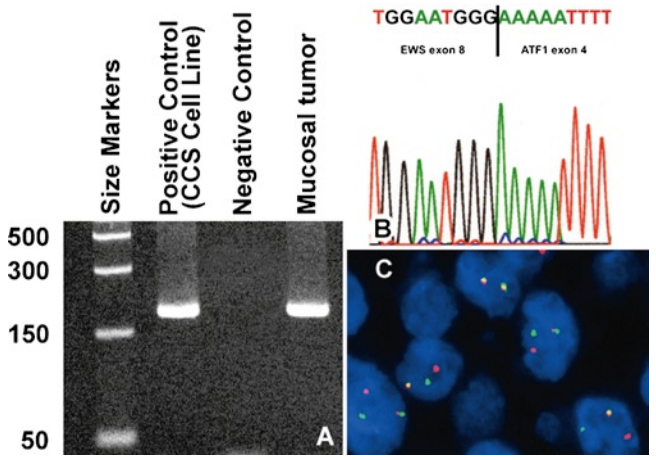

Fig. 8.3 Molecular analysis of clear cell sarcoma (*CCS*). (**a**) Reverse transcription polymerase chain reaction (*RT-PCR*) demonstration of the *EWS-ATF1* fusion transcript that results from the t(12;22)(q13;q12) translocation, a characteristic of CCS. (**b**) The partial nucleotide sequence of the RT-PCR product from the mucosal tumor shows an in-frame fusion between *EWS* and *ATF1*; the vertical line indicates the region derived from *EWS* exon 8 and the region derived from *ATF1* exon 4. (**c**) Break-apart FISH for t(12;22). The probe centromeric to *EWS* at 22q12 is labeled *red*; the probe telomeric to *EWS* is labeled *green*. The presence of a pair of split *red* and *green* signals in the majority of nuclei indicates a rearrangement of the *EWS* locus, consistent with the presence of the t(12;22) (Panel C, courtesy of Dr. Alejandro Gru, Washington University School of Medicine, St. Louis, MO)

of the translocation breakpoints within the two genes give rise to structural heterogeneity in the resulting *EWS-ATF1* fusion transcripts. The t(2;22)(q33;q12) translocation is present in <10% of cases, and results in an *EWS-CREB1* fusion gene [92–94]. Additional recurrent cytogenetic changes that have been described in CCS include trisomy 7, trisomy 8, and structural and numerical aberrations (other than the characteristic translocation) of chromosome 22 [90, 95, 96]. However, the significance of these latter aberrations remains to be defined.

The EWS-ATF1 chimeric protein consists of the N-terminal transcriptional activation domain of EWS fused to the bZIP DNA-binding and dimerization domain of the ATF1 transcription factor. Expression of ATF1 is normally regulated by cyclic adenosine monophosphate (cAMP); however, the chimeric EWS-ATF1 protein is constitutively expressed, as it is under the control of the strongly and broadly active *EWS* promoter [97, 98]. Consequently, the EWS-ATF1 protein likely leads to dysregulated expression of genes normally controlled by cAMP [97–99]. The structure of the EWS-CREB1 chimeric protein is similar, in that the strong transcriptional activation domain in the N-terminal region of the native EWS is fused to the bZIP DNA-binding and dimerization domain of the C-terminal region of CREB1 [94, 100].

The distinction of CCS and cutaneous melanoma is important due to different treatment regimens and prognoses (i.e., 5-year survival rates of 48–67% for CCS). However, the differentiation of these two tumors can be extremely difficult, since they are usually indistinguishable by morphologic (i.e., presence of melanin), histochemical, immunohistochemical (i.e., S100+/HMB-45+), and ultrastructural (i.e., presence of pre-melanosomes) features. However, neither t(12;22) nor t(2;22) translocations, and/or their resulting fusion genes, have been documented in cutaneous melanoma [101, 102]. Consequently, the presence of the *EWS-ATF1* or *EWS-CREB1* fusion gene can be used to definitively differentiate primary CCS from cutaneous melanoma [102].

The *EWS-ATF1* and *EWS-CREB1* fusions can also be found in angiomatoid fibrous histiocytoma (AFH). The presence of the exact same molecular alteration in these two distinct tumor types has obvious implications for test interpretation. In addition, a subset of cases of the recently described

osteoclast-rich tumor of the gastrointestinal tract also show the t(12;22)(q13;q12) translocation [103]. A link between this latter tumor and conventional CCS, if any, remains to be determined.

Molecular testing. RT-PCR has been widely used to detect the *EWS-ATF1* fusion transcripts [101, 104–107]. When fresh tissue is available for testing, a fusion transcript can be demonstrated in virtually 100% of cases. RT-PCR adapted for use with FFPE tissue demonstrates the *EWS-ATF1* fusion transcript in >85% of cases (Fig. 8.3) [93, 101, 102, 104]. Because of the structural heterogeneity of *EWS-ATF1* transcripts, maximum test sensitivity is only achieved when multiple primer sets that can detect all possible fusion transcript variants are employed in the assay.

FISH-based methods can also be used to reliably detect the t(12;22) translocation in metaphase chromosomes [84, 85] and interphase nuclei in tissue sections (Fig. 8.3) [102, 106]. In one comparative study, FISH showed higher sensitivity than RT-PCR [93].

Prognostic features. The aggressiveness of CCS does not appear to correlate with either (a) differences in the position of the translocation breakpoint within the *EWS-ATF1* gene fusion or (b) which gene fusion is present (*EWS-ATF1 vs. EWS-CREB1*) [92].

Extrarenal Malignant Rhabdoid Tumor

Neoplasms with rhabdoid features have been reported in a variety of anatomic sites [108, 109]. In most cases, particularly those that arise in adults, a rhabdoid phenotype represents merely a poorly differentiated component of what is otherwise an easily classified tumor. In contrast, the malignant rhabdoid tumor (MRT) is an extremely aggressive neoplasm of infants and young children that has virtually pure rhabdoid morphology, and which characteristically involves the kidney, central nervous system, or occasionally soft tissues (Fig. 8.1). Rare cases can arise in the skin [110–113]. Congenital cases can manifest as the so-called blueberry muffin baby [114].

Genetics. The characteristic genetic abnormality in MRT is somatic bi-allelic alteration of the *hSNF5/INI1* tumor suppressor gene at chromosome band 22q11.2 [115]. Abnormalities include: (a) homozygous deletion; (b) hemizygous deletion with inactivation of the remaining allele by nonsense, frameshift, or intragenic mutation; and (c) bi-allelic mutation without evidence of chromosome 22q11.2 deletion [115, 116]. *hSNF5/INI1* encodes a protein that is a member of the ATP-dependent chromatin-remodeling complex [117, 118]. However, the precise mechanism by which loss-of-function mutations in *hSNF5/INI1* promote oncogenesis remains unknown.

Most MRTs are non-syndromic. In these sporadic cases: (a) mutations of *hSNF5/INI1* are somatic events; and (b) although specific mutations are nonrandomly associated with tumor site, individual mutations do not correlate with prognosis [116]. A subset of MRTs arises in the setting of the *rhabdoid predisposition syndrome*, in which mutations of *hSNF5/INI1* are germline. Patients with this syndrome are not only predisposed to renal or extrarenal MRT, but also to a variety of other tumors of the central nervous system, including central EWS/PNET, medulloblastoma, and choroid plexus carcinoma [119, 120].

Bi-allelic loss/inactivation of *hSNF5/INI1* has not been described as a recurring feature of any other skin or soft tissue tumor, except for proximal-type epithelioid sarcoma (see below). Given the wide variety of cutaneous tumor types that can show rhabdoid differentiation, including carcinomas, sarcomas, and melanoma, molecular testing for alterations at this locus is clearly helpful in the diagnosis of MRT.

Molecular testing. Conventional cytogenetic analysis, interphase FISH, and PCR-based microsatellite loss of heterozygosity (LOH) analysis have all been used to demonstrate deletions of the long arm of chromosome 22 [115, 116, 120 –123]. Analysis of FFPE tissue may be associated with a loss of sensitivity using PCR-based testing [116].

Regardless of the testing approach, approximately 75% of MRT (outside of the kidney and central nervous system) show homozygous deletion of the *hSNF5/INI1* locus. In the subset of MRT with hemizygous deletion of 22q11.2, exhaustive DNA sequence analysis demonstrates nonsense or frameshift mutations of the retained copy of the *hSNF5/INI1* gene in 75–100% of cases [15, 115, 116, 119, 120–123]. (Note: the location of mutations within *hSNF5/INI1* does not show significant clustering, and thus it is necessary to sequence all nine exons of the gene, and/or the complete cDNA from the *hSNF5/INI1* transcript). Since the vast majority of cases of MRT with hemizygous deletion of *hSNF5/INI1* harbor inactivating mutations of the retained copy of the gene, the demonstration of hemizygous loss of 22q11.2 is considered to be indicative of bi-allelic inactivation of *hSNF5/INI1*.

Immunohistochemistry. Nuclear hSNF5/INI1 protein expression is absent in virtually 100% of extrarenal MRT [124]. Of note, there is 100% correlation between molecular evidence of *hSNF5/INI1* deletion/mutation and immunohistochemical demonstration of protein loss. Although some cases of extraskeletal myxoid chondrosarcoma, myoepithelial carcinoma, and epithelioid malignant peripheral nerve sheath tumor (MPNST) also do not express this protein, the finding that nuclear immunoreactivity is retained in EWS/PNET, Wilms tumor, desmoplastic small round cell tumor, CCS, synovial sarcoma (SS), and rhabdomyosarcoma suggests that immunohistochemical testing may be a substitute for molecular analysis in cases of suspected MRT [125].

Epithelioid Sarcoma

Epithelioid sarcoma typically arises in young adults in their 2nd to 4th decade, presenting as one or more slowly growing, tan-white nodules with an infiltrating margin. The tumor usually arises in the subcutaneous or soft tissue, although rare cases may occur in the dermis [126, 127]. Recently, epithelioid sarcoma has been divided into two distinct subtypes: (a) the distal-type and (b) the more aggressive proximal-type.

Distal-type epithelioid sarcoma (DTES) involves fingers, hands, or wrists, and equivalent sites in the distal lower extremity. Microscopically, the tumor is composed of nodules of uniform polygonal cells with eosinophilic cytoplasm, which may transition into areas showing spindled morphology. The nodules can undergo central necrosis resembling a necrobiotic granuloma (Fig. 8.4).

The more aggressive proximal-type epithelioid sarcoma (PTES) involves the pelvis, perineum, and/or genital tract [127]. Microscopically, PTES is characterized by a lack of a granuloma-like pattern, instead the tumor is composed of cells with prominent epithelioid or rhabdoid morphology [128]. Based on the histopathologic and genetic similarities (see below) between PTES and extrarenal MRT, some authors have proposed that these two tumor types actually represent a single disease entity [129].

Genetics. The characteristic genetic feature of PTES is inactivation of the *hSNF5/INI1* tumor suppressor gene at chromosome band 22q11.2 by either chromosomal deletion or sequence mutation [130, 131]. The frequency of *hSNF5/INI1* inactivation in PTES ranges from 25% to 100% in published studies [131–133]. Despite this marked variation in mutation prevalence (likely an artifact of the small number of cases that have been evaluated, and compounded by different testing approaches), the cumulative data indicate that the frequency of *hSNF5/INI1* inactivation in PTES seems to be significantly lower than in extrarenal MRT. While *hSNF5/INI1* gene alterations have been described in occasional cases of other tumor types, including extraskeletal myxoid chondrosarcoma, pediatric undifferentiated soft tissue sarcoma, and uterine carcinosarcoma [134], these tumor types are not usually in the differential diagnosis of epithelioid sarcoma. Alterations in the *hSNF5/INI1* gene are not a feature of DTES [131–133].

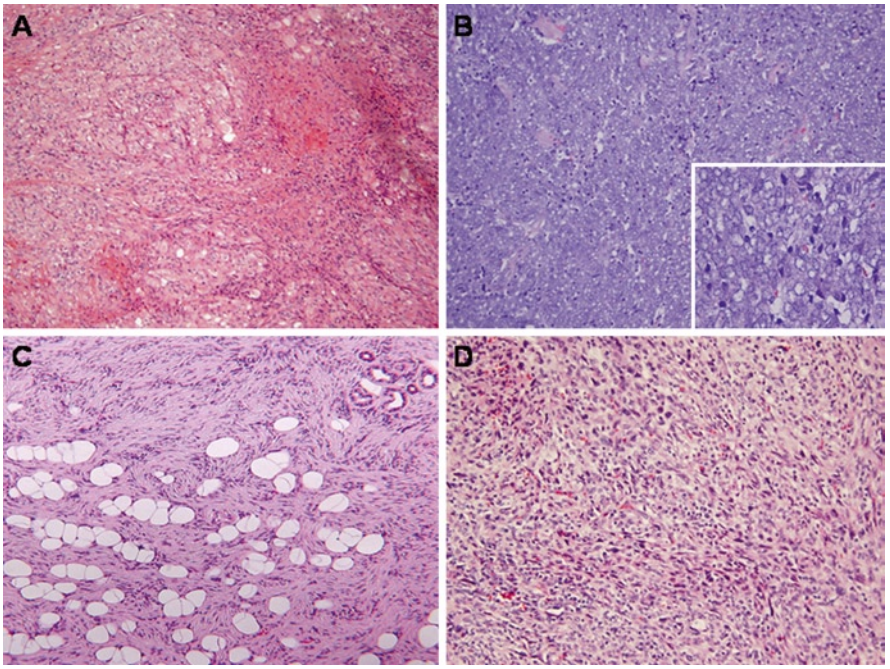

Fig. 8.4 (**a**) Distal-type epithelioid sarcoma (*DTES*). (**b**) Merkel cell carcinoma (*MCC*). (**c**) Dermatofibrosarcoma protuberans (*DFSP*). (**d**) Inflammatory myofibroblastic tumor (*IMT*)

Molecular Testing. A variety of techniques have been used to evaluate the *hSNF5/INI1* locus in epithelioid sarcomas. Conventional cytogenetic analysis, metaphase FISH, interphase FISH, and comparative genomic hybridization (CGH) have all been shown to be reliable strategies to demonstrate chromosomal deletions [130, 131, 133]. However, given that many of the inactivating alterations of the *hSNF5/INI1* locus are either frameshift or missense mutations in the coding regions of the gene, the most sensitive molecular testing approach is DNA sequence analysis [130, 131]. Since there is no evidence that sequence mutations cluster in specific regions, the entire gene must be evaluated in order to ensure high test sensitivity.

Immunohistochemistry. Despite the fact that *hSNF5/INI1* gene alterations are characteristic of PTES, but not DTES, loss of INI1 protein expression is seen in both types of epithelioid sarcoma [133, 135, 136]. In the largest study to date, 95% of PTES showed a complete loss of INI1 expression, as did 91% of DTES [136]. Loss of INI1 protein expression was not observed in a broad range of other tumor types, including metastatic carcinomas, melanomas, epithelioid mesotheliomas, and angiosarcomas. However, the specificity of absent INI1 expression was not absolute, since ~50% of cases of epithelioid MPNST, 9% of cases of myoepithelial carcinoma, and virtually 100% of MRT showed a complete absence of INI1 expression [136]. Lack of protein expression is also found in some cases of extraskeletal myxoid chondrosarcoma [125].

Alveolar Soft Part Sarcoma

Alveolar soft part sarcoma (ASPS) usually occurs in the 2nd or 3rd decade of life. In adults, the tumor most often develops in the deep soft tissues of the extremities, especially the thigh, while in

children the tumor preferentially involves the head and neck region, especially the orbit and tongue. Morphologically, ASPS is composed of uniform large epithelioid cells with abundant eosinophilic granular cytoplasm, arranged in a characteristic architectural pattern of cell nests separated by delicate sinusoidal vessels. While primary skin examples have not been reported, initial presentation of ASPS with cutaneous involvement, or as a cutaneous metastasis, is well documented (Fig. 9.6) [137, 138].

Genetics. The nonreciprocal der[17]t(X;17)(p11;q25) translocation is a characteristic genetic feature of ASPS. This translocation results in the formation of an *ASPL-TFE3* fusion gene [139], that encodes a chimeric protein in which (a) the N-terminal region of ASPL (also known as RCC17) is linked to (b) the basic helix-loop-helix and leucine zipper DNA-binding and multimerization domains of TFE3 [139, 140]. Given that the *ASPL* promoter is apparently constitutively activated, high-level expression of ASPL-TFE3 is believed to cause the transcriptional deregulation that leads to tumor development [139, 141, 142].

ASPL-TFE3 fusion genes are also characteristic of a distinctive subgroup of primary renal neoplasms in children and young adults [140, 143]; however, in these tumors, the fusion gene is the result of a balanced t(X;17)(p11.2;q25) translocation. From a practical point of view, the fact that both soft tissue and renal tumors can harbor the same *ASPL-TFE3* fusion usually does not create diagnostic difficulty, since the two tumor types have such different clinicopathologic features. However, if the diagnosis is in doubt, for example, in the setting of metastatic disease, demonstration of the balanced or unbalanced nature of the rearrangement permits definitive classification [141].

Molecular testing. In most ASPS, the unbalanced der[17]t(X;17)(p11;q25) can be demonstrated by routine cytogenetic analysis; although, the karyotype can be ambiguous as the translocation is not reciprocal [141]. FISH is a more straightforward approach for identifying this genomic abnormality, using either metaphase chromosomes or interphase nuclei [139, 141]. RT-PCR can also be used to detect *ASPL-TFE3* rearrangements (Fig. 9.6). When used to test RNA extracted from frozen tissue, RT-PCR has a sensitivity of virtually 100% in cases of ASPS [139]. The sensitivity of RT-PCR in FFPE samples is unknown.

Immunohistochemistry. Use of a polyclonal antibody directed against the C-terminal portion of the native TFE3 component of the ASPL-TFE3 chimeric protein has a sensitivity of >97% and a specificity of >99% [144]. However, the ubiquitous expression of TFE3 mandates that the assay must first be optimized on known positive and negative cases, in order to adjust assay sensitivity in an effort to minimize false-positive results.

Undifferentiated Soft Tissue Sarcoma

Undifferentiated soft tissue sarcomas (USTS), also often termed poorly differentiated malignant round cell neoplasms, undifferentiated sarcomas, or small round cell sarcomas of indeterminate type, are a group of tumors which show a diffuse hypercellular pattern, consisting of sheets of medium-sized cells that have minimal to moderate amounts of cytoplasm and variable nuclear morphologies [145, 146]. Consistent with the lack of identifying histopathological features, these tumors do not have a diagnostic histochemical or immunohistochemical profile, and have no characteristic ultrastructural changes [145, 147].

Molecular testing. When RT-PCR is used to screen USTS, a fusion transcript characteristic of a specific sarcoma type can be detected in ~40% of cases, regardless of whether fresh or FFPE tissue is used for testing [8, 36, 77]. This finding can be used to guide patient management, even if

a more precise histopathological classification has not been reached [36]. Nonetheless, it is important to recognize that prospective clinical trials have yet to demonstrate that USTS classified as a specific sarcoma type solely on the basis of molecular analysis have the same prognosis and/or response to therapy as corresponding sarcomas diagnosed by light microscopy and immunohistochemistry.

Merkel Cell Carcinoma

Merkel cell carcinoma (MCC) is a rare and often lethal cutaneous malignancy that generally develops in sun-exposed skin. The cell of origin was traditionally thought to be the slow-acting mechanoreceptor Merkel cell located in the epidermis [148], but recent studies have cast doubt on this assumption [149, 150]. Microscopically, MCC is composed of small-to-intermediate sized round blue cells that have an oval nucleus and scant cytoplasm, arranged in sheets or nests within the dermis, and often extending into the subcutis (Figs. 8.4 and 9.5). Immunohistochemically, MCC typically demonstrates positivity for epithelial (CK8, CK20) and neuroendocrine (chromogranin, synaptophysin) markers, in addition to reactivity for neurofilaments. Immunostaining for CK20 usually shows a characteristic "dot-like" perinuclear staining pattern [151].

Genetics. It has recently been demonstrated that the majority of MCC (~80%) harbor a novel polyomavirus, aptly named the Merkel Cell Polyomavirus (MCPyV) [152, 153]. The virus has a ~5,300 bp double-stranded DNA genome that shares a high degree of homology with African green monkey lymphotropic polyomavirus. Like other polyomaviruses that infect humans, MCPyV expresses SV40 large T antigen.

Although details of the MCPyV oncogenic pathway remain to be fully elucidated, the virus is clonally inserted into the tumor cells, and truncating deletions of the large T antigen gene that eliminate viral DNA replication capacity are consistently present [154]. Interestingly, the large T antigen deletions preserve the retinoblastoma (Rb)-binding domain of the protein, which may lead to unchecked S-phase cell cycle entry by tumor cells.

The oncogenic potential of MCPyV is primarily limited to MCC. Several studies have demonstrated that the virus is present in only a small subset of other cutaneous neoplasms or primary tumors of other sites [155–159]. In particular, MCPyV is rarely found in primary visceral high-grade neuroendocrine tumors that have overlapping architectural, cytologic, and immunophenotypic features with MCC, including small cell carcinoma of the lung [160, 161].

Molecular testing. PCR-based testing is a straightforward approach for demonstration of MCPyV in MCC. The virus is detected in ~80% of cases, in both primary tumors and metastases, and regardless of whether fresh or FFPE tissue is used for analysis [152, 153, 160, 162]. However, high test sensitivity requires that primer sets are optimized for differentially processed tissues [160, 162]. Monoclonal integration of MCPyV in MCC tumor cells can also be demonstrated by Southern blot (Fig. 3.1). The high prevalence of MCPyV in MCC, together with the rarity of virus detection in other high-grade neuroendocrine carcinomas, highlights the potential clinical utility of molecular analysis in problematic cases [160, 161]. A recently developed monoclonal antibody CM2B4 can now also be used for immunohistochemical-based identification of MCPyV. In addition, recent evidence suggests that viral abundance in MCC tumors may be associated with distinct clinical features, including age at diagnosis and survival outcomes [153].

While molecular testing for MCPyV may have a role in the diagnosis and prognostication of MCC, until such time as the natural history of MCPyV infection is more well defined (i.e., route of infection, disease prevalence), it remains unclear as to whether or not viral testing may be useful in screening of at-risk populations.

Spindle Cell Tumors

Dermatofibrosarcoma Protuberans

Dermatofibrosarcoma protuberans (DFSP) is a nodular or plaque-like fibrohistiocytic tumor of low-grade or intermediate malignant potential, typically showing diffuse infiltration of the dermis and subcutaneous tissue. DFSP shows a propensity for local recurrence, but distant metastasis is rare (Fig. 8.4). Fibrosarcomatous change in DFSP, although a well-described phenomenon, is actually uncommon [163].

Genetics. Two cytogenetic changes are characteristic of DFSP (Table 8.2): (a) the reciprocal translocation t(17;22)(q22;q13) and (b) supernumerary ring chromosomes derived from this translocation [164, 165]. Both structural changes produce a fusion gene in which the collagen type Iα1 gene (*COLIA1*) on chromosome 17 is fused with the platelet-derived growth factor β-chain gene (*PDGFB*) on chromosome 22 (Fig. 8.5) [166]. Chromosomal breakpoints have been identified in about 20 different *COLIA1* introns. Therefore, marked structural heterogeneity in *COLIA1-PDGFB* fusion genes is seen, although the breakpoint is almost always within intron 1 of *PDGFB* [167]. In addition, many DFSP show gains or amplifications of the *PDGFB* gene, although it is currently unknown if these copy number changes correlate with prognosis or response to therapy [168]. A variety of other recurrent structural and numerical chromosomal abnormalities have also been described in DFSP, including trisomy 8 (present in about one third of cases) and trisomy 5 (less frequent) [91, 169].

The *COLIA1-PDGFB* rearrangement replaces the strong, negative regulatory sequences that are normally upstream of the *PDGFB* gene with the promoter of the *COLIA1* gene [170, 171]. The ensuing unregulated production of PDGFB is thought to promote tumorigenesis via autocrine stimulation of the PDGF receptor [166, 169, 172].

As expected, the *COLIA1-PDGFB* fusion gene is a characteristic feature of the pigmented (Bednar tumor) [169, 173] and granular cell [174] variants of DFSP. The fusion gene is also present in fibrosarcomatous areas that may evolve in these tumors [175]. Of note, the *COLIA1-PDGFB*

Table 8.2 Summary of recurring genetic aberrations in spindle cell tumors of the skin

Tumor	Aberration	Gene(s) involved	Estimated prevalence
Synovial sarcoma	t(X;18)(p11;q11)	*SYT-SSX1*	65%
	t(X;18)(p11;q11)	*SYT-SSX2*	35%
	t(X;18)(p11;q11)	*SYT-SSX4*	Rare
Inflammatory myofibroblastic tumor	Rearrangements of 2p23	*ALK*	47%
Malignant peripheral nerve sheath tumor	Complex changes	Unknown	–
Dermatofibrosarcoma protuberans	t(17;22)(q22;q13) and derivative ring chromosomes	*COLIA1-PDGFB*	>95%
Giant cell fibroblastoma (juvenile form of DFSP)	t(17;22)(q22;q13) and derivative ring chromosomes	*COLIA1-PDGFB*	>95%
Angiomatoid fibrous histiocytoma	t(2;22)(q33;q12)	*EWS-CREB1*	75%
	t(12;22)(q13;q12)	*EWS-ATF1*	20%
	t(12;16)(q13;p11)	*TLS-ATF1*	5%
Leiomyosarcoma	Complex changes	Unknown	–

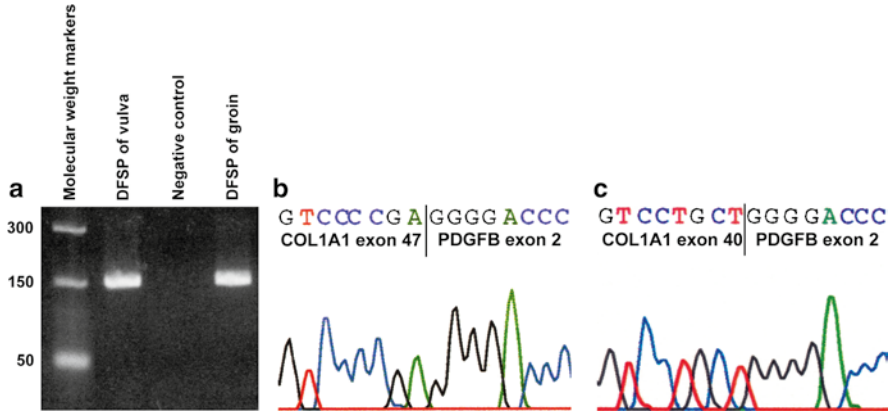

Fig. 8.5 Molecular analysis of dermatofibrosarcoma protuberans (*DFSP*). (**a**) Multiplex reverse transcription polymerase chain reaction (*RT-PCR*) demonstration of *COLIA1-PDGFB* fusion transcripts that result from the t(17;22)(q22;q13) translocation, a characteristic of DFSP. (**b**) The partial nucleotide sequence of the RT-PCR product from the vulvar tumor shows an in-frame fusion between *COLIA1* and *PDGFB*; the *vertical line* indicates the region derived from *COLIA1* exon 47 fused to the region derived from *PDGFB* exon 2. (**c**) The partial nucleotide sequence of the RT-PCR product from the groin tumor shows an in-frame fusion between *COLIA1* exon 40 and *PDGFB* exon 2 (Note that the multiplex design of the RT-PCR assay yields bands of similar size, despite the different translocation breakpoints in the vulvar and groin tumors)

fusion gene is also identified in giant cell fibroblastoma (GCF). This observation, together with the similar clinical and histopathologic features of DFSP and GCF, indicates that the two tumors are merely adult and pediatric presentations, respectively, of a single tumor entity [166, 167, 169, 176, 177]. At the molecular level, the pattern of *COLIA1-PDGFB* fusion genes in GCF and DFSP is indistinguishable. *COLIA1-PDGFB* fusion transcripts, with structural features typical of those occurring in DFSP, have also been demonstrated in superficial adult fibrosarcomas that lack the microscopic changes of conventional DFSP [178]. Based on this finding, it has been suggested that superficial adult fibrosarcomas represent a higher grade and more advanced stage of DFSP with fibrosarcomatous transformation, but this hypothesis awaits confirmation by analysis of additional cases. *COLIA1-PDGFB* gene fusions have not been detected in a number of other tumors that mimic DFSP, including conventional fibrosarcoma, congenital/infantile fibrosarcoma, dermatofibroma, or malignant fibrous histiocytoma [173].

Molecular testing. FISH-based analysis can identify the *COLIA1-PDGFB* rearrangement in metaphase chromosomes, using either spectral karyotyping or probes that are specific for the *PDGFB* and *COLIA1* loci [166, 179]. Interphase FISH, using probes that bracket the *PDGFB* gene in a break-apart strategy, is another approach that has made a much larger number of cases amenable to testing [180, 181].

RT-PCR is also a reliable method for detecting *COLIA1-PDGFB* fusion transcripts and has been shown to be at least as sensitive as interphase FISH [180]. When fresh tissue is used as the substrate for analysis, *COLIA1-PDGFB* fusion transcripts can be detected in ~97% of cases of DFSP (as well as in GCF and Bednar tumor) [173, 182]. Fusion transcripts can be detected in ~85% of FFPE examples [173, 182]. Considerable heterogeneity in the location of the *COLIA1* breakpoint necessitates the use of multiple primers spanning virtually the entire *COLIA1* gene. This is easily accomplished in a multiplex format (Fig. 8.5) [166, 167]. Since the many regions of sequence similarity between the various *COLIA1* exons can give rise to spurious PCR products, confirmation of the amplicon's identity by DNA sequence analysis is advisable to exclude false-positive results (Figs. 8.5 and 9.4) [169].

Prognostic features. There appears to be no correlation between the location of the translocation breakpoint within *COLIA1* and patient age, tumor site, histopathological pattern, likelihood of fibrosarcomatous or metastatic evolution, or prognosis [169].

Since the PDGF receptor is a tyrosine kinase that shows a high level of specific inhibition by imatinib mesylate (Gleevec®), the autocrine loop that results from unregulated production of PDGFB provides a rationale for use of this drug in the treatment of DFSP. Recent clinical reports confirm that patients whose tumors harbor the t(17;22), whether localized or metastatic, often show at least a partial therapeutic response, but that patients whose tumors lack the gene fusion do not respond to this drug [181, 183–185]. This indicates that molecular analysis to detect the *COLIA1-PDGFB* fusion, even if not required for diagnosis, has a role in treatment decisions.

Inflammatory Myofibroblastic Tumor

Inflammatory myofibroblastic tumor (IMT) is composed of spindled myofibroblastic cells accompanied by an inflammatory infiltrate of plasma cells, lymphocytes, and eosinophils (Fig. 8.4). The tumor arises primarily in the soft tissue and viscera of children and young adults, although cases also occur throughout adulthood. Numerous examples of IMT involving the skin have been reported [186–188].

Genetics. Chromosomal rearrangements of the *ALK* tyrosine kinase receptor gene at chromosome band 2p23 are a characteristic feature of IMT in children and young adults (Table 8.2) [189]. These rearrangements involve a variety of partner genes, paralleling the genetics of anaplastic large cell lymphoma (ALCL), in which translocations involving *ALK* were first described [190]. In fact, IMT and ALCL share at least four identical gene fusions, including *TPM3-ALK*, *TPM4-ALK*, *CLTC-ALK*, and *ATIC-ALK* [191–194]. In IMT, the characteristic rearrangements only occur in the myofibroblasts, a finding that indicates that these cells are the neoplastic component of the tumor.

ALK rearrangements are much less frequent in IMT that arise in adults over 40 years of age [195–197]. The molecular abnormality in these cases remains unknown, although a chromosomal rearrangement involving the *HMGA2* locus on chromosome 12 has been described in a single case [198].

The *ALK* fusion genes encode proteins in which the N-terminal dimerization or oligomerization domain of a strongly or ubiquitously expressed protein is fused to the C-terminal tyrosine kinase domain of ALK. In all cases, the chimeric proteins are cytoplasmic, and not membrane-bound. Their oligomerization with subsequent upregulation of ALK tyrosine kinase activity is thought to mimic the kinase activation normally mediated by ligand binding to the native membrane-bound ALK protein.

The fact that *ALK* fusions can be detected in only a subset of IMT, even in cases arising in children and young adults, constrains the utility of testing whether by FISH, RT-PCR, or immunohistochemistry (as discussed below). While the positive predictive value of an *ALK* rearrangement is high, the negative predictive value is quite low. Consequently, the results of testing for *ALK* rearrangements must be interpreted in the context of the clinical and morphologic features of each individual case.

In addition to sharing the same fusion gene, both IMT and ALCL can demonstrate ALK immunoreactivity. Diagnostic concerns are also highlighted by the broad morphologic spectrum of ALCL. The latter includes a fibroblastic as well as a sarcomatoid variant with spindle-shaped lymphoma cells [199, 200]. The recent report of a sarcomatoid variant of ALCL that expressed both cytoplasmic ALK and α-smooth muscle actin further emphasizes this point [201].

Molecular testing. Given the genetic heterogeneity of IMT, interphase FISH analysis of the *ALK* locus using a break-apart approach is a very practical way to test for the presence of a fusion gene

[189, 191–193, 202]. *ALK* rearrangements are found in ~47% of IMT cases [195]. Since break-apart FISH will detect *ALK* rearrangements regardless of the fusion partner, novel rearrangements that have yet to be fully characterized will also be detected by the method. Such rearrangements almost certainly exist, given the oncogenic mechanism of *ALK* fusion genes.

While RT-PCR can be used to detect fusion transcripts that arise from *ALK* rearrangements [191–193, 202], this approach is cumbersome. Optimal test sensitivity requires a panel of PCR primers in order to identify all the possible *ALK* fusion genes described in IMT. Even with a comprehensive panel of primers, novel ALK rearrangements may still not be detected.

Immunohistochemistry. Since all the chimeric proteins characteristic of IMT contain the C-terminal protein kinase domain of native ALK, immunohistochemistry targeting this region provides another method to demonstrate the presence of *ALK* rearrangements. Three distinct patterns of cytoplasmic immunohistochemical staining are observed and appear to correlate with specific ALK fusion protein types [197, 203]. However, the proportion of cases that show cytoplasmic immunopositivity varies widely between different studies, ranging from 0% to 62% [189, 195–198, 203, 204]. This may reflect non-standardized assay conditions or differences in other biological parameters (i.e., age distribution of patients) of the tumors tested. Nonetheless, immunohistochemistry is highly specific; for example, one study demonstrated a 90% correlation between immunohistochemical expression of ALK and the presence of an *ALK* rearrangement based on break-apart FISH [195].

The lack of cytoplasmic immunopositivity for the C-terminal protein kinase domain of ALK in other lesions, such as nodular fasciitis, desmoid fibromatosis, gastrointestinal stromal tumors, infantile myofibromatosis, synovial sarcoma, leiomyoma, and myofibrosarcoma, indicates that immunohistochemistry for ALK is useful in the differential diagnosis of IMT [197, 205]. However, ALK immunopositivity is clearly not specific for IMT, since a significant percentage of cases of MPNST, rhabdomyosarcoma, leiomyosarcoma, and malignant fibrous histiocytoma also show ALK immunoreactivity [205].

Prognostic features. There does not appear to be any statistical association between patient sex, tumor site, tumor histology, tumor recurrence, or malignant transformation and the presence of an *ALK* rearrangement [195].

Angiomatoid Fibrous Histiocytoma

Angiomatoid fibrous histiocytoma (AFH) is a slowly-growing hemorrhagic, multicystic soft tissue tumor that usually arises in the lower dermis or subcutaneous fibroadipose tissue of the limbs, trunk, or head and neck of children and young adults (Fig. 8.6) [206]. Rare cases are encountered in older individuals. Morphologically, the tumor is composed of solid-to-lobulated sheets of plump-to-spindled cells adjacent to areas of hemorrhage.

Genetics. An *EWS-CREB1* fusion gene is present in ~75% of AFH, and results from a translocation involving *EWS* at 22q12 with *CREB1* at 2q33 [100, 207]. About 20% of cases harbor the t(12;22)(q13;q12) translocation, that forms a chimeric *EWS-ATF1* fusion gene [100, 207]; and ~5% of tumors contain the balanced translocation t(12;16)(q13;p11), which produces a *TLS-ATF1* fusion gene [208, 209]. Given the functional homology between TLS and EWS [14, 15], and between CREB1 and ATF1 [100], the presence of this spectrum of fusion genes is not unexpected.

Regardless of the particular fusion gene involved, the general structure of the chimeric protein is the same. A strong transcriptional activation domain in the N-terminal region of the native EWS or TLS protein [14, 15] is fused to the bZIP DNA-binding and dimerization domain of the C-terminal region of CREB1 or ATF1 [100].

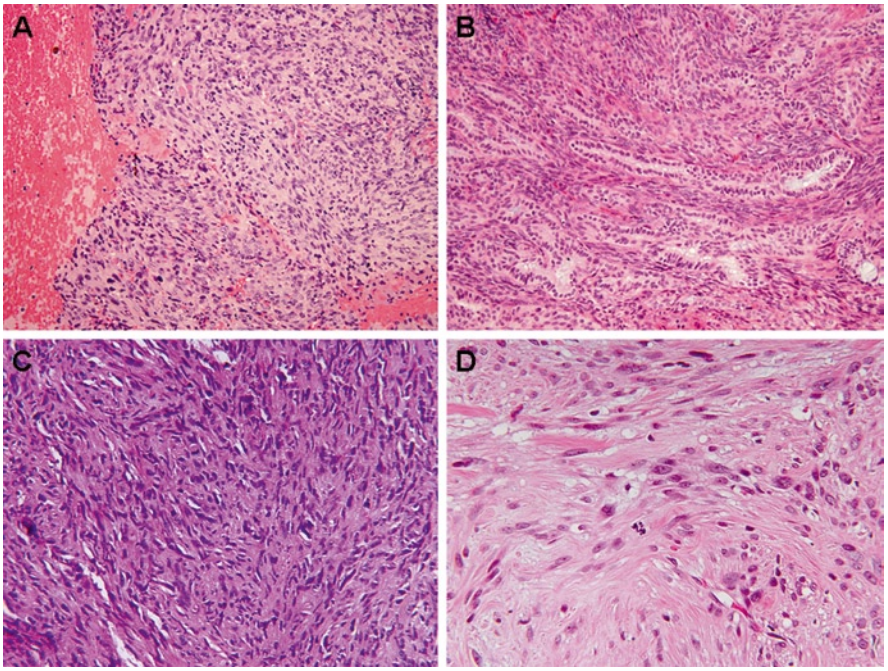

Fig. 8.6 (**a**) Angiomatoid fibrous histiocytoma (*AFH*). (**b**) Synovial sarcoma (*SS*). (**c**) Malignant peripheral nerve sheath tumor (*MPNST*). (**d**) Leiomyosarcoma

These *EWS-CREB1* and *EWS-ATF1* fusions can also be found in CCS, as previously discussed (Fig. 8.3). Regardless of the implications of this observation as far as the oncobiology of these two tumor types are concerned (i.e., the need for additional tumor-specific mutations or divergent differentiation programs in the putative precursor cell populations) [100, 207], the presence of the exact same molecular alteration in both AFH and CCS has obvious significance for test interpretation. Furthermore, rearrangements of *EWS* are also characteristic of a number of other malignancies that fall within the differential diagnosis of AFH, indicating that analyses limited to interphase FISH break-apart testing of the *EWS* locus alone may be insufficient for definitive diagnosis [100]. These observations emphasize that a diagnosis of AFH should be based on all the clinical and histopathological features of a case, rather than the molecular genetic test result alone [100].

Molecular testing. RT-PCR has been shown to be a useful method for demonstrating fusion gene transcripts in both fresh and FFPE AFH tumor samples [100, 207–209]. However, given the number of different fusion genes that are characteristic of this tumor, the sensitivity of RT-PCR approaches clearly depends on the comprehensiveness of testing protocols.

Interphase FISH has emerged as the most useful method for demonstrating the chromosomal rearrangements in AFH, especially via a probe break-apart strategy. There is excellent agreement between interphase FISH and RT-PCR in the evaluation of AFH; the two techniques yield results that are concordant in 83–100% of cases [100, 207, 210].

Synovial Sarcoma

Synovial sarcoma (SS) is historically described as a tumor that arises in the periarticular regions of adolescents and young adults; however, despite the name, no biological or pathologic relationship

between SS and synovium has been demonstrated. Microscopically, SS is divided into two major subtypes: (a) Biphasic SS and (b) Monophasic SS. Biphasic SS contains both spindle and epithelioid cells often arranged in glandular structures (Fig. 8.6). Immunohistochemical analysis shows that the epithelioid component expresses epithelial membrane antigen (EMA) and less frequently cytokeratins. Monophasic SS is composed entirely of spindle cells. Molecular characterization of the genetic abnormalities characteristic of SS has markedly expanded the clinicopathologic spectrum of this tumor. SS is now known to occur in patients of all ages (including newborns and the elderly) and in a wide variety of anatomic sites, including the skin.

Genetics. The t(X;18)(p11.2;q11) translocation is the genetic hallmark of SS. The translocation fuses the *SYT* gene on chromosome 18 to a member of the *SSX* gene family at Xp11 [211–214]. *SSX1* and *SSX2* are involved in most examples of SS [212, 213]. Biphasic tumors usually harbor a *SYT-SSX1* fusion gene, while most tumors with a *SYT-SSX2* transcript are monophasic subtypes [215, 216]. There is little heterogeneity in the structure of *SYT-SSX* fusion transcripts, and the rare variant transcripts that have been reported reflect unique and non-recurring breakpoints [212, 213, 217–219]. Although the t(X;18) is the sole recurring cytogenetic abnormality in SS, more complex translocations and aneuploidy are often also present. Loss of chromosome 3, and gains of chromosomes 7, 8, and 12, may be detected

SYT-SSX fusion genes encode a chimeric protein in which a transcription activation domain of SYT replaces the Kruppel-associated box (KRAB) transcription repression domain in the N-terminal region of the SSX protein [220]. The resulting chimeric SYT-SSX protein is thought to be oncogenic, as a result of aberrant transcriptional regulation mediated through protein–protein interactions. However, the identity of dysregulated genes is largely unknown.

Non-SS tumors rarely harbor *SYT-SSX* rearrangements, making molecular genetic analysis for *SYT-SSX* fusion genes very useful, especially in problematic cases [221–226]. Nonetheless, *SYT-SSX* fusion transcripts have been detected by RT-PCR in MPNST [227, 228] and rare cases of EWS/PNET [229]. Because such *SYT-SSX*-containing tumors are relatively uncommon, the "unexpected" finding of a *SYT-SSX* fusion in any neoplasm should lead to a review of all the clinical and histopathological aspects of the case, to ensure that the tumor is not simply a misdiagnosed SS.

Molecular testing. RT-PCR-based demonstration of *SYT-SSX* chimeric transcripts is a highly sensitive and widely used method to detect the fusion genes characteristic of SS, even in those cases that demonstrate a masked translocation by conventional cytogenetic analysis [230]. Fusion transcripts can be detected in >90% of SS when either fresh or FFPE tissue is used for analysis [32, 36, 224, 231]. RT-PCR can be performed using consensus primers that will detect both *SYT-SSX1* and *SYT-SSX2* fusions. These can then be distinguished by either direct sequence testing, restriction enzyme digestion of PCR products, or melting curve analysis. Alternatively, RT-PCR or real time PCR can be performed using primers that are specific for the different *SSX* genes [213, 223].

FISH may be employed to demonstrate the presence of the t(X;18) translocation in SS, using either metaphase chromosomes or interphase nuclei [41, 216, 221, 222, 226]. With appropriately designed probes, FISH can even be used to determine which *SSX* gene is involved in the translocation [232]. This is an important advantage, given that different *SYT-SSX* fusions may be associated with variable clinical outcomes, as discussed below.

Prognostic features. In most clinical series, the *SYT-SSX2* fusion is associated with a better prognosis than the *SYT-SSX1* fusion. The prognostic implications of *SYT-SSX4* fusions, atypical *SYT-SSX* fusions, and heterogeneous patterns of fusion transcripts, are presently unknown [229, 233–236]. In one study, the *SYT-SSX2* transcript type was the only independent statistically significant factor for overall survival in patients with localized disease at presentation [236]. Cytogenetic complexity, based on karyotypic analysis, has not been shown to predict clinical outcome [234]. In addition, genomic imbalances detected by CGH also show no correlation with overall survival [237].

Detection of minimal disease. In patients with SS, the clinical significance of (a) circulating tumor cells detected by nested RT-PCR [238, 239], and (b) surgical resection margins that show evidence of tumor by RT-PCR, but that are uninvolved by routine microscopic examination [238, 240], remain unclear. This is largely due to the small number of patients thus far evaluated in these studies [238–240].

Immunohistochemistry. Routine immunohistochemistry demonstrates strong nuclear expression of SYT in >85% of SS cases known to harbor *SYT-SSX* gene fusions. SYT immunostaining is a useful adjunct to diagnosis when insufficient tissue is available for RT-PCR or FISH-based testing [241]. However, since a subset of morphologically similar tumors, including EWS/PNET and other malignant sarcomas, can show variable staining for this protein, strict criteria for the interpretation of immunohistochemical studies must be employed [241].

Malignant Peripheral Nerve Sheath Tumor

Malignant peripheral nerve sheath tumor (MPNST) is a spindle cell sarcoma (Fig. 8.6), although several morphologic subtypes have been described, including rhabdoid and epithelioid variants. Traditionally, the tumor is associated with a peripheral nerve, especially in patients with neurofibromatosis. Rare primary cutaneous cases have been described [242].

Genetics. Conventional cytogenetic analysis has shown that cases of MPNST typically have a complex karyotype, with aberrations frequently involving 1p, 7p22, 11q13-23, 20q13, and 22q11-13. Recurrent translocations are not a characteristic finding. However, *SYT-SSX* fusion genes have been detected in rare cases of MPNST, by both RT-PCR and FISH in paired analysis [227, 228]. In addition, the t(2;5)(p23;q35) translocation that is characteristic of ALCL may also be found in MPNST [205]. The significance of these fusion genes in occasional cases of MPNST is unclear [243]. The translocations may merely reflect the chromosomal instability and clonal evolution that is a hallmark of malignancy [33, 34]; in which case the mutations are epiphenomena, unrelated to the genetic alterations responsible for tumor development. On the other hand, given the extensive clinical, morphologic, and immunohistochemical overlap between SS and MPNST [244–246], the presence of the *SYT-SSX* fusion, in cases for which a definitive distinction between MPNST and SS is not possible, may be an indication that the two tumor types are actually related [227]. This latter concept is supported by gene expression profiling studies [247]. In any event, it is clear from the accumulated data that cases of MPNST harboring unexpected fusion genes are uncommon. Consequently, when faced with an unexpected molecular genetic test result, it is prudent to review all the clinical and histopathological aspects of the case to ensure that the diagnosis of MPNST is correct.

Leiomyosarcoma

Leiomyosarcoma is traditionally subdivided into three subgroups. The most common subgroup, and also the most aggressive, comprises tumors that arise in the uterus or deep soft tissues (including the retroperitoneum, abdominal cavity, and extremities). The second subgroup consists of tumors that arise in the walls of blood vessels (often the inferior vena cava). The third subgroup is superficial leiomyosarcomas, composed of subcutaneous lesions (possibly arising from the smooth muscle in the walls of blood vessels) and cutaneous tumors (thought to arise from the arrector pili muscles or the genital dartoic muscles) (Fig. 8.6) [248, 249].

The superficial leiomyosarcoma subgroup accounts for 2–3% of all soft tissue sarcomas. Subcutaneous leiomyosarcomas recur in 50–70% of patients, and metastasize in 30–40% of cases [250]. Cutaneous leiomyosarcomas tend to have a more indolent clinical course; while local recurrence rates are ~30–50%, hematogenous and/or lymphatic metastases are rare [250].

Genetics. Conventional cytogenetic analysis and array-based techniques, such as array-CGH, have demonstrated that leiomyosarcomas are characterized by complex chromosomal aberrations. In fact, no two cases of leiomyosarcoma have ever been shown to have identical karyotypes [251, 252]. The profile of genetic changes in an individual leiomyosarcoma seems to be more related to the tumor's anatomic site of origin or subgroup than to its morphology (i.e., spindle, epithelioid, myxoid, or inflammatory). Clear associations between specific cytogenetic changes and clinical outcome have not been established [251–253].

Despite the complexity of chromosomal aberrations, some recurring patterns have emerged. The tumor suppressor genes *RB1* and *PTEN* appear to have roles in the development of leiomyosarcoma, as suggested by frequent losses at 10q and 13q chromosomal regions. Recurrent gains at 6q and 8q suggest that the *MYC* and *MYB* oncogenes may also be involved. Trisomy 8 is another recurring feature of leiomyosarcoma [248, 251].

There is a weak association between a familial tumor syndrome, known as hereditary cutaneous leiomyomatosis and renal cell cancer (HLRCC), and cutaneous leiomyosarcoma. HLRCC is inherited in an autosomal dominant pattern, and results from heterozygous mutations in the fumarate hydratase gene [254]. Mutations in another, as yet unknown, gene may also be involved [249].

Molecular testing. A role for molecular testing of leiomyosarcoma for any chromosomal abnormality or genetic change has not been established. Diagnosis continues to rely on traditional morphologic and immunohistochemical evaluation [250].

Other Fibrous Proliferations

The cutaneous fibrous proliferations represent a heterogeneous group of lesions that include true neoplasms and fibrohistiocytic hyperplasias. To date, few detailed studies have examined cytogenetic alterations in these entities. Further investigations are needed to determine the frequency and/or types of chromosomal alterations in fibrous proliferations before these observations can be of clinical use.

Nodular fasciitis has long been considered a benign reactive proliferation of fibroblasts, with close histological resemblance to soft tissue sarcomas (Fig. 8.7). The limited studies examining genetic aberrations in nodular fasciitis have identified a number of chromosomal alterations, that include rearrangements involving chromosomes 2, 15, and 16 [255–257]. In a recent comparison of myofibroblastic sarcomas to nodular fasciitis, a majority of the former showed DNA copy number changes as opposed to ~20% of nodular fasciitis cases [258]. The results of this study suggest that, at least in a subset of cases, nodular fasciitis may not simply represent a reactive proliferation, but rather a true neoplasm.

Atypical fibroxanthoma (AFX) is another proliferation of debatable fibrohistiocytic histogenesis and uncertain biological potential (Fig. 8.7). Molecular studies of this lesion are rare. Early investigations demonstrated aneuploidy, especially in the large pleomorphic cells of AFX [259–261]. A more recent study, using CGH analysis, demonstrated DNA copy number changes in 80% of cases [262]. The most common cytogenetic alterations included deletions of 9p and 13q. These were also the most common alterations found in cases of undifferentiated high-grade pleomorphic sarcoma [262].

Fibrous histocytoma, also known as *dermatofibroma*, is a commonly biopsied lesion. Most authors regard dermatofibromas as reactive fibrohistiocytic proliferations. However, a number of studies report that some dermatofibromas are clonal in nature [263, 264].

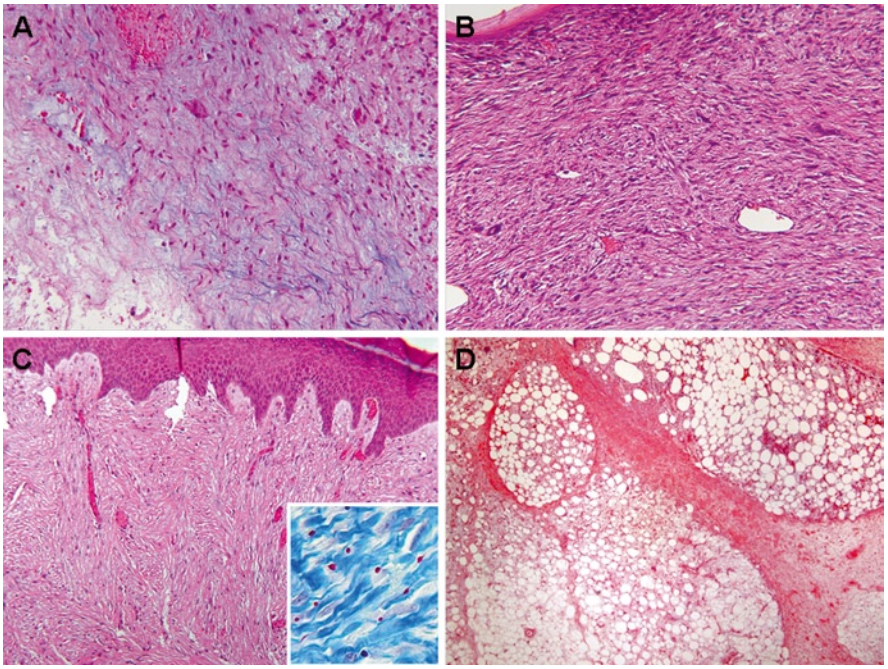

Fig. 8.7 (**a**) Nodular fasciitis. (**b**) Atypical fibroxanthoma (*AFX*). (**c**) Infantile digital fibromatosis. (**d**) Lipoblastoma

Pediatric fibrous proliferations include *infantile digital fibromatosis*, *infantile myofibromatosis*, and *infantile fibrosarcoma*. Infantile digital fibromatosis is a localized myofibroblastic neoplasm that is characterized by spindle cells with distinct paranuclear cytoplasmic inclusions (Fig. 8.7) [265]. Cytogenetic alterations in infantile digital fibromatosis have yet to be documented. Infantile myofibromatosis presents in one of three ways: (a) isolated; (b) multicentric without gastrointestinal involvement; and (c) multicentric with gastrointestinal involvement. Cytogenetic analysis has demonstrated del[6](q12q15), monosomy 9q, and trisomy 16q in this tumor [266, 267]. Infantile fibrosarcoma, a morphologic mimic of infantile myofibromatosis, carries the chromosomal rearrangement t(12;15)(p13;q25), resulting in the *ETV6-NTRK3* fusion gene [268, 269].

Tumors of Adipose Tissue

Lipoblastoma

Lipoblastoma is a benign tumor of adipose tissue that has two patterns of presentation. The localized, well-circumscribed form is referred to as lipoblastoma; the diffuse infiltrative form is termed lipoblastomatosis (Fig. 8.7). Lipoblastoma is usually diagnosed in children less than 5 years old, is more common in males, and occurs most often in the soft tissues of the extremities. Cases arising in the skin have been reported [270]. Histologically, the tumor is composed of an admixture of mature and immature adipocytes, variable numbers of lipoblasts, and stellate mesenchymal cells.

Genetics. The genetic hallmark of lipoblastoma is a rearrangement of chromosomal region 8q12 that generates a *PLAG1* fusion gene [271]. The majority of cases harbor an intrachromosomal 8q

Table 8.3 Summary of recurrent genetic aberrations in lipomatous neoplasms of the skin

Tumor	Aberration	Gene(s) involved	Estimated prevalence
Lipoblastoma	Intrachromosomal 8q rearrangements	*HAS2-PLAG1*	Combined, 8q11-13 aberrations are present in up to 90%
	t(7;8)(q22;q12)	*COLIA2-PLAG1*	
	Low-level amplification of *PLAG1*	*PLAG1*	
	Other rearrangements of 8q11-13; polysomy 8	Unknown	
Atypical lipomatous tumor (ALT/WDLPS)	Supernumerary ring chromosomes; giant marker chromosomes	Amplification of region 12q14-15, including *MDM2, CDK4, HMGA2, SAS, GL1*	Up to 100%
Dedifferentiated liposarcoma	Same as for ALT/WDLPS	Same as for ALT/WDLPS	Up to 100%
Myxoid/round cell liposarcoma	t(12;16)(q13;p11)	*TLS-CHOP*	95%
	t(12;22)(q13;q12)	*EWS-CHOP*	5%
Pleomorphic liposarcoma	Complex changes	Unknown	–

rearrangement that produces an *HAS2-PLAG1* fusion, in which the partner is the hyaluronic synthase 2 gene at 8q24 (Table 8.3). A minority of cases harbor translocations that produce a *COLIA2-PLAG1* fusion gene, in which the partner is the collagen1α2 gene at 7q22 [271–273]. Rare tumors contain other (uncharacterized) translocations involving *PLAG1* [274]. Chromosome 8 polysomy or low-level amplification of *PLAG1* are also characteristic features of lipoblastoma, present in a subset of tumors both with and without 8q12 rearrangements [273, 275]. The *PLAG1* alterations are present in many of the different mesenchymal cell types in lipoblastomas, including lipoblasts, mature adipocytes, primitive mesenchymal cells, and fibroblast-like cells, which suggests that lipoblastomas originate from a primitive mesenchymal precursor cell with variable differentiation [273].

The rearrangements of 8q12 are essentially promoter-swapping events, in which the upstream 5′ regulatory elements of *PLAG1* are replaced by the promoter regions of the fusion partner. Since *PLAG1* encodes a zinc finger transcription factor that is normally developmentally regulated [276], the increased expression of PLAG1 that results from the promoter-swapping is believed to be responsible for tumor development, even when the structure of the protein is unaltered by the chromosomal rearrangement [271, 272]. The *PLAG1* dosage alterations that result from polysomy 8 are thought to represent an alternative mechanism for increased PLAG1 expression [273].

Rearrangements of 8q12 and polysomy 8 are uncommon in other tumors of adipose tissue that are likely to enter into the differential diagnosis of lipoblastoma. These include lipoma, atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDLPS), and myxoid/round cell liposarcoma. The utility of molecular testing for rearrangements of *PLAG1*, in order to distinguish lipoblastoma from ALT/WDLPS, has recently been emphasized [274]. It is noteworthy that ~40% of pleomorphic adenomas of the salivary gland also harbor *PLAG1* fusion genes, although the *PLAG1* fusion partners in the latter are different from those in lipoblastoma [272, 277, 278]. While the presence of *PLAG1* fusion genes in such disparate tumors is interesting from the perspective of tumor biology, it obviously has little impact on the diagnostic utility of molecular genetic analysis of fatty tumors.

Molecular testing. FISH, using either metaphase chromosomes or interphase nuclei, can be used to detect rearrangements of *PLAG1*, the 8q12 region, chromosome 8 polysomy, and low-level

amplification of *PLAG1* in ~90% of lipoblastomas, including some cases that show no aberrations of the 8q12 region by routine cytogenetic analysis [273–275]. Given the variability and type of genetic aberrations, RT-PCR is not ideally suited to routine molecular evaluation of lipoblastomas.

Immunohistochemistry. A polyclonal anti-PLAG1 antibody has been used to demonstrate increased nuclear staining in a small number of cases [272]. However, since PLAG1 expression has been identified in other tumor subtypes [279], the utility of PLAG1 immunohistochemistry in the diagnosis of lipoblastomas remains unclear.

Atypical Lipomatous Tumor/Well-Differentiated Liposarcoma

Atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDLPS) is a malignant soft tissue neoplasm that most commonly arises in adults in the 5th to 7th decade of life. Rare cases developing in the skin have been reported [270]. The tumor is composed of cells that show adipocyte differentiation with variation in cell size and at least focal nuclear atypia (Fig. 8.8). Metastatic potential is associated with dedifferentiation, and prognosis of this tumor is largely determined by the site of origin. Lesions arising at surgically amenable locations rarely recur and virtually never metastasize, while lesions that arise at sites where it may be impossible to achieve wide surgical excision (i.e., retroperitoneum or mediastinum) may demonstrate uncontrolled recurrences that can cause death, even in the absence of dedifferentiation or metastasis.

Genetics. The defining cytogenetic features of ALT/WDLPS are the presence of supernumerary ring or giant marker chromosomes [280], or less commonly supernumerary marker chromosomes

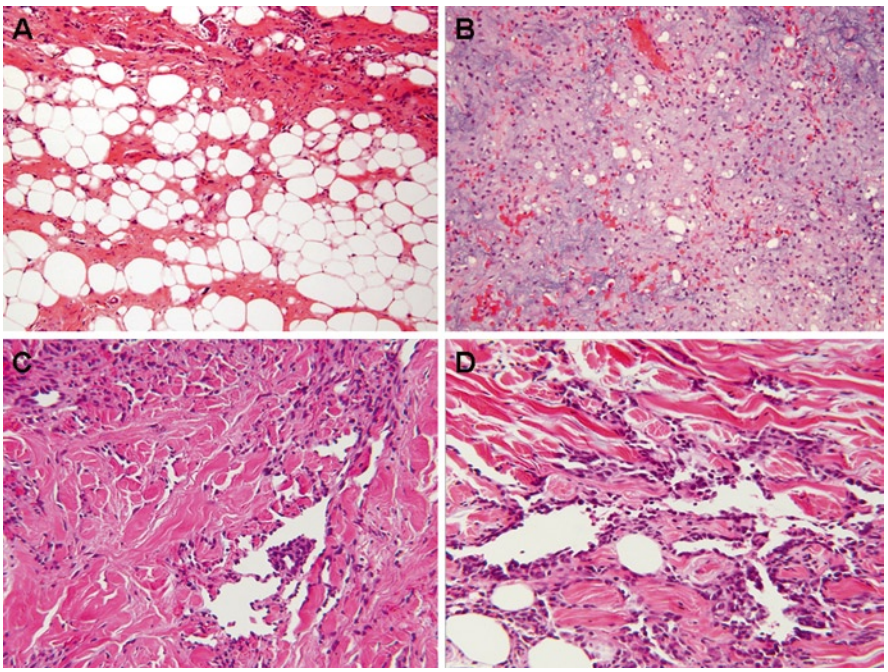

Fig. 8.8 (**a**) Atypical lipomatous tumor/well-differentiated liposarcoma (*ALT/WDLPS*). (**b**) Myxoid liposarcoma (*ML*). (**c**) Kaposi's sarcoma (*KS*). (**d**) Angiosarcoma

[281], which contain amplifications of the 12q14-15 region. This locus contains several putative and established oncogenes, including *MDM2*, *CDK4*, *SAS*, and *HMGA2* [282, 283]. Several other chromosomal regions, the most frequent of which are 12q21-22 and 1q21-25, are often co-amplified with the 12q14-15 region [284, 285].

The exact mechanism of tumorigenesis in ALT/WDLPS remains unclear. No single gene of the *MDM2*, *CDK4*, *SAS*, and *HMGA2* group seems to play a dominant role. It is likely that these genes promote tumor development via direct or indirect control of cell proliferation. Both *MDM2* and *CDK4* encode proteins that are involved in regulation of the cell cycle: MDM2 by binding and inhibiting TP53 activity, and CDK4 by regulating phosphorylation of the retinoblastoma tumor suppressor encoded by the *RB1* gene. In addition, *HMGA2* encodes a protein that modifies chromatin structure and facilitates formation of transcriptional complexes.

Lipomas have a set of characteristic genetic features that generally do not overlap with those of ALT/WDLPS [286]. It is possible that the rare so-called lipomas with *MDM2* amplification and ring chromosomes actually represent early non-diagnosable ALT/WDLPS [287, 288]. Consequently, any fatty tumor with discordant histopathological and molecular genetic features should be thoroughly reviewed for a definitive diagnosis.

Molecular testing. The vast majority of ALT/WDLPS contain supernumerary ring or giant marker chromosomes [283, 284]. Since conventional cytogenetic techniques, such as G- and R-banding, do not reveal the chromosomal origin of the supernumerary chromosomes, FISH can be used to confirm their identity [281, 289]. Interphase FISH is a useful method for demonstration of *MDM2* gene amplification in FFPE tissue sections [290, 291]. In addition, quantitative PCR and RT-PCR analyses have shown that the level of *MDM2* and *CDK4* gene amplification or expression can be used to reliably distinguish lipoma from ALT/WDLPS, even in FFPE tissue [291–294]. It is important to note that supernumerary ring chromosomes are not absolutely specific for ALT/WDLPS and have also been described in some sporadic lipomas, although the ring chromosomes in lipomas do not harbor 12q14-15 rearrangements [282, 284].

Immunohistochemistry. Consistent with quantitative RT-PCR results showing increased transcription of genes in the 12q14-15 region in ALT/WDLPS [293, 294], immunohistochemistry demonstrates overexpression of the corresponding proteins. MDM2 and CDK4 are overexpressed in 50% and 100% of ALT/WDLPS, respectively, but in 0% and only 11% of lipomas, respectively. Therefore, immunohistochemistry has been proposed as a reliable means to distinguish these two tumor types [290, 291, 295–297]. It is important to note that the *MDM2* gene is frequently amplified or overexpressed in several other sarcomas, including malignant fibrous histiocytoma, osteosarcoma, and even rare leiomyosarcomas [287, 294, 298–300].

Dedifferentiated Liposarcoma

Dedifferentiated liposarcomas are malignant neoplasms of adipose tissue demonstrating a transition from either a primary or recurrent ALT/WDLPS to a non-lipogenic sarcoma of variable histopathologic grade. Consistent with this derivation, conventional cytogenetic analysis has shown that most dedifferentiated liposarcomas contain ring or giant marker chromosomes with associated amplification of 12q14-15 [301–303]. The majority of dedifferentiated liposarcomas also show elevated levels of CDK4 and MDM2 proteins [297], although levels of expression vary with the anatomic site of the tumor [302]. Immunostains for PPAR-γ can also be used to demonstrate evidence of adipocytic differentiation [304]. Additional genetic alterations, including mutations of *TP53* and *RB1*, which may be responsible for tumor dedifferentiation, can be present [302, 305–307].

Myxoid Liposarcoma/Round Cell Liposarcoma

Myxoid liposarcoma (ML) is the second most common subtype of liposarcoma. It occurs predominantly in the deep soft tissues of the extremities, and more than two-thirds of cases arise within the musculature of the thigh (Fig. 8.8). Rare cutaneous cases have been reported [270].

Genetics. Approximately 95% of cases of ML contain the t(12;16)(q13;p11) translocation, which fuses the *TLS* gene (also known as *FUS*) at 16p11 with the *CHOP* gene (also known as *DDIT3* and *GADD153*) at 12q13 [308–310]. *TLS-CHOP* gene fusions are also characteristic of round cell liposarcoma (RCL), suggesting that the two tumor types are etiologically related [311]. Variability in the site of the translocation breakpoint can generate structural heterogeneity in the fusion genes [311–313], and rare variant *TLS-CHOP* fusion genes have been described [314–316]. Given the sequence similarity between *TLS* and *EWS* [14, 15], it is not surprising that ~5% of cases of ML/RCL harbor a t(12;22)(q13;q12) translocation, in which *EWS* is fused with *CHOP* [315, 317–319].

The fusion gene encodes a chimeric protein in which the N-terminal region of TLS, which includes a transcriptional activation domain [14, 15], is fused to the full length of CHOP, a member of the bZIP family of transcription factors. Because the *TLS* promoter is strongly and broadly activated, the fusion protein is constitutively expressed. Consistent with the normal function of CHOP in adipocyte differentiation and growth arrest, the fusion protein has been shown to inhibit the differentiation of pre-adipocytes [320–322]. Presumably, EWS-CHOP fusion proteins exert their oncogenic effect through a mechanism analogous to their TLS-CHOP counterparts.

Molecular testing. Interphase FISH, using routinely processed tumor tissue or even cytology samples, can be employed to detect the translocations characteristic of ML/RCL [41, 323–325]. RT-PCR-based testing has been extensively used to demonstrate the presence of *TLS-CHOP* or *EWS-CHOP* fusion transcripts, and is especially helpful in cases showing atypical karyotypes [326]. Fusion transcripts can be detected in up to 95% of cases, using fresh tissue [36]. RT-PCR analysis of FFPE tissue is not associated with a significant loss of sensitivity [36].

Since ML/RCL harbor distinct cytogenetic abnormalities compared with lipomas and the majority of well-differentiated liposarcomas (including those with myxoid change), the latter tumor types do not appear to be oncogenetically related to ML/RCL [310, 327–329]. However, rare examples of well-differentiated and pleomorphic liposarcoma with *TLS-CHOP* fusions have been described [330]. Nonetheless, the rarity of tumors other than ML/RCL that harbor *TLS-CHOP* rearrangements makes molecular analysis for this fusion very useful in problematic cases [330].

Prognostic features of transcript type. Preliminary evidence suggests that tumors harboring *TLS* exon 7 to *CHOP* exon 2 fusions (so-called type I fusions) have a more aggressive clinical course than tumors harboring *TLS* exon 5 to *CHOP* exon 2 fusions (so-called type II fusions) [316].

Detection of minimal disease. The clinical significance of submicroscopic disease in ML/RCL remains unclear. In a retrospective study, PCR-based testing demonstrated evidence of *TLS-CHOP* or *EWS-CHOP* fusions in PB samples of 20% of patients with this tumor, although the molecular result did not correlate with clinical outcome [331]. Similarly, in a prospective study, RT-PCR analysis detected *TLS-CHOP* fusion transcripts in the BM and/or PB of a subset of patients with ML; however, an interpretation of the significance of these results was limited by the small study size and short duration of follow-up [330].

Vascular Tumors

Kaposi's Sarcoma

Kaposi's sarcoma (KS) is a low-grade vascular neoplasm of which four clinical subtypes have been described: (a) an indolent form, that usually involves the lower extremities of elderly men of Mediterranean or eastern European origin; (b) KS associated with long-term immunosuppressive therapy (usually in transplant recipients); (c) as an endemic disease in young adults and children in central Africa; and (d) KS in the setting of human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS) [332]. KS has traditionally been divided into three clinical-histopathological stages: (a) patch, (b) plaque, and (c) nodular, which form a morphologic continuum (Fig. 8.8).

Genetics. Kaposi's sarcoma-associated herpes virus (KSHV), also known as human herpes virus-8 (HHV-8), is the causative agent of KS [333]. The virus has a primary tropism for endothelial cells and B-lymphocytes, but can also infect other cell types with much lower efficiency [334]. As with other members of the gamma herpes virus subfamily, the virus encodes a number of proteins that can result in latent infection [334, 335].

Molecular testing. PCR-based testing of FFPE tissue detects HHV-8 sequences in 88–100% of KS cases, regardless of the clinical form or histopathological stage of disease [336–338]. RT-PCR analysis of FFPE tissue, in which transcripts encoding the latency-associated nuclear antigen (LANA) are the target for amplification, detects HHV-8 in virtually 100% of cases [339]. In situ RT-PCR testing of FFPE tissue has also been described; although technically demanding, it also demonstrates a sensitivity of virtually 100% [340].

HHV-8 infection is also associated with a subset of primary effusion lymphomas [332, 341–343], solid/extra-cavitary lymphomas [344], multicentric Castleman's disease [342, 344], primary pulmonary hypertension [345], and IMT [346]. The virus has even been detected in reactive mesothelium [347]. Since these diseases rarely enter into the differential diagnosis of KS, molecular testing for this virus is useful for confirmation of the latter, especially at an early stage of disease evolution or in patients with an unusual clinical presentation [340, 348].

It is important to note that other endothelial neoplasms have been reported to harbor HHV-8, including angiosarcoma (up to 29% of cases in one series, by PCR and Southern blot analysis) [349, 350], pyogenic granuloma/lobular capillary hemangioma (by PCR and Southern blot analysis) [351], retiform hemangioendothelioma [352], angiolymphoid hyperplasia with eosinophilia [353], and hemangioma [350]. It is possible that the extreme sensitivity of some molecular strategies may be responsible for false-positive test results in lesions other than KS. For example, PCR-based approaches can detect intralesional blood mononuclear cells that are infected with HHV-8, even when the lesional cell (i.e., endothelial) population itself is not [339, 354]. In any event, since the cumulative data indicate that only exceedingly rare vascular lesions (other than KS) harbor HHV-8 [336, 337, 339, 355], a positive molecular result must be interpreted in the context of all the clinical, serological, histopathological, and immunohistochemical features in order to ensure that a lesion is accurately diagnosed.

Immunohistochemistry. Monoclonal antibodies to LANA-1 (HHV-8) strongly stain the nuclei of spindle cells that line the vascular channels of KS. Immunostaining has a sensitivity of 92–100%, with a specificity that is virtually 100%, since immunoreactivity is not seen in other vascular proliferations (including angiosarcoma, Kaposiform hemangioendothelioma, and pyogenic granuloma) or non-vascular lesions (including DFSP and spindle cell melanoma) [355–357]. In parallel analyses, immunohistochemistry shows higher specificity than RT-PCR for *LANA* transcripts [339], indicating how more complete knowledge of the molecular mechanisms underlying tumorigenesis can lead to the development of conventional protein expression assays that are both more accurate and less cumbersome than nucleic acid-based tests.

Angiosarcoma

Angiosarcomas are malignant tumors that are comprised of cells with the morphologic and immunohistochemical features of endothelial cells (Fig. 8.8). Many angiosarcomas occur at cutaneous sites, often in association with long-standing lymphedema. However, a subset of these tumors can develop adjacent to synthetic vascular grafts or other foreign material, following radiation therapy for other malignancies, or in association with benign or malignant nerve sheath tumors (usually in the setting of neurofibromatosis type 1) [358–360]. Angiosarcoma rarely, if ever, arises from pre-existing KS [361].

Genetics. Conventional cytogenetic analysis shows that angiosarcomas typically contain complex non-recurrent cytogenetic aberrations. Sporadic angiosarcomas involving non-hepatic sites demonstrate *TP53* mutations in 11–52% of cases using DNA sequence analysis [362, 363], and TP53 protein accumulation in 53% of cases by immunohistochemistry [363]. Based on the observations that wild-type TP53 can be functionally inactivated via binding to the MDM2 protein, and *MDM2* gene amplification has cellular effects similar to those of mutated *TP53*, deregulation of *MDM2* expression is thought to serve as an alternative mechanism for escape from TP53-regulated growth control [364]. Consistent with this hypothesis, overexpression of MDM2 is found in the majority of sporadic angiosarcomas [363]. Since mutant TP53 induces expression of vascular endothelial growth factor (VEGF) [365], the finding that VEGF levels are increased in ~80% of angiosarcomas is not unexpected [363].

Despite the observed link between *TP53* and *MDM2* mutations, as well as aberrations in several other genes, in the pathogenesis of angiosarcoma, molecular genetic assays to detect these abnormalities have not been evaluated for routine clinical use. The fact that mutations in these genes are present in only a subset of tumors will probably diminish the utility of testing from a diagnostic point of view. Since there is no evidence that these mutations correlate with clinical outcome, there is apparently no value in testing from a prognostic perspective either.

Sarcomas Arising in Other Cutaneous Tumors

Carcinosarcoma, also known as metaplastic carcinoma, represents a rare primary cutaneous tumor with both malignant epithelial and mesenchymal components (Fig. 8.9). The epithelial component often consists of a trichoblastic carcinoma or squamous cell carcinoma [366]. The mesenchymal component is usually quite variable (and sometimes poorly differentiated), often demonstrating fibrous, smooth muscle, skeletal muscle, cartilaginous, or osteogenic components [367, 368]. Molecular diagnostic testing of these tumors has a limited role. To date, recurrent genetic aberrations have not been described within the sarcomatous components of these lesions.

Sarcomas Metastatic to the Skin

Cutaneous metastases have been reported for a myriad of tumors. Although it is uncommon for a sarcoma to present as a cutaneous metastasis, case reports documenting this phenomenon do exist (Fig. 8.9). Sarcomas account for <3% of cutaneous metastases in adults [369], but 50% of non-hematopoietic cutaneous metastases in children [370]. In most instances, the site/differentiation of the primary tumor has already been identified, and skin biopsy is merely performed to document the presence of metastasis. However, molecular analysis of cutaneous tissue may aid in the accurate classification of some tumors (see Chap. 9).

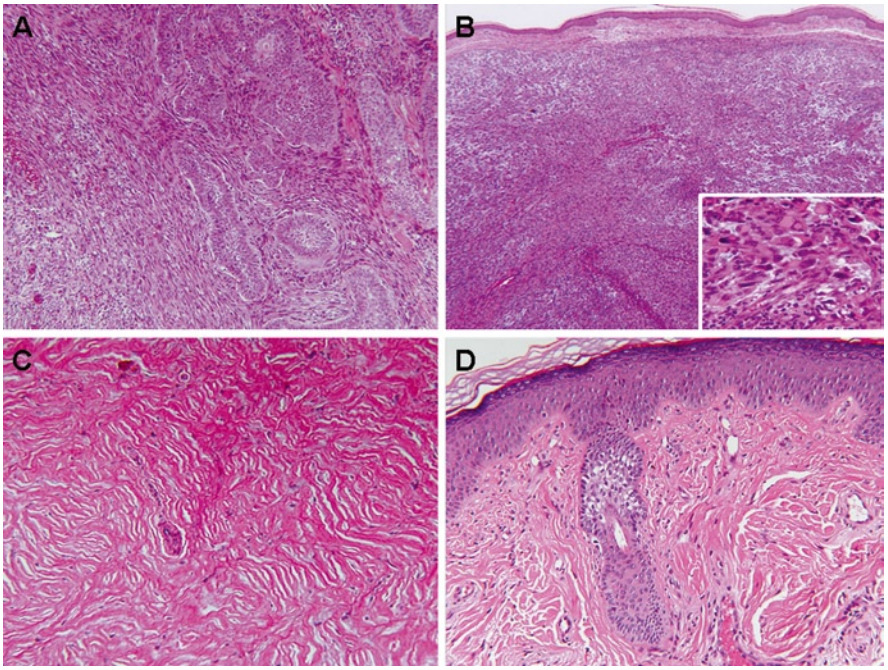

Fig. 8.9 (**a**) Carcinosarcoma. (**b**) Sarcoma with rhabdoid features, metastatic to the skin. (**c**) Sclerotic fibroma. (**d**) Angiofibroma

Syndrome-Associated Mesenchymal Tumors

For years, both dermatologists and pathologists have noted the close association between specific clinical syndromes and certain mesenchymal tumors (Table 8.4). Thus, a pathologist can suggest the possible presence of a syndrome based on the diagnosis of particular tumor. In the appropriate clinical setting, this may direct the clinician to perform syndrome-specific screening for associated genomic abnormalities, or initiate a referral of the patient to a medical geneticist. In general, molecular diagnostic testing of skin specimens is not routinely used to confirm the diagnosis of these syndromes. For illustration purposes, four syndromes and their associated mesenchymal tumors will be discussed: Cowden disease, neurofibromatosis type 1, tuberous sclerosis, and multiple cutaneous and uterine leiomyomatosis.

Cowden disease is caused by a germline mutation in the tumor suppressor gene *PTEN* (Phosphatase and TENsin homologue, chromosome 10) [371]. Associated cutaneous mesenchymal tumors include sclerotic fibromas, lipomas, and neuromas. Sclerotic fibromas are uncommon fibrous tumors characterized by a distinctive "raked earth"-like appearance (Fig. 8.9). A diagnosis of sclerotic fibroma may prompt the pathologist to suggest an association with Cowden disease, initiating appropriate genetic testing [372]. *PTEN* mutations can easily be detected in FFPE tissue specimens. However, it is important to consider that somatic *PTEN* mutations can be found in sporadic tumors [373]. Therefore, non-tumorous tissue must be used for syndrome testing, in order to demonstrate the presence of germline mutations in this gene. For large specimens, this can be achieved simply by testing normal tissue adjacent to any tumorous lesion. However, as is often the case with small skin biopsies, normal tissue may not be available. Cowden disease is now regarded as part of the *PTEN* hamartoma syndrome, which includes Bannayan–Riley–Ruvalcaba syndrome, Proteus syndrome, and Proteus-like syndrome [373].

Table 8.4 Cutaneous syndromes and associated mesenchymal tumors

Syndrome	Inheritance	Gene	Mesenchymal tumor	Other common tumors
Bannayan–Riley–Ruvalcaba[a]	AD	PTEN	Lipoma, hemangioma	Hamartomatous polyp
Basal Cell Nevus	AD	Patched	Medulloblastoma, ovarian fibroma, fibrosarcoma	Basal cell carcinoma, odontogenic cyst
Beckwith–Wiedemann	Sporadic	(KIP2)	Wilms tumor, hepatoblastoma, rhabdomyosarcoma, neuroblastoma	Adrenal cortical carcinoma
Birt–Hogg–Dubé	AD	FLCN	Spectrum of cutaneous hamartomas: fibrofolliculoma, angiofibroma, trichodiscoma, perifollicular fibroma	Kidney neoplasms, lung cysts
Carney complex	AD	PRKAR1α	Myxoma, schwannoma	Epithelioid blue nevus, Sertoli and Leydig cell testicular tumors
Costello	AD	HRAS	Rhabdomyosarcoma	–
Cowden	AD	PTEN	Sclerotic fibroma, neuroma, uterine leiomyoma, lipoma	Trichilemmoma, oral papilloma, breast/thyroid/endometrial carcinoma, hamartomatous polyp
Familial mastocytosis	AD	KIT	Gastrointestinal stromal tumor	–
Familial multiple lipomatosis	AD	Unknown	Lipoma	–
Gardner	AD	APC	Lipoma, fibroma, osteoma, leiomyoma, desmoid tumors, fibrosarcoma	Epidermoid cysts with pilomatrical differentiation, colonic adenocarcinoma
Hereditary glomangioma	AD	GLMN	Glomuvenous malformation	–
Klippel–Trenaunay	Sporadic	Unknown	Cutaneous capillary malformation, hypertrophy of bone and soft tissue, lipoma	–
Madelung	(Mitochondrial)	(MT-TK)	Multiple symmetrical lipomatosis, lipoma	–
Maffucci	Sporadic (or AD)	(Parathyroid hormone receptor 1)	Enchondroma, chondrosarcoma, hemangioma	–
MEN1	AD	MEN1	Angiofibroma, collagenoma, lipoma	Pituitary, parathyroid, and pancreatic tumors
MEN2B	AD	RET	Mucosal neuroma	Pheochromocytoma, medullary thyroid carcinoma
Multiple cutaneous and uterine leiomyomatosis	AD	Fumarate hydratase	Leiomyoma	Renal cell carcinoma
Neurofibromatosis type 1	AD	NF1	Neurofibroma, optic glioma, acoustic neuroma, malignant peripheral nerve sheath tumor, rhabdomyosarcoma	Lisch nodule, juvenile myelomonocytic leukemia, juvenile xanthogranuloma, pheochromocytoma
POEMS	Sporadic	Unknown	Glomeruloid hemangioma	–

(continued)

Table 8.4 (continued)

Syndrome	Inheritance	Gene	Mesenchymal tumor	Other common tumors
Proteus	Sporadic	PTEN	Lipoma, connective tissue nevus, vascular malformations	Epidermal nevus
Rothmund–Thompson	AR	RECQL4	Osteosarcoma, fibrosarcoma	Squamous cell carcinoma
Tuberous sclerosis	AD	TSC1/TSC2	Angiofibroma, periungual and gingival fibromas, shagreen patch, renal angiomyolipoma, pulmonary lymphangioleiomyomatosis, cardiac rhabdomyoma, astrocytoma, glioblastoma multiforme	Retinal hamartoma, rectal polyps
Werner	AR	WRN/RECQL2	Osteosarcoma, fibrosarcoma	–

[a]Significant clinical overlap between Bannayan–Riley–Ruvalcaba syndrome, Cowden syndrome, and even Lhermitte–Duclos disease has led to the suggestion that these entities be collectively referred to as the PTEN hamartomatous syndrome

( ) proposed, *AD* autosomal dominant, *AR* autosomal recessive, *MEN* multiple endocrine neoplasia, *POEMS* polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes

Neurofibromatosis type 1 (NF1) is caused by a mutation in the *NF1* gene. Neurofibromas are the most common mesenchymal tumors in patients with NF1. Although sporadic neurofibromas commonly occur in non-NF1 individuals, plexiform neurofibromas are considered pathognomonic of NF1. Neurofibromas in patients with NF1 have a risk of malignant transformation to MPNST. The latter are characterized by multiple complex cytogenetic aberrations, frequently involving chromosomes 1p, 7p22, 11q13-23, 20q13, and 22q11-13 (as discussed previously).

The common variant of NF1, termed "segmental neurofibromatosis," is caused by a post-zygotic somatic mutation in *NF1*. In the typical scenario, a patient presents with unilateral neurofibromas, without the other clinical findings of NF1. The patient's risk of germline involvement is related to the extent and location of the segmental neurofibromas.

Tuberous sclerosis demonstrates several skin findings, including ashleaf spots, shagreen patches, ungual/subungual fibromas, and facial angiofibromas ("adenoma sebaceum") (Fig. 8.9). Non-cutaneous mesenchymal tumors include glioblastoma multiforme and other neural tumors, renal angiomyolipoma, and pulmonary lymphangioleiomyomatosis. Tuberous sclerosis is caused by mutations in the *TSC1* or *TSC2* genes. Similar to NF1, somatic and germline mosacism can produce a pattern of disease characterized by a segmental distribution of tumors [374–378].

Multiple cutaneous and uterine leiomyomatosis, and the closely related condition *hereditary leiomyomatosis and renal cell cancer*, are syndromes caused by mutations in the fumarate hydratase gene [379]. Based on a high frequency of germline fumarate hydratase mutations in patients with multiple leiomyomata, some authors have suggested that screening tests for aberrations in this gene should be part of the standard clinical work-up in such instances. Screening could be performed on lesional tissue, and would likely have a high level of clinical utility, since sporadic leiomyomata (and sporadic leiomyosarcomas) do not appear to harbor fumarate hydratase mutations [380]. Examples of segmental cutaneous leiomyomatosis have been described, demonstrating that this syndrome can also present in a mosaic form [381–384].

References

1. Toro JR, Travis LB, Wu HJ, et al. Incidence patterns of soft tissue sarcomas, regardless of primary site, in the surveillance, epidemiology and end results program, 1978–2001: an analysis of 26, 758 cases. Int J Cancer. 2006;119:2922–30.
2. Gulley ML, Kaiser-Rogers KA. A rational approach to genetic testing for sarcoma. Diagn Mol Pathol. 2009;18:1–10.
3. Pfeifer JD. Molecular genetic testing in surgical pathology. Philadelphia, PA: Lippincott, Williams & Wilkins; 2006.
4. O'Sullivan MJ, Perlman EJ, Furman J, et al. Visceral primitive peripheral neuroectodermal tumors: a clinico-pathologic and molecular study. Hum Pathol. 2001;32:1109–15.
5. Hasegawa SL, Davison JM, Rutten A, et al. Primary cutaneous Ewing's sarcoma: immunophenotypic and molecular cytogenetic evaluation of five cases. Am J Surg Pathol. 1998;22:310–8.
6. Lee CS, Southey MC, Slater H, et al. Primary cutaneous Ewing's sarcoma/peripheral primitive neuroectodermal tumors in childhood. A molecular, cytogenetic, and immunohistochemical study. Diagn Mol Pathol. 1995; 4:174–81.
7. Terrier-Lacombe MJ, Guillou L, Chibon F, et al. Superficial primitive Ewing's sarcoma: a clinicopathologic and molecular cytogenetic analysis of 14 cases. Mod Pathol. 2009;22:87–94.
8. Delattre O, Zucman J, Melot T, et al. The Ewing family of tumors–a subgroup of small-round-cell tumors defined by specific chimeric transcripts. N Engl J Med. 1994;331:294–9.
9. Delattre O, Zucman J, Plougastel B, et al. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. Nature. 1992;359:162–5.
10. Zucman J, Melot T, Desmaze C, et al. Combinatorial generation of variable fusion proteins in the Ewing family of tumours. EMBO J. 1993;12:4481–7.
11. Sorensen PH, Lessnick SL, Lopez-Terrada D, et al. A second Ewing's sarcoma translocation, t(21;22), fuses the EWS gene to another ETS-family transcription factor, ERG. Nat Genet. 1994;6:146–51.

12. Shing DC, McMullan DJ, Roberts P, et al. FUS/ERG gene fusions in Ewing's tumors. Cancer Res. 2003;63:4568–76.
13. Ng TL, O'Sullivan MJ, Pallen CJ, et al. Ewing sarcoma with novel translocation t(2;16) producing an in-frame fusion of FUS and FEV. J Mol Diagn. 2007;9:459–63.
14. Morohoshi F, Arai K, Takahashi EI, et al. Cloning and mapping of a human RBP56 gene encoding a putative RNA binding protein similar to FUS/TLS and EWS proteins. Genomics. 1996;38:51–7.
15. Bertolotti A, Lutz Y, Heard DJ, et al. hTAF(II)68, a novel RNA/ssDNA-binding protein with homology to the pro-oncoproteins TLS/FUS and EWS is associated with both TFIID and RNA polymerase II. EMBO J. 1996;15:5022–31.
16. Wang L, Bhargava R, Zheng T, et al. Undifferentiated small round cell sarcomas with rare EWS gene fusions: identification of a novel EWS-SP3 fusion and of additional cases with the EWS-ETV1 and EWS-FEV fusions. J Mol Diagn. 2007;9:498–509.
17. Barr FG, Womer RB. Molecular diagnosis of Ewing family tumors: too many fusions...? J Mol Diagn. 2007;9:437–40.
18. Giovannini M, Biegel JA, Serra M, et al. EWS-erg and EWS-Fli1 fusion transcripts in Ewing's sarcoma and primitive neuroectodermal tumors with variant translocations. J Clin Invest. 1994;94:489–96.
19. Ida K, Kobayashi S, Taki T, et al. EWS-FLI-1 and EWS-ERG chimeric mRNAs in Ewing's sarcoma and primitive neuroectodermal tumor. Int J Cancer. 1995;63:500–4.
20. Zielenska M, Zhang ZM, Ng K, et al. Acquisition of secondary structural chromosomal changes in pediatric Ewing sarcoma is a probable prognostic factor for tumor response and clinical outcome. Cancer. 2001;91:2156–64.
21. Hattinger CM, Zoubek A, Ambros PF. Molecular cytogenetics in Ewing tumors: diagnostic and prognostic information. Onkologie. 2000;23:416–22.
22. Lin PP, Brody RI, Hamelin AC, et al. Differential transactivation by alternative EWS-FLI1 fusion proteins correlates with clinical heterogeneity in Ewing's sarcoma. Cancer Res. 1999;59:1428–32.
23. de Alava E, Panizo A, Antonescu CR, et al. Association of EWS-FLI1 type 1 fusion with lower proliferative rate in Ewing's sarcoma. Am J Pathol. 2000;156:849–55.
24. May WA, Lessnick SL, Braun BS, et al. The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol Cell Biol. 1993;13:7393–8.
25. Katz RL, Quezado M, Senderowicz AM, et al. An intra-abdominal small round cell neoplasm with features of primitive neuroectodermal and desmoplastic round cell tumor and a EWS/FLI-1 fusion transcript. Hum Pathol. 1997;28:502–9.
26. Sorensen PH, Shimada H, Liu XF, et al. Biphenotypic sarcomas with myogenic and neural differentiation express the Ewing's sarcoma EWS/FLI1 fusion gene. Cancer Res. 1995;55:1385–92.
27. Thorner P, Squire J, Chilton-MacNeil S, et al. Is the EWS/FLI-1 fusion transcript specific for Ewing sarcoma and peripheral primitive neuroectodermal tumor? A report of four cases showing this transcript in a wider range of tumor types. Am J Pathol. 1996;148:1125–38.
28. Thorner P. Intra-abdominal polyphenotypic tumor. Pediatr Pathol Lab Med. 1996;16:161–9.
29. de Alava E, Lozano MD, Sola I, et al. Molecular features in a biphenotypic small cell sarcoma with neuroectodermal and muscle differentiation. Hum Pathol. 1998;29:181–4.
30. Hattinger CM, Rumpler S, Kovar H, et al. Fine-mapping of cytogenetically undetectable EWS/ERG fusions on DNA fibers of Ewing tumors. Cytogenet Cell Genet. 2001;93:29–35.
31. Sainati L, Scapinello A, Montaldi A, et al. A mesenchymal chondrosarcoma of a child with the reciprocal translocation (11;22)(q24;q12). Cancer Genet Cytogenet. 1993;71:144–7.
32. Fritsch MK, Bridge JA, Schuster AE, et al. Performance characteristics of a reverse transcriptase-polymerase chain reaction assay for the detection of tumor-specific fusion transcripts from archival tissue. Pediatr Dev Pathol. 2003;6:43–53.
33. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature. 1998;396:643–9.
34. Michor F, Iwasa Y, Nowak MA. Dynamics of cancer progression. Nat Rev Cancer. 2004;4:197–205.
35. Ladanyi M, Bridge JA. Contribution of molecular genetic data to the classification of sarcomas. Hum Pathol. 2000;31:532–8.
36. Hill DA, O'Sullivan MJ, Zhu X, et al. Practical application of molecular genetic testing as an aid to the surgical pathologic diagnosis of sarcomas: a prospective study. Am J Surg Pathol. 2002;26:965–77.
37. Pfeifer JD, Hill DA, O'Sullivan MJ, et al. Diagnostic gold standard for soft tissue tumours: morphology or molecular genetics? Histopathology. 2000;37:485–500.
38. Peter M, Gilbert E, Delattre O. A multiplex real-time pcr assay for the detection of gene fusions observed in solid tumors. Lab Invest. 2001;81:905–12.
39. Greer CE, Peterson SL, Kiviat NB, et al. PCR amplification from paraffin-embedded tissues. Effects of fixative and fixation time. Am J Clin Pathol. 1991;95:117–24.

40. Jin L, Majerus J, Oliveira A, et al. Detection of fusion gene transcripts in fresh-frozen and formalin-fixed paraffin-embedded tissue sections of soft-tissue sarcomas after laser capture microdissection and rt-PCR. Diagn Mol Pathol. 2003;12:224–30.
41. Yoshida H, Nagao K, Ito H, et al. Chromosomal translocations in human soft tissue sarcomas by interphase fluorescence in situ hybridization. Pathol Int. 1997;47:222–9.
42. Kumar S, Pack S, Kumar D, et al. Detection of EWS-FLI-1 fusion in Ewing's sarcoma/peripheral primitive neuroectodermal tumor by fluorescence in situ hybridization using formalin-fixed paraffin-embedded tissue. Hum Pathol. 1999;30:324–30.
43. Gardner LJ, Ayala AG, Monforte HL, et al. Ewing sarcoma/peripheral primitive neuroectodermal tumor: adult abdominal tumors with an Ewing sarcoma gene rearrangement demonstrated by fluorescence in situ hybridization in paraffin sections. Appl Immunohistochem Mol Morphol. 2004;12:160–5.
44. Qian X, Jin L, Shearer BM, Ketterling RP, et al. Molecular diagnosis of Ewing's sarcoma/primitive neuroectodermal tumor in formalin-fixed paraffin-embedded tissues by RT-PCR and fluorescence in situ hybridization. Diagn Mol Pathol. 2005;14:23–8.
45. Bridge RS, Rajaram V, Dehner LP, et al. Molecular diagnosis of Ewing sarcoma/primitive neuroectodermal tumor in routinely processed tissue: a comparison of two FISH strategies and RT-PCR in malignant round cell tumors. Mod Pathol. 2006;19:1–8.
46. Zoubek A, Dockhorn-Dworniczak B, Delattre O, et al. Does expression of different EWS chimeric transcripts define clinically distinct risk groups of Ewing tumor patients? J Clin Oncol. 1996;14:1245–51.
47. de Alava E, Kawai A, Healey JH, et al. EWS-FLI1 fusion transcript structure is an independent determinant of prognosis in Ewing's sarcoma. J Clin Oncol. 1998;16:1248–55.
48. West DC, Grier HE, Swallow MM, et al. Detection of circulating tumor cells in patients with Ewing's sarcoma and peripheral primitive neuroectodermal tumor. J Clin Oncol. 1997;15:583–8.
49. Zoubek A, Ladenstein R, Windhager R, et al. Predictive potential of testing for bone marrow involvement in Ewing tumor patients by RT-PCR: a preliminary evaluation. Int J Cancer. 1998;79:56–60.
50. Fagnou C, Michon J, Peter M, et al. Presence of tumor cells in bone marrow but not in blood is associated with adverse prognosis in patients with Ewing's tumor. Societe Francaise d'Oncologie Pediatrique. J Clin Oncol. 1998;16:1707–11.
51. de Alava E, Ladanyi M, Rosai J, et al. Detection of chimeric transcripts in desmoplastic small round cell tumor and related developmental tumors by reverse transcriptase polymerase chain reaction. A specific diagnostic assay. Am J Pathol. 1995;147:1584–91.
52. Zoubek A, Kovar H, Kronberger M, et al. Mobilization of tumour cells during biopsy in an infant with Ewing sarcoma. Eur J Pediatr. 1996;155:373–6.
53. Mhawech-Fauceglia P, Herrmann F, Penetrante R, et al. Diagnostic utility of FLI-1 monoclonal antibody and dual-colour, break-apart probe fluorescence in situ (FISH) analysis in Ewing's sarcoma/primitive neuroectodermal tumour (EWS/PNET). A comparative study with CD99 and FLI-1 polyclonal antibodies. Histopathology. 2006;49:569–75.
54. Llombart B, Monteagudo C, Lopez-Guerrero JA, et al. Clinicopathological and immunohistochemical analysis of 20 cases of Merkel cell carcinoma in search of prognostic markers. Histopathology. 2005;46:622–34.
55. Folpe AL, Chand EM, Goldblum JR, et al. Expression of Fli-1, a nuclear transcription factor, distinguishes vascular neoplasms from potential mimics. Am J Surg Pathol. 2001;25:1061–6.
56. Rossi S, Orvieto E, Furlanetto A, et al. Utility of the immunohistochemical detection of FLI-1 expression in round cell and vascular neoplasm using a monoclonal antibody. Mod Pathol. 2004;17:547–52.
57. Ragsdale BD, Lee JP, Mines J. Alveolar rhabdomyosarcoma on the external ear: a case report. J Cutan Pathol. 2009;36:267–9.
58. Nakagawa N, Tsuda T, Yamamoto M, et al. Adult cutaneous alveolar rhabdomyosarcoma on the face diagnosed by the expression of PAX3-FKHR gene fusion transcripts. J Dermatol. 2008;35:462–7.
59. Brecher AR, Reyes-Mugica M, Kamino H, et al. Congenital primary cutaneous rhabdomyosarcoma in a neonate. Pediatr Dermatol. 2003;20:335–8.
60. Gong Y, Chao J, Bauer B, et al. Primary cutaneous alveolar rhabdomyosarcoma of the perineum. Arch Pathol Lab Med. 2002;126:982–4.
61. Setterfield J, Sciot R, Debiec-Rychter M, et al. Primary cutaneous epidermotropic alveolar rhabdomyosarcoma with t(2;13) in an elderly woman: case report and review of the literature. Am J Surg Pathol. 2002;26:938–44.
62. Kuroiwa M, Sakamoto J, Shimada A, et al. Manifestation of alveolar rhabdomyosarcoma as primary cutaneous lesions in a neonate with Beckwith-Wiedemann syndrome. J Pediatr Surg. 2009;44:e31–5.
63. Godambe SV, Rawal J. Blueberry muffin rash as a presentation of alveolar cell rhabdomyosarcoma in a neonate. Acta Paediatr. 2000;89:115–7.
64. Shapiro DN, Sublett JE, Li B, et al. Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. Cancer Res. 1993;53:5108–12.

65. Galili N, Davis RJ, Fredericks WJ, et al. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet. 1993;5:230–5.
66. Davis RJ, D'Cruz CM, Lovell MA, et al. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. Cancer Res. 1994;54:2869–72.
67. Davis RJ, Bennicelli JL, Macina RA, et al. Structural characterization of the FKHR gene and its rearrangement in alveolar rhabdomyosarcoma. Hum Mol Genet. 1995;4:2355–62.
68. Barr FG, Qualman SJ, Macris MH, et al. Genetic heterogeneity in the alveolar rhabdomyosarcoma subset without typical gene fusions. Cancer Res. 2002;62:4704–10.
69. Wachtel M, Dettling M, Koscielniak E, et al. Gene expression signatures identify rhabdomyosarcoma subtypes and detect a novel t(2;2)(q35;p23) translocation fusing PAX3 to NCOA1. Cancer Res. 2004;64:5539–45.
70. Bennicelli JL, Edwards RH, Barr FG. Mechanism for transcriptional gain of function resulting from chromosomal translocation in alveolar rhabdomyosarcoma. Proc Natl Acad Sci USA. 1996;93:5455–9.
71. Downing JR, Khandekar A, Shurtleff SA, et al. Multiplex RT-PCR assay for the differential diagnosis of alveolar rhabdomyosarcoma and Ewing's sarcoma. Am J Pathol. 1995;146:626–34.
72. Dockhorn-Dworniczak B, Schafer KL, Blasius S, et al. Assessment of molecular genetic detection of chromosome translocations in the differential diagnosis of pediatric sarcomas. Klin Padiatr. 1997;209:156–64.
73. Barr FG, Xiong QB, Kelly K. A consensus polymerase chain reaction-oligonucleotide hybridization approach for the detection of chromosomal translocations in pediatric bone and soft tissue sarcomas. Am J Clin Pathol. 1995;104:627–33.
74. Biegel JA, Nycum LM, Valentine V, et al. Detection of the t(2;13)(q35;q14) and PAX3-FKHR fusion in alveolar rhabdomyosarcoma by fluorescence in situ hybridization. Genes Chromosomes Cancer. 1995;12:186–92.
75. McManus AP, O'Reilly MA, Jones KP, et al. Interphase fluorescence in situ hybridization detection of t(2;13)(q35;q14) in alveolar rhabdomyosarcoma–a diagnostic tool in minimally invasive biopsies. J Pathol. 1996;178:410–4.
76. Mehra S, de la Roza G, Tull J, et al. Detection of FOXO1 (FKHR) gene break-apart by fluorescence in situ hybridization in formalin-fixed, paraffin-embedded alveolar rhabdomyosarcomas and its clinicopathologic correlation. Diagn Mol Pathol. 2008;17:14–20.
77. Barr FG, Chatten J, D'Cruz CM, et al. Molecular assays for chromosomal translocations in the diagnosis of pediatric soft tissue sarcomas. JAMA. 1995;273:553–7.
78. Anderson J, Gordon T, McManus A, et al. Detection of the PAX3-FKHR fusion gene in paediatric rhabdomyosarcoma: a reproducible predictor of outcome? Br J Cancer. 2001;85:831–5.
79. Sorensen PH, Lynch JC, Qualman SJ, et al. PAX3-FKHR and PAX7-FKHR gene fusions are prognostic indicators in alveolar rhabdomyosarcoma: a report from the children's oncology group. J Clin Oncol. 2002;20:2672–9.
80. Kelly KM, Womer RB, Sorensen PH, et al. Common and variant gene fusions predict distinct clinical phenotypes in rhabdomyosarcoma. J Clin Oncol. 1997;15:1831–6.
81. Kelly KM, Womer RB, Barr FG. Minimal disease detection in patients with alveolar rhabdomyosarcoma using a reverse transcriptase-polymerase chain reaction method. Cancer. 1996;78:1320–7.
82. Fukuda T, Kakihara T, Baba K, et al. Clear cell sarcoma arising in the transverse colon. Pathol Int. 2000;50:412–6.
83. Kindblom LG, Lodding P, Angervall L. Clear-cell sarcoma of tendons and aponeuroses. An immunohistochemical and electron microscopic analysis indicating neural crest origin. Virchows Arch A Pathol Anat Histopathol. 1983;401:109–28.
84. Pauwels P, Debiec-Rychter M, Sciot R, et al. Clear cell sarcoma of the stomach. Histopathology. 2002;41:526–30.
85. Rubin BP, Fletcher JA, Renshaw AA. Clear cell sarcoma of soft parts: report of a case primary in the kidney with cytogenetic confirmation. Am J Surg Pathol. 1999;23:589–94.
86. Saw D, Tse CH, Chan J, et al. Clear cell sarcoma of the penis. Hum Pathol. 1986;17:423–5.
87. Ansai S, Takeda H, Koseki S, et al. A patient with rhabdomyosarcoma and clear cell sarcoma of the skin. J Am Acad Dermatol. 1994;31:871–6.
88. Bridge JA, Borek DA, Neff JR, et al. Chromosomal abnormalities in clear cell sarcoma. Implications for histogenesis. Am J Clin Pathol. 1990;93:26–31.
89. Reeves BR, Fletcher CD, Gusterson BA. Translocation t(12;22)(q13;q13) is a nonrandom rearrangement in clear cell sarcoma. Cancer Genet Cytogenet. 1992;64:101–3.
90. Rodriguez E, Sreekantaiah C, Reuter VE, et al. t(12;22)(q13;q13) and trisomy 8 are nonrandom aberrations in clear-cell sarcoma. Cancer Genet Cytogenet. 1992;64:107–10.
91. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors. Dermatofibrosarcoma protuberans and giant cell fibroblastoma. Cancer Genet Cytogenet. 2003;140:1–12.
92. Hisaoka M, Ishida T, Kuo TT, et al. Clear cell sarcoma of soft tissue: a clinicopathologic, immunohistochemical, and molecular analysis of 33 cases. Am J Surg Pathol. 2008;32:452–60.

93. Wang WL, Mayordomo E, Zhang W, et al. Detection and characterization of EWSR1/ATF1 and EWSR1/CREB1 chimeric transcripts in clear cell sarcoma (melanoma of soft parts). Mod Pathol. 2009;22:1201–9.
94. Antonescu CR, Nafa K, Segal NH, et al. EWS-CREB1: a recurrent variant fusion in clear cell sarcoma–association with gastrointestinal location and absence of melanocytic differentiation. Clin Cancer Res. 2006;12:5356–62.
95. Limon J, Debiec-Rychter M, Nedoszytko B, et al. Aberrations of chromosome 22 and polysomy of chromosome 8 as non-random changes in clear cell sarcoma. Cancer Genet Cytogenet. 1994;72:141–5.
96. Travis JA, Bridge JA. Significance of both numerical and structural chromosomal abnormalities in clear cell sarcoma. Cancer Genet Cytogenet. 1992;64:104–6.
97. Brown AD, Lopez-Terrada D, Denny C, et al. Promoters containing ATF-binding sites are de-regulated in cells that express the EWS/ATF1 oncogene. Oncogene. 1995;10:1749–56.
98. Fujimura Y, Ohno T, Siddique H, et al. The EWS-ATF-1 gene involved in malignant melanoma of soft parts with t(12;22) chromosome translocation, encodes a constitutive transcriptional activator. Oncogene. 1996;12:159–67.
99. Li KK, Lee KA. MMSP tumor cells expressing the EWS/ATF1 oncogene do not support cAMP-inducible transcription. Oncogene. 1998;16:1325–31.
100. Antonescu CR, Dal Cin P, Nafa K, et al. EWSR1-CREB1 is the predominant gene fusion in angiomatoid fibrous histiocytoma. Genes Chromosomes Cancer. 2007;46:1051–60.
101. Antonescu CR, Tschernyavsky SJ, Woodruff JM, et al. Molecular diagnosis of clear cell sarcoma: detection of EWS-ATF1 and MITF-M transcripts and histopathological and ultrastructural analysis of 12 cases. J Mol Diagn. 2002;4:44–52.
102. Covinsky M, Gong S, Rajaram V, et al. EWS-ATF1 fusion transcripts in gastrointestinal tumors previously diagnosed as malignant melanoma. Hum Pathol. 2005;36:74–81.
103. Zambrano E, Reyes-Mugica M, Franchi A, et al. An osteoclast-rich tumor of the gastrointestinal tract with features resembling clear cell sarcoma of soft parts: reports of 6 cases of a GIST simulator. Int J Surg Pathol. 2003;11:75–81.
104. Panagopoulos I, Mertens F, Debiec-Rychter M, et al. Molecular genetic characterization of the EWS/ATF1 fusion gene in clear cell sarcoma of tendons and aponeuroses. Int J Cancer. 2002;99:560–7.
105. Pellin A, Monteagudo C, Lopez-Gines C, et al. New type of chimeric fusion product between the EWS and ATFI genes in clear cell sarcoma (malignant melanoma of soft parts). Genes Chromosomes Cancer. 1998;23:358–60.
106. Speleman F, Delattre O, Peter M, et al. Malignant melanoma of the soft parts (clear-cell sarcoma): confirmation of EWS and ATF-1 gene fusion caused by a t(12;22) translocation. Mod Pathol. 1997;10:496–9.
107. Zucman J, Delattre O, Desmaze C, et al. EWS and ATF-1 gene fusion induced by t(12;22) translocation in malignant melanoma of soft parts. Nat Genet. 1993;4:341–5.
108. Parham DM, Weeks DA, Beckwith JB. The clinicopathologic spectrum of putative extrarenal rhabdoid tumors. An analysis of 42 cases studied with immunohistochemistry or electron microscopy. Am J Surg Pathol. 1994;18:1010–29.
109. Wick MR, Ritter JH, Dehner LP. Malignant rhabdoid tumors: a clinicopathologic review and conceptual discussion. Semin Diagn Pathol. 1995;12:233–48.
110. Sert MB, Onsrud M, Perrone T, et al. Malignant rhabdoid tumor of the vulva. Case report. Eur J Gynaecol Oncol. 1999;20:258–61.
111. Dabbs DJ, Park HK. Malignant rhabdoid skin tumor: an uncommon primary skin neoplasm. Ultrastructural and immunohistochemical analysis. J Cutan Pathol. 1988;15:109–15.
112. Petitt M, Doeden K, Harris A, et al. Cutaneous extrarenal rhabdoid tumor with myogenic differentiation. J Cutan Pathol. 2005;32:690–5.
113. Boscaino A, Donofrio V, Tornillo L, et al. Primary rhabdoid tumour of the skin in a 14-month-old child. Dermatology. 1994;188:322–5.
114. Perlman EJ, Ali SZ, Robinson R, et al. Infantile extrarenal rhabdoid tumor. Pediatr Dev Pathol. 1998;1:149–52.
115. Versteege I, Sevenet N, Lange J, et al. Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. Nature. 1998;394:203–6.
116. Biegel JA, Tan L, Zhang F, et al. Alterations of the hSNF5/INI1 gene in central nervous system atypical teratoid/rhabdoid tumors and renal and extrarenal rhabdoid tumors. Clin Cancer Res. 2002;8:3461–7.
117. Roberts CW, Orkin SH. The SWI/SNF complex–chromatin and cancer. Nat Rev Cancer. 2004;4:133–42.
118. Roberts CW, Biegel JA. The role of SMARCB1/INI1 in development of rhabdoid tumor. Cancer Biol Ther. 2009;8:412–6.
119. Lee HY, Yoon CS, Sevenet N, et al. Rhabdoid tumor of the kidney is a component of the rhabdoid predisposition syndrome. Pediatr Dev Pathol. 2002;5:395–9.
120. Sevenet N, Sheridan E, Amram D, et al. Constitutional mutations of the hSNF5/INI1 gene predispose to a variety of cancers. Am J Hum Genet. 1999;65:1342–8.
121. Rosty C, Peter M, Zucman J, et al. Cytogenetic and molecular analysis of a t(1;22)(p36;q11.2) in a rhabdoid tumor with a putative homozygous deletion of chromosome 22. Genes Chromosomes Cancer. 1998;21:82–9.

122. Rousseau-Merck MF, Versteege I, Legrand I, et al. hSNF5/INI1 inactivation is mainly associated with homozygous deletions and mitotic recombinations in rhabdoid tumors. Cancer Res. 1999;59:3152–6.
123. Kusafuka T, Miao J, Yoneda A, et al. Novel germ-line deletion of SNF5/INI1/SMARCB1 gene in neonate presenting with congenital malignant rhabdoid tumor of kidney and brain primitive neuroectodermal tumor. Genes Chromosomes Cancer. 2004;40:133–9.
124. Hoot AC, Russo P, Judkins AR, et al. Immunohistochemical analysis of hSNF5/INI1 distinguishes renal and extra-renal malignant rhabdoid tumors from other pediatric soft tissue tumors. Am J Surg Pathol. 2004;28:1485–91.
125. Kohashi K, Oda Y, Yamamoto H, et al. SMARCB1/INI1 protein expression in round cell soft tissue sarcomas associated with chromosomal translocations involving EWS: a special reference to SMARCB1/INI1 negative variant extraskeletal myxoid chondrosarcoma. Am J Surg Pathol. 2008;32:1168–74.
126. Pai KK, Pai SB, Sripathi H, et al. Epithelioid sarcoma: a diagnostic challenge. Indian J Dermatol Venereol Leprol. 2006;72:446–8.
127. Hasegawa T, Matsuno Y, Shimoda T, et al. Proximal-type epithelioid sarcoma: a clinicopathologic study of 20 cases. Mod Pathol. 2001;14:655–63.
128. Guillou L, Wadden C, Coindre JM, et al. "Proximal-type" epithelioid sarcoma, a distinctive aggressive neoplasm showing rhabdoid features. Clinicopathologic, immunohistochemical, and ultrastructural study of a series. Am J Surg Pathol. 1997;21:130–46.
129. Argenta PA, Thomas S, Chura JC. Proximal-type epithelioid sarcoma vs malignant rhabdoid tumor of the vulva: a case report, review of the literature, and an argument for consolidation. Gynecol Oncol. 2007;107:130–5.
130. Lualdi E, Modena P, Debiec-Rychter M, et al. Molecular cytogenetic characterization of proximal-type epithelioid sarcoma. Genes Chromosomes Cancer. 2004;41:283–90.
131. Modena P, Lualdi E, Facchinetti F, et al. SMARCB1/INI1 tumor suppressor gene is frequently inactivated in epithelioid sarcomas. Cancer Res. 2005;65:4012–9.
132. Kohashi K, Izumi T, Oda Y, et al. Infrequent SMARCB1/INI1 gene alteration in epithelioid sarcoma: a useful tool in distinguishing epithelioid sarcoma from malignant rhabdoid tumor. Hum Pathol. 2009;40:349–55.
133. Flucke U, Slootweg PJ, Mentzel T, et al. Direct evidence of mutational inactivation of SMARCB1/INI1 in epithelioid sarcoma. Hum Pathol. 2009;40:1361–2. Correspondence – author reply 2–4.
134. Kohashi K, Oda Y, Tsunoda T. Direct evidence of mutational inactivation of SMARCB1/INI1 in epithelioid sarcoma-Reply. Hum Pathol. 2009;40:1362–4.
135. Orrock JM, Abbott JJ, Gibson LE, et al. INI1 and GLUT-1 expression in epithelioid sarcoma and its cutaneous neoplastic and nonneoplastic mimics. Am J Dermatopathol. 2009;31:152–6.
136. Hornick JL, Dal Cin P, Fletcher CD. Loss of INI1 expression is characteristic of both conventional and proximal-type epithelioid sarcoma. Am J Surg Pathol. 2009;33:542–50.
137. Kacerovska D, Michal M, Nemcova J, et al. Crystal-deficient alveolar soft-part sarcoma with cutaneous involvement: a case report. Am J Dermatopathol. 2009;31:272–7.
138. Moyano S, Aguilera P, Petit A, et al. Alveolar soft part sarcoma presenting with cutaneous metastases: report of a case with immunohistochemical and molecular characterization. J Am Acad Dermatol. 2009;61:117–20.
139. Ladanyi M, Lui MY, Antonescu CR, et al. The der(17)t(X;17)(p11;q25) of human alveolar soft part sarcoma fuses the TFE3 transcription factor gene to ASPL, a novel gene at 17q25. Oncogene. 2001;20:48–57.
140. Heimann P, El Housni H, Ogur G, et al. Fusion of a novel gene, RCC17, to the TFE3 gene in t(X;17)(p11.2;q25.3)-bearing papillary renal cell carcinomas. Cancer Res. 2001;61:4130–5.
141. Sandberg AA, Bridge JA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: alveolar soft part sarcoma. Cancer Genet Cytogenet. 2002;136:1–9.
142. Weterman MA, van Groningen JJ, Jansen A, et al. Nuclear localization and transactivating capacities of the papillary renal cell carcinoma-associated TFE3 and PRCC (fusion) proteins. Oncogene. 2000;19:69–74.
143. Argani P, Antonescu CR, Illei PB, et al. Primary renal neoplasms with the ASPL-TFE3 gene fusion of alveolar soft part sarcoma: a distinctive tumor entity previously included among renal cell carcinomas of children and adolescents. Am J Pathol. 2001;159:179–92.
144. Argani P, Lal P, Hutchinson B, et al. Aberrant nuclear immunoreactivity for TFE3 in neoplasms with TFE3 gene fusions: a sensitive and specific immunohistochemical assay. Am J Surg Pathol. 2003;27:750–61.
145. Pawel BR, Hamoudi AB, Asmar L, et al. Undifferentiated sarcomas of children: pathology and clinical behavior–an Intergroup Rhabdomyosarcoma study. Med Pediatr Oncol. 1997;29:170–80.
146. Alaggio R, Bisogno G, Rosato A, et al. Undifferentiated sarcoma: does it exist? A clinicopathologic study of 7 pediatric cases and review of literature. Hum Pathol. 2009;40:1600–10.
147. Kempson RL, Hendrickson MR. An approach to the diagnosis of soft tissue tumors. Monogr Pathol. 1996;38:1–36.
148. Tang CK, Toker C. Trabecular carcinoma of the skin: an ultrastructural study. Cancer. 1978;42:2311–21.
149. Plaza JA, Suster S. The Toker tumor: spectrum of morphologic features in primary neuroendocrine carcinomas of the skin (Merkel cell carcinoma). Ann Diagn Pathol. 2006;10:376–85.

150. Sibley RK, Dahl D. Primary neuroendocrine (Merkel cell?) carcinoma of the skin. II. An immunocytochemical study of 21 cases. Am J Surg Pathol. 1985;9:109–16.
151. LeBoit PE, Burg G, Weedon D, Sarasin A. Pathology and genetics of skin tumors: World Health Organization. Lyon: IARC Press; 2006.
152. Feng H, Shuda M, Chang Y, et al. Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science. 2008;319:1096–100.
153. Bhatia K, Goedert JJ, Modali R, et al. Immunological detection of viral large T antigen identifies a subset of merkel cell carcinoma tumors with higher viral abundance and better clinical outcome. Int J Cancer. 2010;127:1493–6.
154. zur Hausen H. A specific signature of Merkel cell polyomavirus persistence in human cancer cells. Proc Natl Acad Sci USA. 2008;105:16063–4.
155. Giraud G, Ramqvist T, Ragnarsson-Olding B, et al. DNA from BK virus and JC virus and from KI, WU, and MC polyomaviruses as well as from simian virus 40 is not detected in non-UV-light-associated primary malignant melanomas of mucous membranes. J Clin Microbiol. 2008;46:3595–8.
156. Andres C, Belloni B, Puchta U, et al. Is Merkel cell polyomavirus also prevalent in non-Merkel cell carcinoma (MCC) tumors of sun exposed skin? A study of 66 patinets. J Cutan Pathol. 2010;37:28–34.
157. Katano H, Ito H, Suzuki Y, et al. Detection of Merkel cell polyomavirus in Merkel cell carcinoma and Kaposi's sarcoma. J Med Virol. 2009;81:1951–8.
158. Bluemn EG, Paulson KG, Higgins EE, et al. Merkel cell polyomavirus is not detected in prostate cancers, surrounding stroma, or benign prostate controls. J Clin Virol. 2009;44:164–6.
159. Sastre-Garau X, Peter M, Avril MF, et al. Merkel cell carcinoma of the skin: pathological and molecular evidence for a causative role of MCV in oncogenesis. J Pathol. 2009;218:48–56.
160. Duncavage EJ, Le BM, Wang D, et al. Merkel cell polyomavirus: a specific marker for Merkel cell carcinoma in histologically similar tumors. Am J Surg Pathol. 2009;33:1771–7.
161. Andres C, Ihrler S, Puchta U, et al. Merkel cell polymavirus is prevalent in a subset of small cell lung cancer: a study of 31 patients. Thorax. 2009;64:1007–8.
162. Duncavage EJ, Zehnbauer BA, Pfeifer JD. Prevalence of Merkel cell polyomavirus in Merkel cell carcinoma. Mod Pathol. 2009;22:516–21.
163. Mentzel T, Beham A, Katenkamp D, et al. Fibrosarcomatous ("high-grade") dermatofibrosarcoma protuberans: clinicopathologic and immunohistochemical study of a series of 41 cases with emphasis on prognostic significance. Am J Surg Pathol. 1998;22:576–87.
164. Pedeutour F, Simon MP, Minoletti F, et al. Translocation, t(17;22)(q22;q13), in dermatofibrosarcoma protuberans: a new tumor-associated chromosome rearrangement. Cytogenet Cell Genet. 1996;72:171–4.
165. Pedeutour F, Simon MP, Minoletti F, et al. Ring 22 chromosomes in dermatofibrosarcoma protuberans are low-level amplifiers of chromosome 17 and 22 sequences. Cancer Res. 1995;55:2400–3.
166. Simon MP, Pedeutour F, Sirvent N, et al. Deregulation of the platelet-derived growth factor B-chain gene via fusion with collagen gene COL1A1 in dermatofibrosarcoma protuberans and giant-cell fibroblastoma. Nat Genet. 1997;15:95–8.
167. O'Brien KP, Seroussi E, Dal Cin P, et al. Various regions within the alpha-helical domain of the COL1A1 gene are fused to the second exon of the PDGFB gene in dermatofibrosarcomas and giant-cell fibroblastomas. Genes Chromosomes Cancer. 1998;23:187–93.
168. Abbott JJ, Erickson-Johnson M, Wang X, et al. Gains of COL1A1-PDGFB genomic copies occur in fibrosarcomatous transformation of dermatofibrosarcoma protuberans. Mod Pathol. 2006;19:1512–8.
169. Sirvent N, Maire G, Pedeutour F. Genetics of dermatofibrosarcoma protuberans family of tumors: from ring chromosomes to tyrosine kinase inhibitor treatment. Genes Chromosomes Cancer. 2003;37:1–19.
170. Dirks RP, Jansen HJ, Onnekink C, et al. DNase-I-hypersensitive sites located far upstream of the human c-sis/PDGF-B gene comap with transcriptional enhancers and a silencer and are preceded by (part of) a new transcription unit. Eur J Biochem. 1993;216:487–95.
171. Rao CD, Pech M, Robbins KC, et al. The 5′ untranslated sequence of the c-sis/platelet-derived growth factor 2 transcript is a potent translational inhibitor. Mol Cell Biol. 1988;8:284–92.
172. Greco A, Fusetti L, Villa R, et al. Transforming activity of the chimeric sequence formed by the fusion of collagen gene COL1A1 and the platelet derived growth factor b-chain gene in dermatofibrosarcoma protuberans. Oncogene. 1998;17:1313–9.
173. Wang J, Hisaoka M, Shimajiri S, et al. Detection of COL1A1-PDGFB fusion transcripts in dermatofibrosarcoma protuberans by reverse transcription-polymerase chain reaction using archival formalin-fixed, paraffin-embedded tissues. Diagn Mol Pathol. 1999;8:113–9.
174. Maire G, Pedeutour F, Coindre JM. COL1A1-PDGFB gene fusion demonstrates a common histogenetic origin for dermatofibrosarcoma protuberans and its granular cell variant. Am J Surg Pathol. 2002;26:932–7.
175. Wang J, Morimitsu Y, Okamoto S, et al. COL1A1-PDGFB fusion transcripts in fibrosarcomatous areas of six dermatofibrosarcomas protuberans. J Mol Diagn. 2000;2:47–52.

176. Dal Cin P, Sciot R, de Wever I, et al. Cytogenetic and immunohistochemical evidence that giant cell fibroblastoma is related to dermatofibrosarcoma protuberans. Genes Chromosomes Cancer. 1996;15:73–5.
177. Maire G, Martin L, Michalak-Provost S, et al. Fusion of COL1A1 exon 29 with PDGFB exon 2 in a der(22) t(17;22) in a pediatric giant cell fibroblastoma with a pigmented Bednar tumor component. Evidence for age-related chromosomal pattern in dermatofibrosarcoma protuberans and related tumors. Cancer Genet Cytogenet. 2002;134:156–61.
178. Sheng WQ, Hashimoto H, Okamoto S, et al. Expression of COL1A1-PDGFB fusion transcripts in superficial adult fibrosarcoma suggests a close relationship to dermatofibrosarcoma protuberans. J Pathol. 2001;194:88–94.
179. Vanni R, Faa G, Dettori T, et al. A case of dermatofibrosarcoma protuberans of the vulva with a COL1A1/PDGFB fusion identical to a case of giant cell fibroblastoma. Virchows Arch. 2000;437:95–100.
180. Patel KU, Szabo SS, Hernandez VS, et al. Dermatofibrosarcoma protuberans COL1A1-PDGFB fusion is identified in virtually all dermatofibrosarcoma protuberans cases when investigated by newly developed multiplex reverse transcription polymerase chain reaction and fluorescence in situ hybridization assays. Hum Pathol. 2008;39:184–93.
181. McArthur GA, Demetri GD, van Oosterom A, et al. Molecular and clinical analysis of locally advanced dermatofibrosarcoma protuberans treated with imatinib: Imatinib Target Exploration Consortium Study B2225. J Clin Oncol. 2005;23:866–73.
182. Gokden N, Dehner LP, Zhu X, et al. Dermatofibrosarcoma protuberans of the vulva and groin: detection of COL1A1-PDGFB fusion transcripts by RT-PCR. J Cutan Pathol. 2003;30:190–5.
183. Maki RG, Awan RA, Dixon RH, et al. Differential sensitivity to imatinib of 2 patients with metastatic sarcoma arising from dermatofibrosarcoma protuberans. Int J Cancer. 2002;100:623–6.
184. Rubin BP, Schuetze SM, Eary JF, et al. Molecular targeting of platelet-derived growth factor B by imatinib mesylate in a patient with metastatic dermatofibrosarcoma protuberans. J Clin Oncol. 2002;20:3586–91.
185. McArthur G. Dermatofibrosarcoma protuberans: recent clinical progress. Ann Surg Oncol. 2007;14:2876–86.
186. Coffin CM, Watterson J, Priest JR, et al. Extrapulmonary inflammatory myofibroblastic tumor (inflammatory pseudotumor). A clinicopathologic and immunohistochemical study of 84 cases. Am J Surg Pathol. 1995;19:859–72.
187. Vadmal MS, Pellegrini AE. Inflammatory myofibroblastic tumor of the skin. Am J Dermatopathol. 1999;21:449–53.
188. Frey J, Huerter C, Shehan J. Inflammatory pseudotumor of the skin: a case report and review of the literature. Internet J Dermatol. 2007;6(1).
189. Griffin CA, Hawkins AL, Dvorak C, et al. Recurrent involvement of 2p23 in inflammatory myofibroblastic tumors. Cancer Res. 1999;59:2776–80.
190. Lamant L, Gascoyne RD, Duplantier MM, et al. Non-muscle myosin heavy chain (MYH9): a new partner fused to ALK in anaplastic large cell lymphoma. Genes Chromosomes Cancer. 2003;37:427–32.
191. Debiec-Rychter M, Marynen P, Hagemeijer A, et al. ALK-ATIC fusion in urinary bladder inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2003;38:187–90.
192. Lawrence B, Perez-Atayde A, Hibbard MK, et al. TPM3-ALK and TPM4-ALK oncogenes in inflammatory myofibroblastic tumors. Am J Pathol. 2000;157:377–84.
193. Bridge JA, Kanamori M, Ma Z, et al. Fusion of the ALK gene to the clathrin heavy chain gene, CLTC, in inflammatory myofibroblastic tumor. Am J Pathol. 2001;159:411–5.
194. Reading NS, Jenson SD, Smith JK, et al. 5′-(RACE) identification of rare ALK fusion partner in anaplastic large cell lymphoma. J Mol Diagn. 2003;5:136–40.
195. Coffin CM, Patel A, Perkins S, et al. ALK1 and p80 expression and chromosomal rearrangements involving 2p23 in inflammatory myofibroblastic tumor. Mod Pathol. 2001;14:569–76.
196. Chan JK, Cheuk W, Shimizu M. Anaplastic lymphoma kinase expression in inflammatory pseudotumors. Am J Surg Pathol. 2001;25:761–8.
197. Cook JR, Dehner LP, Collins MH, et al. Anaplastic lymphoma kinase (ALK) expression in the inflammatory myofibroblastic tumor: a comparative immunohistochemical study. Am J Surg Pathol. 2001;25:1364–71.
198. Kazmierczak B, Dal Cin P, Sciot R, et al. Inflammatory myofibroblastic tumor with HMGIC rearrangement. Cancer Genet Cytogenet. 1999;112:156–60.
199. Chan JK, Buchanan R, Fletcher CD. Sarcomatoid variant of anaplastic large-cell Ki-1 lymphoma. Am J Surg Pathol. 1990;14:983–8.
200. Stein H, Foss HD, Durkop H, et al. CD30(+) anaplastic large cell lymphoma: a review of its histopathologic, genetic, and clinical features. Blood. 2000;96:3681–95.
201. Suzuki R, Seto M, Nakamura S, et al. Sarcomatoid variant of anaplastic large cell lymphoma with cytoplasmic ALK and alpha-smooth muscle actin expression: a mimic of inflammatory myofibroblastic tumor. Am J Pathol. 2001;159:383–4.
202. Cools J, Wlodarska I, Somers R, et al. Identification of novel fusion partners of ALK, the anaplastic lymphoma kinase, in anaplastic large-cell lymphoma and inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2002;34:354–62.

203. Ma Z, Hill DA, Collins MH, et al. Fusion of ALK to the Ran-binding protein 2 (RANBP2) gene in inflammatory myofibroblastic tumor. Genes Chromosomes Cancer. 2003;37:98–105.
204. Kapusta LR, Weiss MA, Ramsay J, et al. Inflammatory myofibroblastic tumors of the kidney: a clinicopathologic and immunohistochemical study of 12 cases. Am J Surg Pathol. 2003;27:658–66.
205. Cessna MH, Zhou H, Sanger WG, et al. Expression of ALK1 and p80 in inflammatory myofibroblastic tumor and its mesenchymal mimics: a study of 135 cases. Mod Pathol. 2002;15:931–8.
206. Koletsa T, Hytiroglou P, Semoglou C, et al. Angiomatoid fibrous histiocytoma with cystic structures of sweat duct origin. Pathol Int. 2007;57:513–6.
207. Rossi S, Szuhai K, Ijszenga M, et al. EWSR1-CREB1 and EWSR1-ATF1 fusion genes in angiomatoid fibrous histiocytoma. Clin Cancer Res. 2007;13:7322–8.
208. Waters BL, Panagopoulos I, Allen EF. Genetic characterization of angiomatoid fibrous histiocytoma identifies fusion of the FUS and ATF-1 genes induced by a chromosomal translocation involving bands 12q13 and 16p11. Cancer Genet Cytogenet. 2000;121:109–16.
209. Raddaoui E, Donner LR, Panagopoulos I. Fusion of the FUS and ATF1 genes in a large, deep-seated angiomatoid fibrous histiocytoma. Diagn Mol Pathol. 2002;11:157–62.
210. Dunham C, Hussong J, Seiff M, et al. Primary intracerebral angiomatoid fibrous histiocytoma: report of a case with a t(12;22)(q13;q12) causing type 1 fusion of the EWS and ATF-1 genes. Am J Surg Pathol. 2008; 32:478–84.
211. de Leeuw B, Balemans M, Geurts van Kessel A. A novel Kruppel-associated box containing the SSX gene (SSX3) on the human X chromosome is not implicated in t(X;18)-positive synovial sarcomas. Cytogenet Cell Genet. 1996;73:179–83.
212. Crew AJ, Clark J, Fisher C, et al. Fusion of SYT to two genes, SSX1 and SSX2, encoding proteins with homology to the Kruppel-associated box in human synovial sarcoma. EMBO J. 1995;14:2333–40.
213. de Leeuw B, Balemans M, Olde Weghuis D, et al. Identification of two alternative fusion genes, SYT-SSX1 and SYT-SSX2, in t(X;18)(p11.2;q11.2)-positive synovial sarcomas. Hum Mol Genet. 1995;4:1097–9.
214. Chand A, Clark J, Cooper CS, et al. Long-range organization of reiterated sequences, including the SSX1 cDNA at the OATL1 cluster in Xp11.23. Genomics. 1995;30:545–52.
215. Antonescu CR, Kawai A, Leung DH, et al. Strong association of SYT-SSX fusion type and morphologic epithelial differentiation in synovial sarcoma. Diagn Mol Pathol. 2000;9:1–8.
216. de Leeuw B, Suijkerbuijk RF, Olde Weghuis D, et al. Distinct Xp11.2 breakpoint regions in synovial sarcoma revealed by metaphase and interphase FISH: relationship to histologic subtypes. Cancer Genet Cytogenet. 1994;73:89–94.
217. Fligman I, Lonardo F, Jhanwar SC, et al. Molecular diagnosis of synovial sarcoma and characterization of a variant SYT-SSX2 fusion transcript. Am J Pathol. 1995;147:1592–9.
218. Safar A, Wickert R, Nelson M, et al. Characterization of a variant SYT-SSX1 synovial sarcoma fusion transcript. Diagn Mol Pathol. 1998;7:283–7.
219. O'Sullivan MJ, Humphrey PA, Dehner LP, et al. t(X;18) reverse transcriptase-polymerase chain reaction demonstrating a variant transcript. J Mol Diagn. 2002;4:178–80.
220. Lim FL, Soulez M, Koczan D, et al. A KRAB-related domain and a novel transcription repression domain in proteins encoded by SSX genes that are disrupted in human sarcomas. Oncogene. 1998;17:2013–8.
221. Yang P, Hirose T, Hasegawa T, et al. Dual-colour fluorescence in situ hybridization analysis of synovial sarcoma. J Pathol. 1998;184:7–13.
222. Poteat HT, Corson JM, Fletcher JA. Detection of chromosome 18 rearrangement in synovial sarcoma by fluorescence in situ hybridization. Cancer Genet Cytogenet. 1995;84:76–81.
223. Hill DA, Riedley SE, Patel AR, et al. Real-time polymerase chain reaction as an aid for the detection of SYT-SSX1 and SYT-SSX2 transcripts in fresh and archival pediatric synovial sarcoma specimens: report of 25 cases from St. Jude Children's Research Hospital. Pediatr Dev Pathol. 2003;6:24–34.
224. Argani P, Zakowski MF, Klimstra DS, et al. Detection of the SYT-SSX chimeric RNA of synovial sarcoma in paraffin-embedded tissue and its application in problematic cases. Mod Pathol. 1998;11:65–71.
225. Shipley J, Crew J, Birdsall S, et al. Interphase fluorescence in situ hybridization and reverse transcription polymerase chain reaction as a diagnostic aid for synovial sarcoma. Am J Pathol. 1996;148:559–67.
226. Lee W, Han K, Harris CP, et al. Use of FISH to detect chromosomal translocations and deletions. Analysis of chromosome rearrangement in synovial sarcoma cells from paraffin-embedded specimens. Am J Pathol. 1993;143:15–9.
227. O'Sullivan MJ, Kyriakos M, Zhu X, et al. Malignant peripheral nerve sheath tumors with t(X;18). A pathologic and molecular genetic study. Mod Pathol. 2000;13:1336–46.
228. Vang R, Biddle DA, Harrison WR, et al. Malignant peripheral nerve sheath tumor with a t(X;18). Arch Pathol Lab Med. 2000;124:864–7.
229. Yang K, Lui WO, Xie Y, et al. Co-existence of SYT-SSX1 and SYT-SSX2 fusions in synovial sarcomas. Oncogene. 2002;21:4181–90.

230. Geurts van Kessel A, de Bruijn D, Hermsen L, et al. Masked t(X;18)(p11;q11) in a biphasic synovial sarcoma revealed by FISH and RT-PCR. Genes Chromosomes Cancer. 1998;23:198–201.
231. Bijwaard KE, Fetsch JF, Przygodzki R, et al. Detection of SYT-SSX fusion transcripts in archival synovial sarcomas by real-time reverse transcriptase-polymerase chain reaction. J Mol Diagn. 2002;4:59–64.
232. Surace C, Panagopoulos I, Palsson E, et al. A novel FISH assay for SS18-SSX fusion type in synovial sarcoma. Lab Invest. 2004;84:1185–92.
233. Inagaki H, Nagasaka T, Otsuka T, et al. Association of SYT-SSX fusion types with proliferative activity and prognosis in synovial sarcoma. Mod Pathol. 2000;13:482–8.
234. Panagopoulos I, Mertens F, Isaksson M, et al. Clinical impact of molecular and cytogenetic findings in synovial sarcoma. Genes Chromosomes Cancer. 2001;31:362–72.
235. Kawai A, Woodruff J, Healey JH, et al. SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. N Engl J Med. 1998;338:153–60.
236. Ladanyi M, Antonescu CR, Leung DH, et al. Impact of SYT-SSX fusion type on the clinical behavior of synovial sarcoma: a multi-institutional retrospective study of 243 patients. Cancer Res. 2002;62:135–40.
237. Skytting BT, Szymanska J, Aalto Y, et al. Clinical importance of genomic imbalances in synovial sarcoma evaluated by comparative genomic hybridization. Cancer Genet Cytogenet. 1999;115:39–46.
238. Willeke F, Mechtersheimer G, Schwarzbach M, et al. Detection of SYT-SSX1/2 fusion transcripts by reverse transcriptase-polymerase chain reaction (RT-PCR) is a valuable diagnostic tool in synovial sarcoma. Eur J Cancer. 1998;34:2087–93.
239. Hashimoto N, Myoui A, Araki N, et al. Detection of SYT-SSX fusion gene in peripheral blood from a patient with synovial sarcoma. Am J Surg Pathol. 2001;25:406–10.
240. Nakasone J, Shimizu T, Gomyo H, et al. Assessment of microinvasion with reverse transcriptase polymerase chain reaction in a case of synovial sarcoma. J Orthop Sci. 2004;9:162–5.
241. He R, Patel RM, Alkan S, et al. Immunostaining for SYT protein discriminates synovial sarcoma from other soft tissue tumors: analysis of 146 cases. Mod Pathol. 2007;20:522–8.
242. Morgan MB, Stevens L, Patterson J, et al. Cutaneous epithelioid malignant nerve sheath tumor with rhabdoid features: a histologic, immunohistochemical, and ultrastructural study of three cases. J Cutan Pathol. 2000;27:529–34.
243. de Alava E. Transcripts, transcripts, everywhere. Adv Anat Pathol. 2001;8:264–72.
244. van de Rijn M, Barr FG, Xiong QB, et al. Poorly differentiated synovial sarcoma: an analysis of clinical, pathologic, and molecular genetic features. Am J Surg Pathol. 1999;23:106–12.
245. Folpe AL, Schmidt RA, Chapman D, et al. Poorly differentiated synovial sarcoma: immunohistochemical distinction from primitive neuroectodermal tumors and high-grade malignant peripheral nerve sheath tumors. Am J Surg Pathol. 1998;22:673–82.
246. Guillou L, Wadden CF, Kraus MD, et al. S-100 protein reactivity in synovial sarcomas-a potentially frequent diagnostic pitfall: immunohistochemical analysis of 100 cases. Appl Immunohistochem. 1996;4:167–75.
247. Nagayama S, Katagiri T, Tsunoda T, et al. Genome-wide analysis of gene expression in synovial sarcomas using a cDNA microarray. Cancer Res. 2002;62:5859–66.
248. Yang J, Du X, Chen K, et al. Genetic aberrations in soft tissue leiomyosarcoma. Cancer Lett. 2009;275:1–8.
249. Badeloe S, van Geest AJ, van Marion AM, et al. Absence of fumarate hydratase mutation in a family with cutaneous leiomyosarcoma and renal cancer. Int J Dermatol. 2008;47 Suppl 1:18–20.
250. Holst VA, Junkins-Hopkins JM, Elenitsas R. Cutaneous smooth muscle neoplasms: clinical features, histologic findings, and treatment options. J Am Acad Dermatol. 2002;46:477–90. quiz, 91-4.
251. Sandberg AA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: leiomyosarcoma. Cancer Genet Cytogenet. 2005;161:1–19.
252. Wang R, Lu YJ, Fisher C, et al. Characterization of chromosome aberrations associated with soft-tissue leiomyosarcomas by twenty-four-color karyotyping and comparative genomic hybridization analysis. Genes Chromosomes Cancer. 2001;31:54–64.
253. Mandahl N, Fletcher CD, Dal Cin P, et al. Comparative cytogenetic study of spindle cell and pleomorphic leiomyosarcomas of soft tissues: a report from the CHAMP Study Group. Cancer Genet Cytogenet. 2000;116:66–73.
254. Tomlinson IP, Alam NA, Rowan AJ, et al. Germline mutations in FH predispose to dominantly inherited uterine fibroids, skin leiomyomata and papillary renal cell cancer. Nat Genet. 2002;30:406–10.
255. Velagaleti GV, Tapper JK, Panova NE, et al. Cytogenetic findings in a case of nodular fasciitis of subclavicular region. Cancer Genet Cytogenet. 2003;141:160–3.
256. Donner LR, Silva T, Dobin SM. Clonal rearrangement of 15p11.2, 16p11.2, and 16p13.3 in a case of nodular fasciitis: additional evidence favoring nodular fasciitis as a benign neoplasm and not a reactive tumefaction. Cancer Genet Cytogenet. 2002;139:138–40.
257. Weibolt VM, Buresh CJ, Roberts CA, et al. Involvement of 3q21 in nodular fasciitis. Cancer Genet Cytogenet. 1998;106:177–9.

258. Meng GZ, Zhang HY, Zhang Z, et al. Myofibroblastic sarcoma vs nodular fasciitis: a comparative study of chromosomal imbalances. Am J Clin Pathol. 2009;131:701–9.
259. Michie BA, Reid RP, Fallowfield ME. Aneuploidy in atypical fibroxanthoma: DNA content quantification of 10 cases by image analysis. J Cutan Pathol. 1994;21:404–7.
260. Worrell JT, Ansari MQ, Ansari SJ, et al. Atypical fibroxanthoma: DNA ploidy analysis of 14 cases with possible histogenetic implications. J Cutan Pathol. 1993;20:211–5.
261. Oshiro Y, Fukuda T, Tsuneyoshi M. Atypical fibroxanthoma versus benign and malignant fibrous histiocytoma. A comparative study of their proliferative activity using MIB-1, DNA flow cytometry, and p53 immunostaining. Cancer. 1995;75:1128–34.
262. Mihic-Probst D, Zhao J, Saremaslani P, et al. CGH analysis shows genetic similarities and differences in atypical fibroxanthoma and undifferentiated high grade pleomorphic sarcoma. Anticancer Res. 2004;24:19–26.
263. Hui P, Glusac EJ, Sinard JH, et al. Clonal analysis of cutaneous fibrous histiocytoma (dermatofibroma). J Cutan Pathol. 2002;29:385–9.
264. Chen TC, Kuo T, Chan HL. Dermatofibroma is a clonal proliferative disease. J Cutan Pathol. 2000;27:36–9.
265. Laskin WB, Miettinen M, Fetsch JF. Infantile digital fibroma/fibromatosis: a clinicopathologic and immunohistochemical study of 69 tumors from 57 patients with long-term follow-up. Am J Surg Pathol. 2009;33:1–13.
266. Stenman G, Nadal N, Persson S, et al. del(6)(q12q15) as the sole cytogenetic anomaly in a case of solitary infantile myofibromatosis. Oncol Rep. 1999;6:1101–4.
267. Sirvent N, Perrin C, Lacour JP, et al. Monosomy 9q and trisomy 16q in a case of congenital solitary infantile myofibromatosis. Virchows Arch. 2004;445:537–40.
268. Sheng WQ, Hisaoka M, Okamoto S, et al. Congenital-infantile fibrosarcoma. A clinicopathologic study of 10 cases and molecular detection of the ETV6-NTRK3 fusion transcripts using paraffin-embedded tissues. Am J Clin Pathol. 2001;115:348–55.
269. Alaggio R, Barisani D, Ninfo V, et al. Morphologic overlap between infantile myofibromatosis and infantile fibrosarcoma: a pitfall in diagnosis. Pediatr Dev Pathol. 2008;11:355–62.
270. Dei Tos AP, Mentzel T, Fletcher CD. Primary liposarcoma of the skin: a rare neoplasm with unusual high grade features. Am J Dermatopathol. 1998;20:332–8.
271. Hibbard MK, Kozakewich HP, Dal Cin P, et al. PLAG1 fusion oncogenes in lipoblastoma. Cancer Res. 2000;60:4869–72.
272. Astrom A, D'Amore ES, Sainati L, et al. Evidence of involvement of the PLAG1 gene in lipoblastomas. Int J Oncol. 2000;16:1107–10.
273. Gisselsson D, Hibbard MK, Dal Cin P, et al. PLAG1 alterations in lipoblastoma: involvement in varied mesenchymal cell types and evidence for alternative oncogenic mechanisms. Am J Pathol. 2001;159:955–62.
274. Sciot R, De Wever I, Debiec-Rychter M. Lipoblastoma in a 23-year-old male: distinction from atypical lipomatous tumor using cytogenetic and fluorescence in-situ hybridization analysis. Virchows Arch. 2003;442:468–71.
275. Ropke A, Kalinski T, Kluba U, et al. PLAG1 activation in lipoblastoma coinciding with low-level amplification of a derivative chromosome 8 with a deletion del(8)(q13q21.2). Cytogenet Genome Res. 2007;119:33–8.
276. Van Dyck F, Declercq J, Braem CV, et al. PLAG1, the prototype of the PLAG gene family: versatility in tumour development (review). Int J Oncol. 2007;30:765–74.
277. Kas K, Voz ML, Roijer E, et al. Promoter swapping between the genes for a novel zinc finger protein and beta-catenin in pleiomorphic adenomas with t(3;8)(p21;q12) translocations. Nat Genet. 1997;15:170–4.
278. Voz ML, Astrom AK, Kas K, et al. The recurrent translocation t(5;8)(p13;q12) in pleomorphic adenomas results in upregulation of PLAG1 gene expression under control of the LIFR promoter. Oncogene. 1998;16:1409–16.
279. Astrom AK, Voz ML, Kas K, et al. Conserved mechanism of PLAG1 activation in salivary gland tumors with and without chromosome 8q12 abnormalities: identification of SII as a new fusion partner gene. Cancer Res. 1999;59:918–23.
280. Dal Cin P, Kools P, Sciot R, et al. Cytogenetic and fluorescence in situ hybridization investigation of ring chromosomes characterizing a specific pathologic subgroup of adipose tissue tumors. Cancer Genet Cytogenet. 1993;68:85–90.
281. Forus A, Bjerkehagen B, Sirvent N, et al. A well-differentiated liposarcoma with a new type of chromosome 12-derived markers. Cancer Genet Cytogenet. 2001;131:13–8.
282. Pedeutour F, Forus A, Coindre JM, et al. Structure of the supernumerary ring and giant rod chromosomes in adipose tissue tumors. Genes Chromosomes Cancer. 1999;24:30–41.
283. Rubin BP, Dal Cin P. The genetics of lipomatous tumors. Semin Diagn Pathol. 2001;18:286–93.
284. Mandahl N, Hoglund M, Mertens F, et al. Cytogenetic aberrations in 188 benign and borderline adipose tissue tumors. Genes Chromosomes Cancer. 1994;9:207–15.
285. Nilsson M, Meza-Zepeda LA, Mertens F, et al. Amplification of chromosome 1 sequences in lipomatous tumors and other sarcomas. Int J Cancer. 2004;109:363–9.

286. Sandberg AA. Updates on the cytogenetics and molecular genetics of bone and soft tissue tumors: lipoma. Cancer Genet Cytogenet. 2004;150:93–115.
287. Nakayama T, Toguchida J, Wadayama B, et al. MDM2 gene amplification in bone and soft-tissue tumors: association with tumor progression in differentiated adipose-tissue tumors. Int J Cancer. 1995;64:342–6.
288. Nilbert M, Rydholm A, Mitelman F, et al. Characterization of the 12q13-15 amplicon in soft tissue tumors. Cancer Genet Cytogenet. 1995;83:32–6.
289. Storlazzi CT, Mertens F, Domanski H, et al. Ring chromosomes and low-grade gene amplification in an atypical lipomatous tumor with minimal nuclear atypia. Int J Oncol. 2003;23:67–71.
290. Weaver J, Downs-Kelly E, Goldblum JR, et al. Fluorescence in situ hybridization for MDM2 gene amplification as a diagnostic tool in lipomatous neoplasms. Mod Pathol. 2008;21:943–9.
291. Sirvent N, Coindre JM, Maire G, et al. Detection of MDM2-CDK4 amplification by fluorescence in situ hybridization in 200 paraffin-embedded tumor samples: utility in diagnosing adipocytic lesions and comparison with immunohistochemistry and real-time PCR. Am J Surg Pathol. 2007;31:1476–89.
292. Shimada S, Ishizawa T, Ishizawa K, et al. The value of MDM2 and CDK4 amplification levels using real-time polymerase chain reaction for the differential diagnosis of liposarcomas and their histologic mimickers. Hum Pathol. 2006;37:1123–9.
293. Hostein I, Pelmus M, Aurias A, et al. Evaluation of MDM2 and CDK4 amplification by real-time PCR on paraffin wax-embedded material: a potential tool for the diagnosis of atypical lipomatous tumours/well-differentiated liposarcomas. J Pathol. 2004;202:95–102.
294. Miyajima K, Tamiya S, Oda Y, et al. Relative quantitation of p53 and MDM2 gene expression in leiomyosarcoma; real-time semi-quantitative reverse transcription-polymerase chain reaction. Cancer Lett. 2001;164:177–88.
295. Boltze C, Schneider-Stock R, Jager V, et al. Distinction between lipoma and liposarcoma by MDM2 alterations: a case report of simultaneously occurring tumors and review of the literature. Pathol Res Pract. 2001;197:563–8.
296. Pilotti S, Della GT, Mezzelani A, et al. The expression of MDM2/CDK4 gene product in the differential diagnosis of well differentiated liposarcoma and large deep-seated lipoma. Br J Cancer. 2000;82:1271–5.
297. Binh MB, Sastre-Garau X, Guillou L, et al. MDM2 and CDK4 immunostainings are useful adjuncts in diagnosing well-differentiated and dedifferentiated liposarcoma subtypes: a comparative analysis of 559 soft tissue neoplasms with genetic data. Am J Surg Pathol. 2005;29:1340–7.
298. Cordon-Cardo C, Latres E, Drobnjak M, et al. Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. Cancer Res. 1994;54:794–9.
299. Nilbert M, Rydholm A, Willen H, et al. MDM2 gene amplification correlates with ring chromosome in soft tissue tumors. Genes Chromosomes Cancer. 1994;9:261–5.
300. Reid AH, Tsai MM, Venzon DJ, et al. MDM2 amplification, P53 mutation, and accumulation of the P53 gene product in malignant fibrous histiocytoma. Diagn Mol Pathol. 1996;5:65–73.
301. Meis-Kindblom JM, Sjogren H, Kindblom LG, et al. Cytogenetic and molecular genetic analyses of liposarcoma and its soft tissue simulators: recognition of new variants and differential diagnosis. Virchows Arch. 2001;439:141–51.
302. Pilotti S, Della GT, Lavarino C, et al. Distinct mdm2/p53 expression patterns in liposarcoma subgroups: implications for different pathogenetic mechanisms. J Pathol. 1997;181:14–24.
303. Sirvent N, Forus A, Lescaut W, et al. Characterization of centromere alterations in liposarcomas. Genes Chromosomes Cancer. 2000;29:117–29.
304. Horvai AE, Schaefer JT, Nakakura EK, et al. Immunostaining for peroxisome proliferator gamma distinguishes dedifferentiated liposarcoma from other retroperitoneal sarcomas. Mod Pathol. 2008;21:517–24.
305. Dei Tos AP, Doglioni C, Piccinin S, et al. Molecular abnormalities of the p53 pathway in dedifferentiated liposarcoma. J Pathol. 1997;181:8–13.
306. Schneider-Stock R, Walter H, Radig K, et al. MDM2 amplification and loss of heterozygosity at Rb and p53 genes: no simultaneous alterations in the oncogenesis of liposarcomas. J Cancer Res Clin Oncol. 1998;124:532–40.
307. Takahira T, Oda Y, Tamiya S, et al. Alterations of the RB1 gene in dedifferentiated liposarcoma. Mod Pathol. 2005;18:1461–70.
308. Crozat A, Aman P, Mandahl N, et al. Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature. 1993;363:640–4.
309. Rabbitts TH, Forster A, Larson R, et al. Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. Nat Genet. 1993;4:175–80.
310. Tallini G, Akerman M, Dal Cin P, et al. Combined morphologic and karyotypic study of 28 myxoid liposarcomas. Implications for a revised morphologic typing (a report from the CHAMP Group). Am J Surg Pathol. 1996;20:1047–55.
311. Kuroda M, Ishida T, Horiuchi H, et al. Chimeric TLS/FUS-CHOP gene expression and the heterogeneity of its junction in human myxoid and round cell liposarcoma. Am J Pathol. 1995;147:1221–7.

312. Panagopoulos I, Mandahl N, Mitelman F, et al. Two distinct FUS breakpoint clusters in myxoid liposarcoma and acute myeloid leukemia with the translocations t(12;16) and t(16;21). Oncogene. 1995;11:1133–7.
313. Panagopoulos I, Mandahl N, Ron D, et al. Characterization of the CHOP breakpoints and fusion transcripts in myxoid liposarcomas with the 12;16 translocation. Cancer Res. 1994;54:6500–3.
314. Antonescu CR, Tschernyavsky SJ, Decuseara R, et al. Prognostic impact of P53 status, TLS-CHOP fusion transcript structure, and histological grade in myxoid liposarcoma: a molecular and clinicopathologic study of 82 cases. Clin Cancer Res. 2001;7:3977–87.
315. Xiang H, Wang J, Hisaoka M, et al. Characteristic sequence motifs located at the genomic breakpoints of the translocation t(12;16) and t(12;22) in myxoid liposarcoma. Pathology. 2008;40:547–52.
316. Bode-Lesniewska B, Frigerio S, Exner U, et al. Relevance of translocation type in myxoid liposarcoma and identification of a novel EWSR1-DDIT3 fusion. Genes Chromosomes Cancer. 2007;46:961–71.
317. Dal Cin P, Sciot R, Panagopoulos I, et al. Additional evidence of a variant translocation t(12;22) with EWS/CHOP fusion in myxoid liposarcoma: clinicopathological features. J Pathol. 1997;182:437–41.
318. Hosaka T, Nakashima Y, Kusuzaki K, et al. A novel type of EWS-CHOP fusion gene in two cases of myxoid liposarcoma. J Mol Diagn. 2002;4:164–71.
319. Panagopoulos I, Hoglund M, Mertens F, et al. Fusion of the EWS and CHOP genes in myxoid liposarcoma. Oncogene. 1996;12:489–94.
320. Adelmant G, Gilbert JD, Freytag SO. Human translocation liposarcoma-CCAAT/enhancer binding protein (C/EBP) homologous protein (TLS-CHOP) oncoprotein prevents adipocyte differentiation by directly interfering with C/EBPbeta function. J Biol Chem. 1998;273:15574–81.
321. Kuroda M, Ishida T, Takanashi M, et al. Oncogenic transformation and inhibition of adipocytic conversion of preadipocytes by TLS/FUS-CHOP type II chimeric protein. Am J Pathol. 1997;151:735–44.
322. Ron D, Habener JF. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev. 1992;6:439–53.
323. Aoki T, Hisaoka M, Kouho H, et al. Interphase cytogenetic analysis of myxoid soft tissue tumors by fluorescence in situ hybridization and DNA flow cytometry using paraffin-embedded tissue. Cancer. 1997;79:284–93.
324. Mezzelani A, Sozzi G, Pierotti MA, et al. Rapid differential diagnosis of myxoid liposarcoma by fluorescence in situ hybridisation on cytological preparations. Clin Mol Pathol. 1996;49:M308–9.
325. Downs-Kelly E, Goldblum JR, Patel RM, et al. The utility of fluorescence in situ hybridization (FISH) in the diagnosis of myxoid soft tissue neoplasms. Am J Surg Pathol. 2008;32:8–13.
326. Birch NC, Antonescu CR, Nelson M, et al. Inconspicuous insertion 22;12 in myxoid/round cell liposarcoma accompanied by the secondary structural abnormality der(16)t(1;16). J Mol Diagn. 2003;5:191–4.
327. Antonescu CR, Elahi A, Humphrey M, et al. Specificity of TLS-CHOP rearrangement for classic myxoid/round cell liposarcoma: absence in predominantly myxoid well-differentiated liposarcomas. J Mol Diagn. 2000;2:132–8.
328. Fletcher CD, Akerman M, Dal Cin P, et al. Correlation between clinicopathological features and karyotype in lipomatous tumors. A report of 178 cases from the Chromosomes and Morphology (CHAMP) Collaborative Study Group. Am J Pathol. 1996;148:623–30.
329. Rosai J, Akerman M, Dal Cin P, et al. Combined morphologic and karyotypic study of 59 atypical lipomatous tumors. Evaluation of their relationship and differential diagnosis with other adipose tissue tumors (a report of the CHAMP Study Group). Am J Surg Pathol. 1996;20:1182–9.
330. Willeke F, Ridder R, Mechtersheimer G, et al. Analysis of FUS-CHOP fusion transcripts in different types of soft tissue liposarcoma and their diagnostic implications. Clin Cancer Res. 1998;4:1779–84.
331. Panagopoulos I, Aman P, Mertens F, et al. Genomic PCR detects tumor cells in peripheral blood from patients with myxoid liposarcoma. Genes Chromosomes Cancer. 1996;17:102–7.
332. Hengge UR, Ruzicka T, Tyring SK, et al. Update on Kaposi's sarcoma and other HHV8 associated diseases. Part 1: epidemiology, environmental predispositions, clinical manifestations, and therapy. Lancet Infect Dis. 2002;2:281–92.
333. Chang Y, Cesarman E, Pessin MS, et al. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. Science. 1994;266:1865–9.
334. Verma SC, Robertson ES. Molecular biology and pathogenesis of Kaposi sarcoma-associated herpesvirus. FEMS Microbiol Lett. 2003;222:155–63.
335. Schulz TF. The pleiotropic effects of Kaposi's sarcoma herpesvirus. J Pathol. 2006;208:187–98.
336. Dictor M, Rambech E, Way D, et al. Human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) DNA in Kaposi's sarcoma lesions, AIDS Kaposi's sarcoma cell lines, endothelial Kaposi's sarcoma simulators, and the skin of immunosuppressed patients. Am J Pathol. 1996;148:2009–16.
337. Kazakov DV, Prinz BM, Michaelis S, et al. Study of HHV-8 DNA sequences in archival biopsies from lesional skin of Kaposi's sarcoma, various mesenchymal tumors and related reactive conditions. J Cutan Pathol. 2002;29:279–81.

338. Moore PS, Chang Y. Detection of herpesvirus-like DNA sequences in Kaposi's sarcoma in patients with and without HIV infection. N Engl J Med. 1995;332:1181–5.
339. Hammock L, Reisenauer A, Wang W, et al. Latency-associated nuclear antigen expression and human herpesvirus-8 polymerase chain reaction in the evaluation of Kaposi sarcoma and other vascular tumors in HIV-positive patients. Mod Pathol. 2005;18:463–8.
340. Nuovo M, Nuovo G. Utility of HHV8 RNA detection for differentiating Kaposi's sarcoma from its mimics. J Cutan Pathol. 2001;28:248–55.
341. Cesarman E, Chang Y, Moore PS, et al. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N Engl J Med. 1995;332:1186–91.
342. Dupin N, Fisher C, Kellam P, et al. Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc Natl Acad Sci USA. 1999;96:4546–51.
343. Adiguzel C, Bozkurt SU, Kaygusuz I, et al. Human herpes virus 8-unrelated primary effusion lymphoma-like lymphoma: report of a rare case and review of the literature. APMIS. 2009;117:222–9.
344. Du MQ, Bacon CM, Isaacson PG. Kaposi sarcoma-associated herpesvirus/human herpesvirus 8 and lymphoproliferative disorders. J Clin Pathol. 2007;60:1350–7.
345. Cool CD, Rai PR, Yeager ME, et al. Expression of human herpesvirus 8 in primary pulmonary hypertension. N Engl J Med. 2003;349:1113–22.
346. Gomez-Roman JJ, Ocejo-Vinyals G, Sanchez-Velasco P, et al. Presence of human herpesvirus-8 DNA sequences and overexpression of human IL-6 and cyclin D1 in inflammatory myofibroblastic tumor (inflammatory pseudotumor). Lab Invest. 2000;80:1121–6.
347. Bryant-Greenwood P, Sorbara L, Filie AC, et al. Infection of mesothelial cells with human herpes virus 8 in human immunodeficiency virus-infected patients with Kaposi's sarcoma, Castleman's disease, and recurrent pleural effusions. Mod Pathol. 2003;16:145–53.
348. Insabato L, Di Vizio D, Terracciano LM, et al. Primary Kaposi sarcoma of the bowel in a HIV-negative patient. J Surg Oncol. 2001;76:197–200.
349. Gyulai R, Kemeny L, Kiss M, et al. Herpesvirus-like DNA sequence in angiosarcoma in a patient without HIV infection. N Engl J Med. 1996;334:540–1.
350. McDonagh DP, Liu J, Gaffey MJ, et al. Detection of Kaposi's sarcoma-associated herpesvirus-like DNA sequence in angiosarcoma. Am J Pathol. 1996;149:1363–8.
351. Hisaoka M, Hashimoto H, Iwamasa T. Diagnostic implication of Kaposi's sarcoma-associated herpesvirus with special reference to the distinction between spindle cell hemangioendothelioma and Kaposi's sarcoma. Arch Pathol Lab Med. 1998;122:72–6.
352. Schommer M, Herbst RA, Brodersen JP, et al. Retiform hemangioendothelioma: another tumor associated with human herpesvirus type 8? J Am Acad Dermatol. 2000;42:290–2.
353. Gyulai R, Kemeny L, Adam E, et al. HHV8 DNA in angiolymphoid hyperplasia of the skin. Lancet. 1996; 347:1837.
354. Kazakov DV, Schmid M, Adams V, et al. HHV-8 DNA sequences in the peripheral blood and skin lesions of an HIV-negative patient with multiple eruptive dermatofibromas: implications for the detection of HHV-8 as a diagnostic marker for Kaposi's sarcoma. Dermatology. 2003;206:217–21.
355. Cheuk W, Wong KO, Wong CS, et al. Immunostaining for human herpesvirus 8 latent nuclear antigen-1 helps distinguish Kaposi sarcoma from its mimickers. Am J Clin Pathol. 2004;121:335–42.
356. Patel RM, Goldblum JR, Hsi ED. Immunohistochemical detection of human herpes virus-8 latent nuclear antigen-1 is useful in the diagnosis of Kaposi sarcoma. Mod Pathol. 2004;17:456–60.
357. Robin YM, Guillou L, Michels JJ, et al. Human herpesvirus 8 immunostaining: a sensitive and specific method for diagnosing Kaposi sarcoma in paraffin-embedded sections. Am J Clin Pathol. 2004;121:330–4.
358. Tomasini C, Grassi M, Pippione M. Cutaneous angiosarcoma arising in an irradiated breast. Case report and review of the literature. Dermatology. 2004;209:208–14.
359. Brenn T, Fletcher CD. Postradiation vascular proliferations: an increasing problem. Histopathology. 2006; 48:106–14.
360. Mermershtain W, Cohen AD, Koretz M, et al. Cutaneous angiosarcoma of breast after lumpectomy, axillary lymph node dissection, and radiotherapy for primary breast carcinoma: case report and review of the literature. Am J Clin Oncol. 2002;25:597–8.
361. Schmid H, Zietz C. Human herpesvirus 8 and angiosarcoma: analysis of 40 cases and review of the literature. Pathology. 2005;37:284–7.
362. Naka N, Tomita Y, Nakanishi H, et al. Mutations of p53 tumor-suppressor gene in angiosarcoma. Int J Cancer. 1997;71:952–5.
363. Zietz C, Rossle M, Haas C, et al. MDM-2 oncoprotein overexpression, p53 gene mutation, and VEGF upregulation in angiosarcomas. Am J Pathol. 1998;153:1425–33.
364. Momand J, Zambetti GP. Mdm-2: "Big brother" of p53. J Cell Biochem. 1997;64:343–52.

365. Gospodarowicz D, Abraham JA, Schilling J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci USA. 1989;86:7311–5.
366. Patel NK, McKee PH, Smith NP, et al. Primary metaplastic carcinoma (carcinosarcoma) of the skin. A clinicopathologic study of four cases and review of the literature. Am J Dermatopathol. 1997;19:363–72.
367. Weedon D. Skin pathology. 2nd ed. Edinburgh: Churchill Livingstone; 2002.
368. Syme-Grant J, Syme-Grant NJ, Motta L, et al. Are primary cutaneous carcinisarcomas underdiagnosed? Five cases and a review of the literature. J Plast Reconstr Aesthet Surg. 2006;59:1402–8.
369. Brownstein MH, Helwig EB. Patterns of cutaneous metastasis. Arch Dermatol. 1972;105:862–8.
370. Wesche WA, Khare VK, Chesney TM, et al. Non-hematopoietic cutaneous metastases in children and adolescents: thirty years experience at St. Jude Children's Research Hospital. J Cutan Pathol. 2000;27:485–92.
371. Liaw D, Marsh DJ, Li J, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet. 1997;16:64–7.
372. Requena L, Gutierrez J, Sanchez EY. Multiple sclerotic fibromas of the skin. A cutaneous marker of Cowden's disease. J Cutan Pathol. 1992;19:346–51.
373. Eng C. PTEN: one gene, many syndromes. Hum Mutat. 2003;22:183–98.
374. Verhoef S, Bakker L, Tempelaars AM, et al. High rate of mosaicism in tuberous sclerosis complex. Am J Hum Genet. 1999;64:1632–7.
375. Hall MR, Kovach BT, Miller JL. Unilateral facial angiofibromas without other evidence of tuberous sclerosis: case report and review of the literature. Cutis. 2007;80:284–8.
376. Trauner MA, Ruben BS, Lynch PJ. Segmental tuberous sclerosis presenting as unilateral facial angiofibromas. J Am Acad Dermatol. 2003;49:S164–6.
377. Anliker MD, Dummer R, Burg G. Unilateral agminated angiofibromas: a segmental expression of tuberous sclerosis? Dermatology. 1997;195:176–8.
378. McGrae Jr JD, Hashimoto K. Unilateral facial angiofibromas–a segmental form of tuberous sclerosis. Br J Dermatol. 1996;134:727–30.
379. Alam NA, Barclay E, Rowan AJ, et al. Clinical features of multiple cutaneous and uterine leiomyomatosis: an underdiagnosed tumor syndrome. Arch Dermatol. 2005;141:199–206.
380. Barker KT, Bevan S, Wang R, et al. Low frequency of somatic mutations in the FH/multiple cutaneous leiomyomatosis gene in sporadic leiomyosarcomas and uterine leiomyomas. Br J Cancer. 2002;87:446–8.
381. Konig A, Happle R. Two cases of type 2 segmental manifestation in a family with cutaneous leiomyomatosis. Eur J Dermatol. 2000;10:590–2.
382. Lang K, Reifenberger J, Ruzicka T, et al. Type 1 segmental cutaneous leiomyomatosis. Clin Exp Dermatol. 2002;27:649–50.
383. Badeloe S, van Geel M, van Steensel MA, et al. Diffuse and segmental variants of cutaneous leiomyomatosis: novel mutations in the fumarate hydratase gene and review of the literature. Exp Dermatol. 2006;15:735–41.
384. Suwattee P, Dakin C. Bilateral segmental leiomyomas: a case report and review of the literature. Cutis. 2008;82:33–6.

Chapter 9
Molecular Determination of Tissue Margins, Clonal Origin, and Histogenesis of Skin Cancers

Michael J. Murphy

Technologies are being increasingly employed to identify the biological pathways, genes, and proteins involved in disease pathogenesis and progression. These investigations have uncovered diagnostic and prognostic biomarkers for a variety of cutaneous tumors. Molecular testing may have a role in the evaluation of tissue margins, clonal origin, and histogenesis of skin cancers.

Molecular Evaluation of Tissue Margins of Skin Cancers

The concept of field cancerization, first proposed by Slaughter in 1953, describes a process whereby cells in a particular tissue or organ are sequentially transformed by multiple cumulative genetic and epigenetic alterations, such that a clonal expansion of pre-neoplastic genetically altered, but morphologically normal-appearing cells is present, prior to the development of overt malignancy [1]. Additional genomic aberrations are required for cancer development, but these precursor cells may persist with the malignant cells of a tumor [1]. The skin is the most suitable organ to investigate the mechanisms and potential clinical utility of field cancerization - due to its contiguous nature, accessibility, and ease of removing wide tumor margins. The study of field cancerization in the skin may have a role in: (1) the assessment of tumor risk; (2) the detection of early neoplasia; (3) the study of tumor pathogenesis and progression; and (4) the accurate delineation of "true" tumor margins (i.e., overt tumor and surrounding "field cells"), and as a consequence, the planning of adequate surgical treatment.

The standard treatment of primary melanoma is wide excision with a defined margin of clinically uninvolved skin, in an effort to reduce the risk of local recurrence. It is the presence of occult field cells peripheral to a tumor which explains the efficacy of wide excisions in reducing local recurrences in patients with melanoma [2]. At present, standardized excision recommendations are based on Breslow thickness (i.e., 0.5-cm margins for melanoma in situ, 1-cm margins for invasive melanoma of <2 mm, and 2-cm margins for melanoma of >2 mm) [3]. These guidelines are supported by subsequent histopathological assessment of the microscopic distance between the excision specimen margins and any residual melanocytes which are cytologically atypical, abnormally distributed, and/or increased in number [3]. However, studies have noted that the frequency of local, regional, or distant metastases is not affected by the margins of excision in some patients with melanoma [4].

M.J. Murphy (✉)
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, 21 South Rd., Farmington, CT 06030, USA
e-mail: drmichaelmurphy@netscape.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_9,

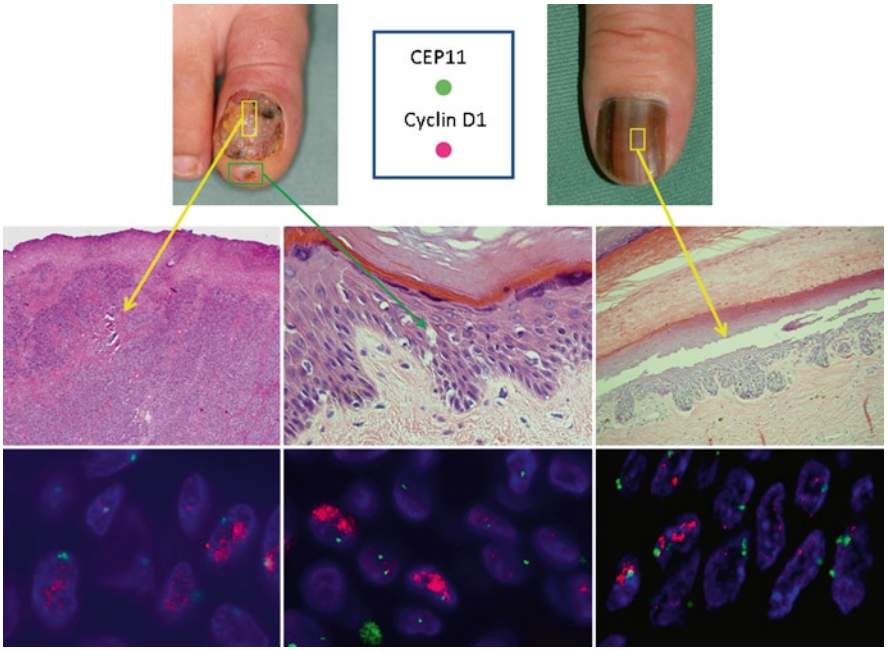

Fig. 9.1 CCND1 (cyclin D1) amplification in acral lentiginous melanoma (*ALM*). Fluorescence in situ hybridization (*FISH*) demonstrating CCND1 (11q13) amplification in an invasive ALM (*left panels*) and an adjacent in situ macular lesion (*middle panels*). An additional case of *ALM* in situ with CCND1 amplification is shown (*right panels*). CCND1 probe, *red signal*; chromosome 11 centromeric probe/CEP11, *green signal* (Courtesy of Dr. Minoru Takata, Shinshu University School of Medicine, Asahi, Matsumoto, Japan)

The development of such melanoma recurrences following recommended excision guidelines could be due to: (1) false-negative microscopic review of the excision specimen (due to different tissue processing techniques, pathologist experience, etc.); or (2) intraepidermal pre-neoplastic or frankly neoplastic melanocytes (i.e., melanocytic field cancerization), which cannot be readily identified by current routine histopathological methods, present at or peripheral to the excision site.

The recent use of fluorescence in situ hybridization (FISH) analysis to determine the presence of overt residual disease and/or field cells in acral lentiginous melanoma (ALM) is a potentially exciting application of a molecular test to guide management and control local recurrence of this disease (Fig. 9.1). Recent studies have demonstrated frequent amplifications of regions on chromosomes 5p15, 22q11–13 and 11q13 (site of CCND1) in ALM [2, 3, 5, 6]. Interestingly, in 84% of cases studied, copy number increases of 11q13 and 5p15 have also been identified in the melanocytic cells of histopathologically normal epidermis at varying distances from the overt ALM tumor margins (mean: 6.1 mm for melanoma in situ, 4.5 mm for invasive melanoma) [3]. However, the extent of these latter findings does not appear to correlate with the Breslow thickness or diameter of the ALM [3]. Both (1) the pattern of aberrations (i.e., stable or progressive increase in gene amplification levels from field cells to in situ to invasive components) and (2) the asymmetric distribution of field cells support the acquirement of additional oncogenic aberrations for progression to frank malignancy [3]. According to the concept of field cancerization, these morphologically normal, but genetically aberrant melanocytes would represent a latent progression phase/early melanoma in situ (supported by gene profiling studies), which precedes a stage of uncontrolled melanocyte proliferation within the epidermis [3, 5, 6]. The results of these FISH studies would suggest that the current recommendations for excision margins based on the Breslow thickness are suboptimal for a subset of melanomas [4]. The routine clinical use of FISH technology to detect field cells in melanoma could help determine the appropriate surgical margins required to minimize the risk

of tumor recurrence. At present, this technique is only applicable to those melanomas with frequent gene amplifications (i.e., ALM and mucosal subtypes).

A number of studies have investigated the use of additional technologies, such as immunohistochemistry (IHC), for margin analysis in melanomas [3, 7, 8]. Protein expression in excision specimens has been evaluated using antibodies directed against cyclin D1 and melanocyte-differentiation markers, such as MART-1, HMB-45, S-100, and Mel-5 [3, 7, 8]. Of note, HMB-45 (an antibody directed against a melanosomal antigen) has been reported in overt in situ and invasive ALM, but can also be identified in field cell areas of these tumors (in up to 56% of cases) [3, 8]. However, HMB-45 is a marker of melanocytic differentiation, and not genetic instability per se, and therefore its ability to reliably distinguish between benign, premalignant or overtly malignant individual melanocytes in the epidermis is questionable. Levels of cyclin D1 protein have also been found to increase from the periphery toward the in situ and invasive portions of ALM [3]. However, the use of IHC analysis for this marker to delineate the extent of the field area in ALM has also been questioned, as cyclin D1 protein expression has not been identified in all field cells that show FISH-determined increased copy numbers of 11q13 [3].

Pathologists often identify changes designated as "atypical melanocytic hyperplasia" (AMH) adjacent to melanomas in tissue sections [9]. However, histopathological and immunohistochemical criteria for distinction of AMH from otherwise benign sun-damaged melanocytes are not fully defined (i.e., such as the number of atypical melanocytes per high-power field/number of keratinocytes). Using loss of heterozygosity (LOH) analysis, Pashaei et al. [9] found increasingly higher defects in the hOGG1 gene progressing from AMH to adjacent melanoma in situ (60% *vs.* 80%) (Fig. 9.2). hOGG1 is an important gene for repair of free radical-induced DNA damage [9].

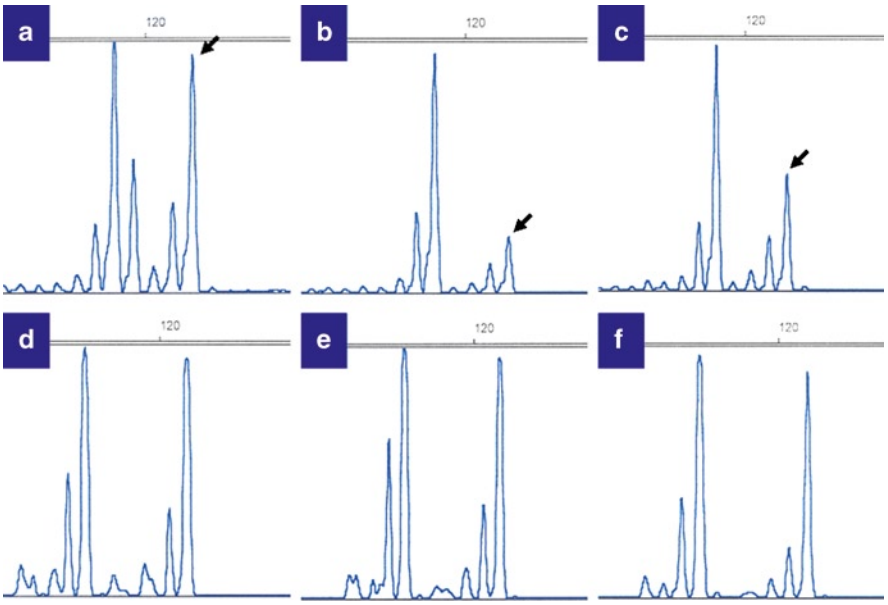

Fig. 9.2 Atypical melanocytic hyperplasia (AMH). DNA samples were subjected to analysis with microsatellite markers D3S1289 (**a**–**c**) for case #1 and D3S1300 (**d**–**f**) for case #2: (**a, d**) normal subcutaneous tissue; (**b**, **e**) melanoma in situ (MIS); (**c**, **f**) AMH. Normal tissues in both cases were heterozygous (informative) for their respective marker, as indicated by the presence of two alleles. The matched DNA samples of case #2 retained heterozygosity in both MIS (**e**) and AMH (**f**), whereas those from case #1 lost one larger allele [i.e., loss of heterozygosity (LOH)] in both MIS (**b**) and AMH (**c**). The allelic loss is illustrated by a shorter second allele in MIS (*arrow in* **b**) and AMH (*arrow in* **c**), as compared with the corresponding allele in normal subcutaneous tissue from the same patient (*arrow in* **a**) (From Pashaei et al. [9]. Reprinted with permission from Wiley, Copyright © 2008)

The authors suggested that AMH could represent an early microscopically-evident stage in melanoma development, and that its presence at the resection margins of melanoma warrants appropriate treatment or close clinical follow-up.

Other studies have used LOH and microsatellite instability testing [10–12], serial analysis of gene expression (SAGE) [13], and whole-genome expression profiling [14], to determine genetic aberrations in melanomas and matched normal (skin) samples.

In the future, the tailoring of surgical management, in order to ensure removal of both overt melanoma and its field cells, could be based on the results of molecular diagnostic tests, and may be particularly useful at those cutaneous sites where function or cosmetic outcome are impacted by current margin guidelines.

The above findings are somewhat analogous to the proposed model for pathogenesis of non-melanoma skin cancers (NMSC), which include actinic keratosis, squamous cell carcinoma (SCC), and basal cell carcinoma (BCC) [1, 6]. As with melanoma, DNA damage and gene mutations play a critical role in the development of these cancers. It is known that high levels of and/or long-term ultraviolet radiation (UVR) exposure induce persistent single-strand nuclear DNA breaks, pyrimidine dimer formation, UVR-signature mutations in TP53, CDKN2A/p16, RAS and PTCH genes, mitochondrial DNA (mtDNA) aberrations, and other types of DNA lesions in human skin [1, 6, 15–19]. Of note, in the absence of pyrimidine dimer repair, mutations in the DNA sequence can occur [19]. Changes in the form of C→T and CC→TT transitions are described as UVR-signature mutations [19]. In addition, epigenetic alterations in skin cancer cells (i.e., promoter hypo-/hypermethylation; histone and chromatin modifications) may also be a response to UVR [19]. These changes are paralleled by altered expression patterns of genes representing a variety of functional classes, including cell cycle control, apoptosis, transcription factors, receptors, transporters, signaling intermediates, growth factors, intermediary metabolism, hormones, translation factors, tumor suppressor genes, and oncogenes [1, 6, 15, 17, 19, 20]. Importantly, these alterations are detectable in chronically sun-exposed, but morphologically normal skin adjacent to both melanomas and other UVR-induced NMSC, and appear related to sunburn history in many cases [1, 15, 17, 19, 20]. For example, clones of morphologically normal p53-expressing keratinocytes are identified in chronic UVR-exposed epidermis, with the size and number of these clones directly proportional to both the level of sun exposure and increasing age (Fig. 9.3) [1, 21]. Approximately 60% of p53 clones in "normal" skin surrounding NMSC have missense TP53 gene mutations (the vast majority of which are UVR-signature mutations), resulting in the translation of an altered protein [21]. While no firm link between epidermal p53 clones and synchronous tumors (including precancers) has been identified, there appears to be some association between these clones and skin cancer development [21]. In addition, from studies of melanoma and NMSC, it has been shown that both tumors and perilesional "normal" tissue contain: (1) identical UVR-induced homoplasmic mtDNA mutations and (2) mtDNA deletions, with the perilesional skin containing different and/or more deletions in some cases (Fig. 9.3) [1, 15, 16]. Therefore, morphologically normal skin surrounding melanoma and NMSC may harbor clonal expansions of cells with mutant mtDNA (i.e., melanocytes and keratinocytes), analogous to p53 clones. Additional, but yet undefined, genetic alterations are required for subsequent development of the recognizable malignant phenotype of these cancers [1, 17, 19, 21].

It is postulated that many of the identified genes with UVR-altered expression play no role in UVR-induced oncogenesis, but may turn out to be useful markers of underlying genomic instability and photo-aging (see Chap. 20) [16, 17]. Nonetheless, the identification of those aberrations that are oncogenic, and their subsequent analysis in morphologically normal (i.e., tumor-free) marginal tissue of skin cancers, could potentially help determine risk for local relapse/recurrence, and thereby, define appropriate excision margins. For instance, Fabricius et al. [20] recently utilized polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA) to investigate the significance of telomerase activity (an RNA-dependent DNA polymerase required for continual division of

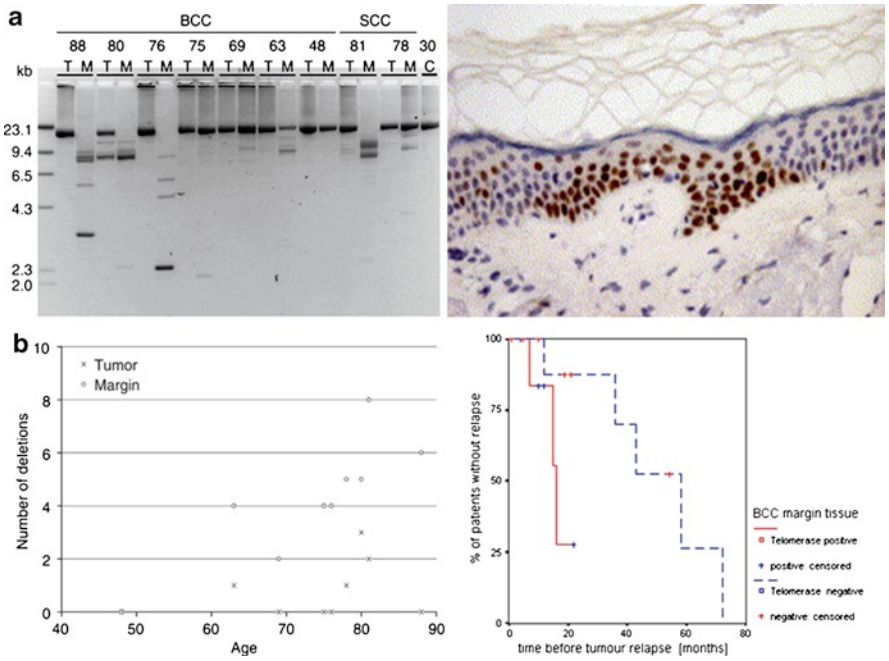

Fig. 9.3 *Left panels*: Mitochondrial DNA (mtDNA) mutations in basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) samples. (**a**) mtDNA isolated from the representative tumor (T) and respective tumor-free photoaged "margin" skin (M) was amplified and resolved. An inverted image of a 0.8% agarose gel is shown. The age of the patient from whom the tumor and margin samples were isolated is indicated above the T and M. DNA from a 30-year-old control patient (**c**) is shown at the *right*. A molecular weight marker is shown on the *left*; sizes of marker fragments are given in kb. Full-length mtDNA is indicated by the amplification of a 16.3 kb product (the only species observed in the control patient). mtDNAs harboring deletions are indicated by the presence of shorter fragments. Tumors contained predominantly or exclusively full-length mtDNA, while margin samples contained readily detectable mtDNA deletions. Older patients had more mtDNA deletions, and sometimes deleted mtDNAs were the only species detected in this assay. (**b**) The number of deleted mtDNA species is plotted versus patient age for the tumor and margin samples in (**a**). The number of deleted mtDNA species in margins, but not in tumors, strongly correlated with patient age (From Eshaghian et al. [16]. Reprinted with permission from Nature Publishing Group, Copyright © 2005). *Upper Right Panel*: High power view of an epidermal p53 clone. The p53 immunoreactive (*brown*), morphologically normal keratinocytes are sharply delineated from the negative epidermis (*blue*) (From Bäckvall et al. [21]. Reprinted with permission from Elsevier, Copyright © 2005). *Lower right panel*: Kaplan–Meier curve showing outcome in 25 patients with BCC in relation to telomerase activity within histopathological tumor-free margin tissue, as determined by PCR-ELISA. Telomerase-positivity was associated with a significantly shorter relapse-free period (From Fabricius et al. [20]. Reprinted with permission from Spandidos Publications, Copyright © 2003)

somatic cells) in the tumor marginal tissue of patients with BCC (Fig. 9.3). In all patients with histopathologically tumor-free margins, telomerase-positivity was associated with a significantly shorter recurrence-free period [20]. In addition to microsatellite instability assays [12], a number of studies have used cDNA/oligonucleotide microarray technology to investigate and compare gene expression profiles in NMSC and "normal" skin (perilesional in many cases) ([22–29], reviewed in [19]). Pathways and biological processes underlying BCC and SCC development were investigated in these studies, but genes dysregulated as a consequence of field cancerization effects were not determined ([22–29], reviewed in [19]). As a function of the methodologies used (i.e., comparative strategies based on tumor and "normal" cDNA co-hybridization), investigators sought to determine and validate (with PCR and IHC) differentially expressed genes, and not common and aberrantly expressed transcripts and proteins [22–29].

Molecular Evaluation of Clonal Origin and Histogenesis of Skin Cancers

The ability to differentiate between a cutaneous metastasis and a primary skin tumor has obvious significance for prognosis, therapy, and survival outcome in individual patients, as well as implications for broader epidemiological studies. Patients with a history of melanoma have an increased risk (1–8%) of developing a second primary tumor, but are also at varying risk (2–20%) of developing cutaneous metastases from any prior lesion [30–33]. In the case of non-melanoma tumors, it may be difficult to distinguish between a skin metastasis originating from a primary NMSC or from an internal malignancy [34].

Currently, a distinction between a second primary tumor and a recurrent/metastatic lesion is based on clinical history, changes on physical examination (i.e., proximity of lesions), and microscopic findings (i.e., similarity of cytomorphological and immunohistochemical features, presence or absence of a precursor lesion, epidermotropism, and/or epidermal connection). However, it can be difficult to definitively categorize a skin cancer as a primary lesion or a metastasis on the basis of comparative histopathological and immunophenotypic findings [34, 35].

A number of studies have investigated whether molecular technologies could be used to delineate a clonal relationship or independent origin among pairs of melanoma samples, and therefore have potential diagnostic utility in the differentiation of metastatic melanomas from second primary melanomas, or from other tumors of different origin/differentiation. In this respect, melanoma samples have been evaluated by techniques such as karyotyping, FISH, comparative genomic hybridization (CGH), X-chromosome inactivation analysis, LOH analysis, and single gene mutational analysis [30–32, 36–44]. These studies are discussed in more detail in Chap. 6. In the future, clinical and histopathological evaluation may be supplemented by molecular diagnostic testing, in order to definitively identify the origin of second malignancies in patients with melanoma.

The origin of non-melanoma cancers in the skin (i.e., second primary tumor *vs.* metastasis) could potentially be determined by molecular analysis, if the particular tumor(s) under investigation are associated with a "signature" genetic aberration. For example, most cases of dermatofibrosarcoma protuberans (DFSP) are associated with the presence of a chimeric COL1A1-PDGFB fusion gene [45]. However, the breakpoints within COL1A1 vary greatly, and are scattered among 20 exons [45]. Combined with the potential for multiple PDGFB exon 2 breakpoints, the likelihood of two DFSPs showing identical mutations would theoretically be 1 in 150,000. Kabumoto et al. [45] recently investigated the relationship between two temporally and anatomically separated DFSPs with divergent histopathological features in the same patient (i.e., interval 23 years, forehead *vs.* occipital scalp, and herringbone *vs.* storiform microscopic pattern). Because both tumors in this patient possessed identical chimeric COL1A1-PDGFB fusion genes, the authors reported that the metastatic nature of the second tumor was confirmed by molecular analysis (Fig. 9.4) [45]. In another study, Nagy et al. [46] confirmed what they described as a second field metachronous Merkel cell carcinoma (MCC) of the lip and the palatine tonsil using array-CGH and real-time PCR analysis (Fig. 9.5). The authors hypothesized that, in addition to comparable microscopic features and close proximity, the partly similar and partly different molecular patterns between the paired-tumors could be interpreted as: (1) indicating a genetic relationship between the cancers; (2) supporting a second field tumor; and (3) excluding the possibility of a metastasis [46]. Schrama et al. [47] reported that sequencing of the recently identified *Merkel cell polyomavirus* (MCPyV) genome was helpful in distinguishing between delayed metastasis and multiple primary MCCs in another patient. Hafner et al. [48], employing a combination of TP53 sequencing and LOH analysis of chromosome 9q, reported that molecular genetic testing demonstrated different genomic alterations between two adjacent BCCs, thereby excluding a possible implantation metastasis mechanism (i.e., direct inoculation of malignant cells to neighboring sites). In a study of patients who developed multiple primary cutaneous SCC following renal transplantation, Blokx et al. [49] reported that a combination of TP53 and

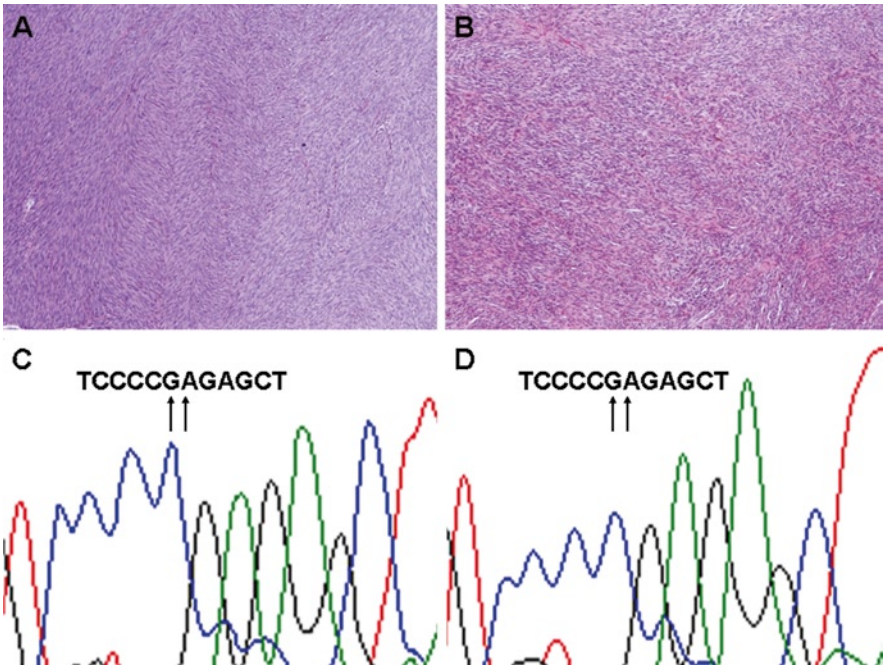

Fig. 9.4 Dermatofibrosarcoma Protuberans (DFSP). (**a**, **b**) Two temporally and anatomically separated DFSPs with divergent histopathological features in the same patient (i.e., interval 23 years; forehead *vs.* occipital scalp; and herringbone *vs.* storiform microscopic pattern). (**c**, **d**) Direct sequencing revealed identical chimeric COL1A1-PDGFB fusion genes (codon 3550 in COL1A1 exon 47 and codon 110 in PDGFB exon 2) in both tumors (Courtesy of Dr. Takenori Kabumoto, Niigata University School of Medicine, Niigata, Japan)

CDKN2A gene mutational analysis was useful in identifying the exact primary tumor responsible for any subsequent metastatic disease. Clonality studies have also been used to determine whether a wide variety of other cutaneous processes are either hyperplastic/hamartomatous or neoplastic in nature (Fig. 9.6). By definition, a hyperplastic or hamartomatous process does not originate from a single cell and is therefore polyclonal [50]. In contrast, neoplasms are regarded as monoclonal in origin [50]. Evidence concerning the possible clonal nature of seborrheic keratoses [50], dermatofibromas [51], and melanocytic nevi [52–55] is conflicting, with both monoclonal and polyclonal results found for all these lesions. For patients with multiple BCC, each tumor is likely to be of monoclonal origin, although individual lesions at anatomically distinct sites may not have arisen from the same progenitor cell [56].

Cutaneous metastases often represent locoregional recurrence, in-transit disease, or distant spread of a primary skin cancer (i.e., melanoma and NMSC). However, metastases to the skin from internal malignancies (reported in up to 10% of cases) must also be considered [34, 57–59]. Although the latter account for a small minority of all skin tumors, and are often a late manifestation of widely disseminated disease associated with a poor prognosis, cutaneous metastases may be the initial clinical presentation in as many as 20% of cases [34, 57–59]. The most common internal malignancies associated with cutaneous metastases are breast, lung, gastrointestinal, ovarian, head and neck, and renal cell carcinomas [34, 57–59]. The clinical and histopathological features of metastases from internal sites can vary depending on the tumor origin, and require differentiation from primary and metastatic cutaneous cancers. It may be difficult to definitively identify the site of the primary tumor on the basis of microscopic features alone, as up to 5% of metastatic tumors do not retain the morphologic characteristics of their organ of origin [34, 59, 60]. Clinical correlation

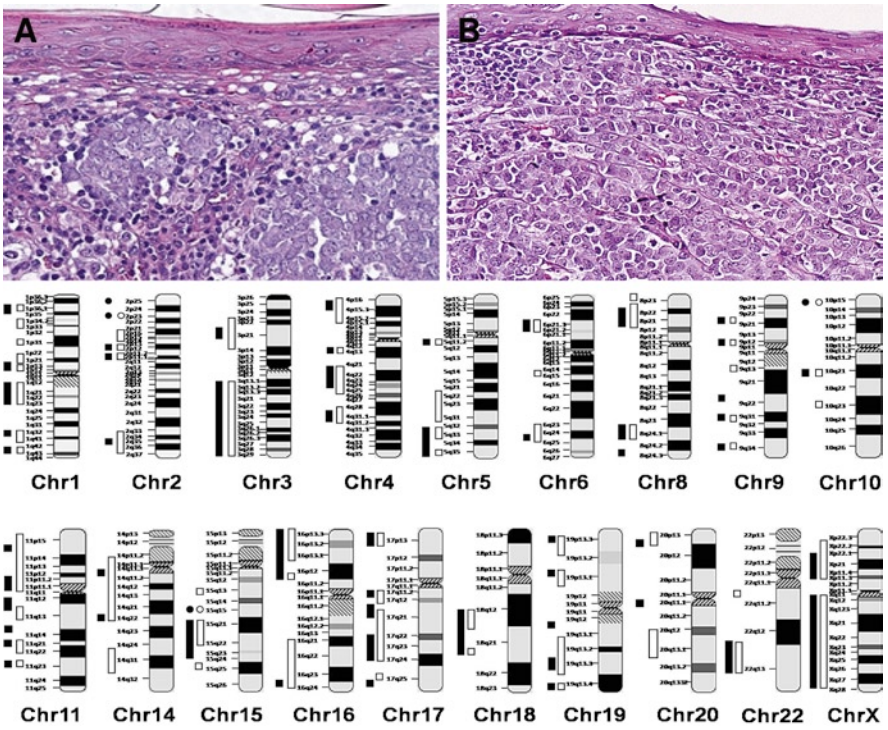

Fig. 9.5 (**a**) Merkel cell carcinoma (*MCC*) of the lip and (**b**) subsequent anaplastic carcinoma of the tonsil in the same patient. Both tumors exhibited chromogranin positivity and paranuclear dot-positivity for cytokeratin-20 (not shown). *Lower panel*: Copy number aberrations found in both tumors using array-based comparative genomic hybridization (*array-CGH*) and real-time polymerase chain reaction (*qPCR*). *Boxes* on the left side of each chromosome ideogram show regions of reduced copy number (losses of DNA in the tumor genome). *Circles* on the left side of each chromosome ideogram show regions of increased copy number (gains of DNA in the tumor genome). *Filled boxes* denote (**a**) MCC of lip and *empty boxes* denote (**b**) tonsillar tumor (Courtesy of Dr. László Puskás, Hungarian Academy of Sciences, Szeged, Hungary)

and adjunct IHC are required in many cases [34, 59, 60]. However, even with the use of a panel of immunohistochemical stains, the success rate in identifying the origin of metastatic tumors only approaches 67% [61]. Molecular technologies may have a role in determining the histogenesis of these tumors. Recently, Moyano et al. [62] reported cutaneous metastases of an alveolar soft part sarcoma, that were diagnosed by reverse transcription (RT)-PCR/sequencing identification of a type 2 alveolar soft part locus-transcription factor E3 (ASPL-TFE3) fusion, secondary to a der[17] t(X;17)(p11.2;q25) translocation (Fig. 9.6). Increasingly, gene expression profiling techniques, such as cDNA/oligonucleotide microarrays and RT-PCR, are being used to classify cancer types and determine diagnostic, prognostic, and drug response signatures [60]. For example, differences between the transcriptional profile of melanoma metastases and other solid tumors have been described [63]. The overall accuracy of transcriptome-based cancer classification appears to be high (~80–90%), based on the premise that metastatic tumors retain the gene expression patterns of their respective primaries [60]. Of note, a number of commercial gene profiling platforms are now available for the molecular classification of metastatic tumors and cancers of unknown primary origin (CUP) [60]. There are some anecdotal reports on the use of these tests (i.e., microarrays, RT-PCR, and microRNA expression) to identify the tissue origin of metastatic tumors and CUP in the skin [61, 64–66]. However, to date, these assays have not been validated for routine diagnostic use in cutaneous specimens.

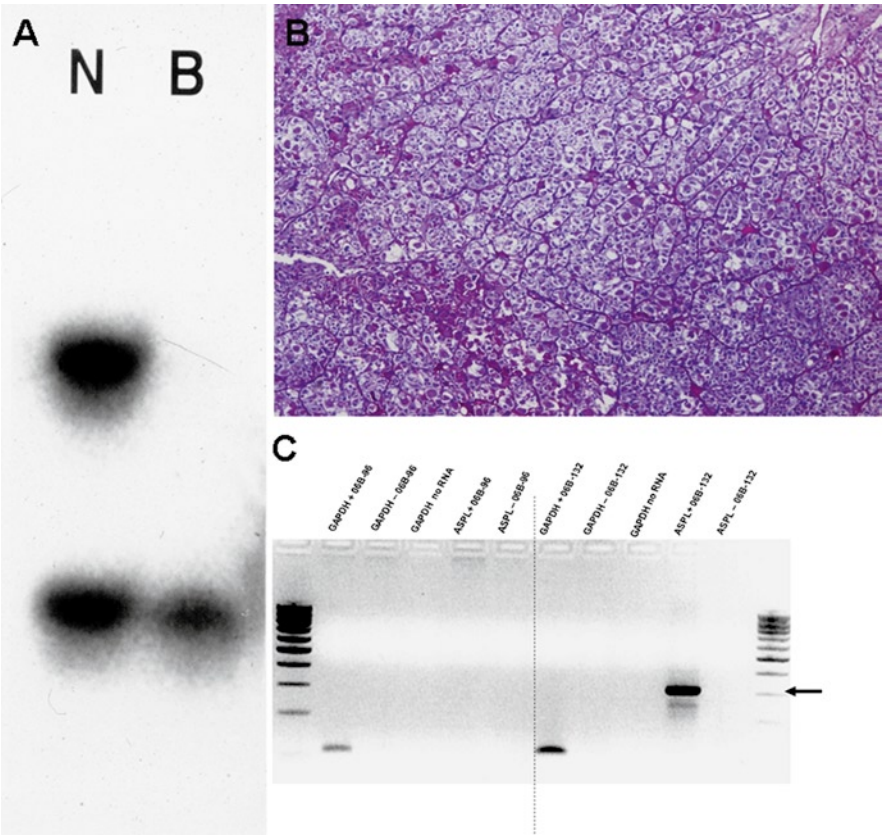

Fig. 9.6 (**a**) Basal cell carcinoma (BCC). Clonal analysis of normal skin (N) and BCC (B) from the same female subject, by a molecular assay depicting X-chromosome inactivation patterns (HUMARA). Absence of a band on upper right in the BCC denotes "non-random" X-chromosome inactivation, indicative of a monoclonal proliferation (Courtesy of Dr. Douglas S. Walsh). (**b**, **c**) Alveolar soft part sarcoma (ASPS). The neoplasm was composed of large cells with abundant clear or eosinophilic cytoplasm, arranged in a nested pattern, with surrounding capillary-sized vascular channels. RT-PCR/sequencing identified a type 2 alveolar soft part locus-transcription factor E3 (ASPL-TFE3) fusion (310 bp, *arrow*), secondary to der[17]t(X;17)(p11.2;q25), which is a specific finding in ASPS (Courtesy of Dr. Llucia Alos, University of Barcelona, Barcelona, Spain; and Dr. Enrique de Alava, University of Salamanca, Salamanca, Spain)

Interestingly, UVR-fingerprint mutation analysis may have some utility in determining the origin of clinically and histopathologically equivocal skin tumors (Fig. 9.7). As previously described, UVR induces a variety of photoproducts in DNA, which have been implicated in human skin carcinogenesis [19, 67, 68]. The two most frequent types are: (1) *cis-syn* cyclobutane pyrimidine dimers (CPD) and (2) pyrimidine pyrimidone photoproducts, or 6-4 photoproducts [19, 67, 68]. NMSC commonly show C→T transitions, and in particular, a high frequency of CC→TT transitions, accounting for up to 39% of the mutations found in SCC, and up to 18% of those in BCC [19, 67, 68]. While C→T transitions can be detected in all tumor types (albeit at a lower frequency than in NMSC), tandem CC→TT transitions are extremely rare in internal malignancies [67]. Theoretically, the detection of C→T and/or CC→TT transitions at dipyrimidine sequences (i.e., UVR-signature mutations) in a test sample would provide strong evidence that UVR played a role in tumor pathogenesis (i.e., the tumor being examined was associated with past exposure to UVR, and therefore most likely originated in the skin).

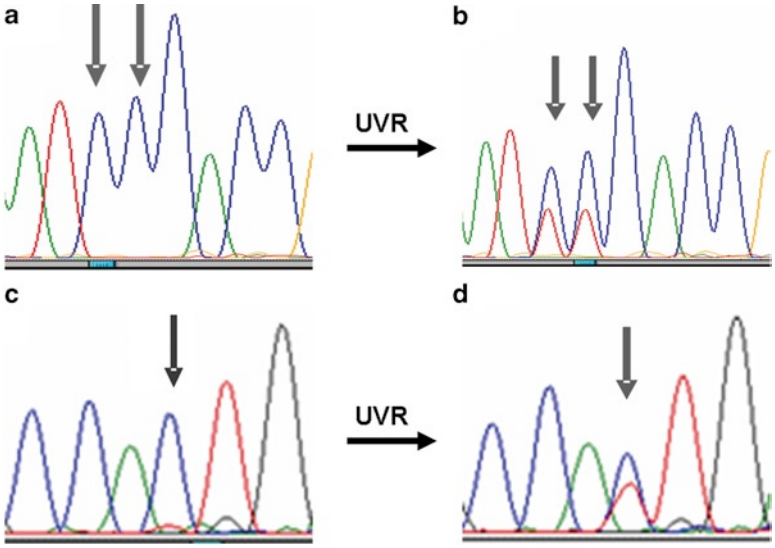

Fig. 9.7 Ultraviolet radiation (UVR)-signature mutations. UVR induces the formation of cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts in DNA. In instances of inappropriate DNA repair mechanisms, typical UVR-fingerprint mutations (C→T, CC→TT transitions) are introduced. *Upper panel*: Electropherogram of DNA from (**a**) normal skin (wild-type) and (**b**) basal cell carcinoma (BCC) showing an UVR-induced CC→TT transition, resulting from a pre-mutagenic CPD lesion. *Lower panel*: Electropherogram of DNA from (**c**) normal skin (wild-type) and (**d**) BCC showing an UVR-induced C→T transition, resulting from a pre-mutagenic 6-4 photoproduct. The detection of UVR-signature mutations provides strong evidence that the tumor originated in the skin (Courtesy of Drs. Peter Wolf and Ellen Heitzer, Medical University of Graz, Graz, Austria)

References

1. Dakubo GD, Jakupciak JP, Birch-Machin MA, et al. Clinical implications and utility of field cancerization. Cancer Cell Int. 2007;7:2.
2. Bastian BC, Kashani-Sabet M, Hamm H, et al. Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. Cancer Res. 2000;60:1968–73.
3. North JP, Kageshita T, Pinkel D, et al. Distribution and significance of occult intraepidermal tumor cells surrounding primary melanoma. J Invest Dermatol. 2008;128:2024–30.
4. Hinshaw M. Use of genetic tools to control tumor margins in melanoma. Arch Dermatol. 2009;145:475–7.
5. Takata M, Murata H, Saida T. Molecular pathogenesis of malignant melanoma: a different perspective from the studies of melanocytic nevus and acral melanoma. Pigment Cell Melanoma Res. 2010;23:64–71.
6. Takata M, Saida T. Early cancers of the skin: clinical, histopathological, and molecular characteristics. Int J Clin Oncol. 2005;10:391–7.
7. Whalen J, Leone D. Mohs micrographic surgery for the treatment of malignant melanoma. Clin Dermatol. 2009;27:597–602.
8. Griego RD, Zitelli JA. Mohs micrographic surgery using HMB-45 for a recurrent acral melanoma. Dermatol Surg. 1998;24:1003–6.
9. Pashaei S, Li L, Zhang H, Spencer HJ, et al. Concordant loss of heterozygosity of DNA repair gene, hOGG1, in melanoma in situ and atypical melanocytic hyperplasia. J Cutan Pathol. 2008;35:525–31.
10. Peris K, Keller G, Chimenti S, et al. Microsatellite instability and loss of heterozygosity in melanoma. J Invest Dermatol. 1995;105:625–8.
11. Funk JO, Schiller PI, Barrett MT, et al. p16INK4a expression is frequently decreased and associated with 9p21 loss of heterozygosity in sporadic melanoma. J Cutan Pathol. 1998;25:291–6.
12. Quinn AG, Healy E, Rehman I, et al. Microsatellite instability in human non-melanoma and melanoma skin cancer. J Invest Dermatol. 1995;104:309–12.
13. Smith AP, Weeraratna AT, Spears JR, et al. SAGE identification and fluorescence imaging analysis of genes and transcripts in melanomas and precursor lesions. Cancer Biol Ther. 2004;3:104–9.

14. Smith AP, Hoek K, Becker D. Whole-genome expression profiling of the melanoma progression pathway reveals marked molecular differences between nevi/melanoma in situ and advanced-stage melanomas. Cancer Biol Ther. 2005;4:1018–29.
15. Hubbard K, Steinberg ML, Hill H, Orlow I. Mitochondrial DNA deletions in skin from melanoma patients. Ethn Dis. 2008;18 Suppl 2:S2-38–43.
16. Eshaghian A, Vleugels RA, Canter JA, et al. Mitochondrial DNA deletions serve as biomarkers of aging in the skin, but are typically absent in nonmelanoma skin cancers. J Invest Dermatol. 2006;126:336–44.
17. Steinberg ML, Hubbard K, Utti C, et al. Patterns of persistent DNA damage associated with sun exposure and the glutathione S-transferase M1 genotype in melanoma patients. Photochem Photobiol. 2009;85:379–86.
18. Brozyna A, Zbytek B, Granese J, et al. Mechanism of UV-related carcinogenesis and its contribution to nevi/melanoma. Exp Rev Dermatol. 2007;2:451–69.
19. Greinert R. Skin cancer: new markers for better prevention. Pathobiology. 2009;76:64–81.
20. Fabricius EM, Bezeluk A, Kruse-Boitschenko U, et al. Clinical significance of telomerase activity in basal cell carcinomas and in tumour-free surgical margins. Int J Oncol. 2003;23:1389–99.
21. Bäckvall H, Asplund A, Gustafsson A, et al. Genetic tumor archeology: microdissection and genetic heterogeneity in squamous and basal cell carcinoma. Mutat Res. 2005;571:65–79.
22. Asplund A, Gry Björklund M, Sundquist C, et al. Expression profiling of microdissected cell populations selected from basal cells in normal epidermis and basal cell carcinoma. Br J Dermatol. 2008;158:527–38.
23. Yu M, Zloty D, Cowan B, et al. Superficial, nodular, and morpheiform basal-cell carcinomas exhibit distinct gene expression profiles. J Invest Dermatol. 2008;128:1797–805.
24. Howell BG, Solish N, Lu C, et al. Microarray profiles of human basal cell carcinoma: insights into tumor growth and behavior. J Dermatol Sci. 2005;39:39–51.
25. Bonifas JM, Pennypacker S, Chuang PT, et al. Activation of expression of hedgehog target genes in basal cell carcinomas. J Invest Dermatol. 2001;116:739–42.
26. O'Driscoll L, McMorrow J, Doolan P, et al. Investigation of the molecular profile of basal cell carcinoma using whole genome microarrays. Mol Cancer. 2006;5:74.
27. Nindl I, Dang C, Forschner T, et al. Identification of differentially expressed genes in cutaneous squamous cell carcinoma by microarray expression profiling. Mol Cancer. 2006;5:30.
28. Dang C, Gottschling M, Manning K, et al. Identification of dysregulated genes in cutaneous squamous cell carcinoma. Oncol Rep. 2006;16:513–9.
29. Serewko MM, Popa C, Dahler AL, et al. Alterations in gene expression and activity during squamous cell carcinoma development. Cancer Res. 2002;62:3759–65.
30. Edinger JT, Radfar A, Jukic DM. Two cutaneous malignant melanomas at the same anatomic site: a case report with molecular evaluation. J Cutan Pathol. 2009;36 Suppl 1:74–9.
31. Bahrami S, Cheng L, Wang M, et al. Clonal relationships between epidermotropic metastatic melanomas and their primary lesions: a loss of heterozygosity and X-chromosome inactivation-based analysis. Mod Pathol. 2007;20:821–7.
32. Katona TM, Jones TD, Wang M, et al. Genetically heterogeneous and clonally unrelated metastases may arise in patients with cutaneous melanoma. Am J Surg Pathol. 2007;31:1029–37.
33. Savoia P, Fava P, Nardò T, et al. Skin metastases of malignant melanoma: a clinical and prognostic survey. Melanoma Res. 2009;19:321–6.
34. Hussein MR. Skin metastasis: a pathologist's perspective. J Cutan Pathol. 2010;37:306–7.
35. Guerriere-Kovach PM, Hunt EL, Patterson JW, et al. Primary melanoma of the skin and cutaneous melanomatous metastases: comparative histologic features and immunophenotypes. Am J Clin Pathol. 2004; 122:70–7.
36. Orlow I, Tommasi DV, Bloom B, et al. Evaluation of the clonal origin of multiple primary melanomas using molecular profiling. J Invest Dermatol. 2009;129:1972–82.
37. Quezado MM, Abati AD, Albuquerque AV, et al. Morphologic diversity in malignant melanoma: the potential use of microdissection and the polymerase chain reaction for diagnosis. Mod Pathol. 1998;11:1010–5.
38. Blokx WA, Lesterhuis WJ, Andriessen MP. CDKN2A (INK4A-ARF) mutation analysis to distinguish cutaneous melanoma metastasis from a second primary melanoma. Am J Surg Pathol. 2007;31:637–41. Erratum in: Am J Surg Pathol. 2007;31:1137. Lesterhuis, Joost J [corrected to Lesterhuis, W Joost]; Punt, Kees J A [corrected to Punt, Cornelis J A].
39. Balaban GB, Herlyn M, Clark Jr WH, et al. Karyotypic evolution in human malignant melanoma. Cancer Genet Cytogenet. 1986;19:113–22.
40. Balázs M, Adám Z, Treszl A, et al. Chromosomal imbalances in primary and metastatic melanomas revealed by comparative genomic hybridization. Cytometry. 2001;46:222–32.
41. Balázs M, Adám Z, Bégány A, et al. Involvement of chromosome losses in the progression and metastasis formation of a human malignant melanoma. Cancer Genet Cytogenet. 1999;109:114–8.

42. Morita R, Fujimoto A, Hatta N, et al. Comparison of genetic profiles between primary melanomas and their metastases reveals genetic alterations and clonal evolution during progression. J Invest Dermatol. 1998;111:919–24.
43. Torres-Cabala CA, Wang WL, Trent J, et al. Correlation between KIT expression and KIT mutation in melanoma: a study of 173 cases with emphasis on the acral-lentiginous/mucosal type. Mod Pathol. 2009;22:1446–56.
44. Begg CB, Eng KH, Hummer AJ. Statistical tests for clonality. Biometrics. 2007;63:522–30.
45. Kabumoto T, Fujiwara H, Kariya N, et al. Skin metastasis of dermatofibrosarcoma protuberans with distinct morphological features, confirmed by COL1A1-PDGFB fusion gene analysis. J Am Acad Dermatol. 2009;61:130–2.
46. Nagy J, Fehér LZ, Sonkodi I, et al. A second field metachronous Merkel cell carcinoma of the lip and the palatine tonsil confirmed by microarray-based comparative genomic hybridisation. Virchows Arch. 2005;446:278–86.
47. Schrama D, Thiemann A, Houben R, et al. Distinction of 2 different primary Merkel cell carcinomas in 1 patient by Merkel cell polyomavirus genome analysis. Arch Dermatol. 2010;146:687–9.
48. Hafner C, Hartmann A, Knuechel R, et al. Molecular genetic analysis excludes implantation metastasis of basal cell carcinomas. Arch Pathol Lab Med. 2003;127:1221–4.
49. Blokx WA, Ruiter DJ, Verdijk MA, et al. INK4-ARF and p53 mutations in metastatic cutaneous squamous cell carcinoma: case report and archival study on the use of Ink4a-ARF and p53 mutation analysis in identification of the corresponding primary tumor. Am J Surg Pathol. 2005;29:125–30.
50. Nakamura H, Hirota S, Adachi S, et al. Clonal nature of seborrheic keratosis demonstrated by using the polymorphism of the human androgen receptor locus as a marker. J Invest Dermatol. 2001;116:506–10.
51. Hui P, Glusac EJ, Sinard JH, et al. Clonal analysis of cutaneous fibrous histiocytoma (dermatofibroma). J Cutan Pathol. 2002;29:385–9.
52. Indsto JO, Cachia AR, Kefford RF, et al. X inactivation, DNA deletion, and microsatellite instability in common acquired melanocytic nevi. Clin Cancer Res. 2001;7:4054–9.
53. Maitra A, Gazdar AF, Moore TO, et al. Loss of heterozygosity analysis of cutaneous melanoma and benign melanocytic nevi: laser capture microdissection demonstrates clonal genetic changes in acquired nevocellular nevi. Hum Pathol. 2002;33:191–7.
54. Harada M, Suzuki M, Ikeda T, et al. Clonality in nevocellular nevus and melanoma: an expression-based clonality analysis at the X-linked genes by polymerase chain reaction. J Invest Dermatol. 1997;109:656–60.
55. Robinson WA, Lemon M, Elefanty A, et al. Human acquired naevi are clonal. Melanoma Res. 1998;8:499–503.
56. Heitzer E, Quehenberger F, Wolf P. Polyclonality of multiple sporadic basal cell carcinomas. J Invest Dermatol. 2009;129:1586–9.
57. Rolz-Cruz G, Kim CC. Tumor invasion of the skin. Dermatol Clin. 2008;26:89–102. viii.
58. Nashan D, Müller ML, Braun-Falco M, et al. Cutaneous metastases of visceral tumours: a review. J Cancer Res Clin Oncol. 2009;135:1–14.
59. Sariya D, Ruth K, Adams-McDonnell R, et al. Clinicopathologic correlation of cutaneous metastases: experience from a cancer center. Arch Dermatol. 2007;143:613–20.
60. Bender RA, Erlander MG. Molecular classification of unknown primary cancer. Semin Oncol. 2009;36:38–43.
61. Ma XJ, Patel R, Wang X, et al. Molecular classification of human cancers using a 92-gene real-time quantitative polymerase chain reaction assay. Arch Pathol Lab Med. 2006;130:465–73.
62. Moyano S, Aguilera P, Petit A, et al. Alveolar soft part sarcoma presenting with cutaneous metastases: report of a case with immunohistochemical and molecular characterization. J Am Acad Dermatol. 2009;61:117–20.
63. Wang E, Panelli MC, Zavaglia K, et al. Melanoma-restricted genes. J Transl Med. 2004;2:34.
64. Bridgewater J, van Laar R, Floore A, et al. Gene expression profiling may improve diagnosis in patients with carcinoma of unknown primary. Br J Cancer. 2008;98:1425–30.
65. Horlings HM, van Laar RK, Kerst JM, et al. Gene expression profiling to identify the histogenetic origin of metastatic adenocarcinomas of unknown primary. J Clin Oncol. 2008;26:4435–41.
66. Rosenfeld N, Aharonov R, Meiri E, et al. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol. 2008;26:462–9.
67. Missero C, D'Errico M, Dotto GP, et al. The molecular basis of skin carcinogenesis. In: Coleman WB, Tsongalis GJ, editors. The molecular basis of human cancer. Totowa: Humana Press; 2002. p. 407–25.
68. Bolshakov S, Walker CM, Strom SS, et al. p53 mutations in human aggressive and nonaggressive basal and squamous cell carcinomas. Clin Cancer Res. 2003;9:228–34.

Chapter 10
Mycosis Fungoides and Related Lesions

Michael J. Murphy

More than 65% of all cutaneous lymphomas are T-cell disorders. Cutaneous T-cell lymphomas (CTCL) represent a heterogeneous group of non-Hodgkin lymphomas that share the common feature of malignant T-cell infiltration of the skin (Table 10.1) [1, 2]. The most common forms of CTCL are mycosis fungoides (MF) and its leukemic counterpart Sézary syndrome (SS), accounting for ~44% and ~3% of cases, respectively. MF evolves through several skin-localized clinical stages (patch, plaque, and tumor stage) with extracutaneous involvement seen as a late manifestation of this disease. SS is characterized by generalized erythroderma, lymphadenopathy, pruritus, and leukemic cells in the peripheral blood, and is associated with a poorer prognosis [1, 2]. Diagnostic and staging systems for MF have historically been based on the clinical and histopathological features of the disease, and include primary tumor, lymph node, metastasis, and peripheral blood (TNMB) parameters (Table 10.2) [3, 4]. Similar to other cutaneous and systemic lymphomas, the prognosis for patients with MF is primarily related to the stage of disease at presentation [4, 5]. However, some cases, particularly early MF lesions, can demonstrate nonspecific clinical and/or histopathological features, and staging systems cannot predict outcome in all patients [4–6]. While many patients have relatively indolent disease, others experience rapidly progressive and often fatal outcomes [4, 5]. It has been proposed that ancillary molecular technologies with high sensitivities and specificities may be useful for more accurate diagnosis, staging, and prognostication of patients with MF. Based on revised International Society for Cutaneous Lymphomas/European Organization of Research and Treatment of Cancer (ISCL/EORTC) guidelines, molecular tests (Southern blot and polymerase chain reaction-based) are now recommended for the initial evaluation/staging of patients with MF [3]. It is anticipated that future studies, which incorporate these molecular techniques, will provide additional prognostic information for patients with CTCL, allowing further refinements of classification and staging guidelines [3].

The purpose of this chapter is to introduce the concepts of molecular testing in patients with CTCL. Studies of T-cell clonality, karyotyping and other molecular technologies, such as in situ hybridization (ISH), comparative genomic hybridization (CGH), array-CGH, loss of heterozygosity (LOH) analysis, and cDNA/oligonucleotide microarrays, will be discussed. The advantages and limitations of these technologies will be described, in addition to the concepts of: (1) clonal dermatoses and cutaneous T-cell lymphoid dyscrasia; (2) pseudomonoclonality; (3) stable clones

M.J. Murphy (✉)
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, 21 South Rd., Farmington, CT 06030, USA
e-mail: drmichaelmurphy@netscape.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_10,

Table 10.1 WHO-EORTC classification of cutaneous lymphomas with primary cutaneous manifestations (2005) (Adapted from Willemze et al. [1])

Cutaneous T-cell and NK-cell lymphomas
Mycosis fungoides (MF)
MF variants and subtypes
Folliculotropic MF
Pagetoid reticulosis
Granulomatous slack skin
Sézary syndrome
Adult T-cell leukemia/lymphoma
Primary cutaneous CD30+ lymphoproliferative disorders
Primary cutaneous anaplastic large cell lymphoma (C-ALCL)
Lymphomatoid papulosis (LyP)
Subcutaneous panniculitis-like T-cell lymphoma
Extranodal NK/T-cell lymphoma, nasal type
Primary cutaneous peripheral T-cell lymphoma, unspecified
Primary cutaneous aggressive epidermotropic CD8+ T-cell lymphoma (provisional)
Cutaneous gamma-delta (γ/δ) T-cell lymphoma (provisional)
Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma (provisional)
Cutaneous B-cell lymphomas
Primary cutaneous marginal zone B-cell lymphoma
Primary cutaneous follicle center lymphoma
Primary cutaneous diffuse large B-cell lymphoma, leg type
Primary cutaneous diffuse large B-cell lymphoma, other
Intravascular large B-cell lymphoma
Precursor hematologic neoplasm
CD4+/CD56+ hematodermic neoplasm (blastic NK-cell lymphoma; now known as blastic plasmacytoid dendritic cell neoplasm)

versus clonal heterogeneity; and (4) composite lymphomas, dual lineage rearrangements and lineage infidelity. Most studies have been performed on samples from patients with MF, and therefore the discussion will be largely directed toward this disease. Nonetheless, the concepts described are theoretically applicable to any form of CTCL. There are numerous reports investigating the molecular biology and pathogenesis of CTCL [2, 7–9]. However, only those studies with direct clinical applicability will be discussed. An exhaustive list of prior studies will not be provided. Rather the scope of this chapter is to provide a focused view of how molecular assays can aid in the diagnosis, staging, and prognostication of CTCL. In addition, the role of such testing in monitoring response to treatment, defining minimal residual disease and remission, detecting early relapse, and identifying novel therapeutic targets in patients with CTCL will be described.

Diagnosis of CTCL

In many instances, the diagnosis of MF can be made with confidence on the basis of combined clinical and histopathological features of the disease [2, 3, 6]. Diagnostic microscopic changes include prominent epidermotropism of cytologically atypical T-cells both singly and in small collections (i.e., Pautrier's microabscesses) and/or a band-like dermal infiltrate of cytologically atypical T-cells [3]. Several different grading systems, purportedly incorporating reliable and reproducible criteria for the diagnosis of early lesions, have been proposed [3, 10–12]. However, the clinical presentation of CTCL can be highly variable, and the microscopic features of early MF are

Table 10.2 ISCL/EORTC revision to the classification of mycosis fungoides (MF) and Sézary syndrome (SS), incorporating results of molecular studies for T-cell clonality (*italics*) (Adapted from Olsen et al. [3])

TNMB stages	
Skin	
T1	Limited patches, papules, and/or plaques covering <10% of the skin surface: T1a (patch only) versus T1b (plaque ± patch)
T2	Patches, papules, or plaques covering ≥10% of the skin surface: T2a (patch only) versus T2b (plaque ± patch)
T3	One or more tumors (≥1 cm diameter)
T4	Confluence of erythema covering ≥80% body surface area
Node	
N0	No clinically abnormal peripheral lymph nodes; biopsy not required
N1	Clinically abnormal peripheral lymph nodes; histopathology Dutch grade 1 or NCI LN0–2
N1a	*Clone negative*
N1b	*Clone positive*
N2	Clinically abnormal peripheral lymph nodes; histopathology Dutch grade 2 or NCI LN3
N2a	*Clone negative*
N2b	*Clone positive*
N3	Clinically abnormal peripheral lymph nodes; histopathology Dutch grades 3–4 or NCI LN4; *clone positive or negative*
Nx	Clinically abnormal peripheral lymph nodes; no histologic confirmation
Visceral	
M0	No visceral organ involvement
M1	Visceral involvement
Blood	
B0	Absence of significant blood involvement: ≤5% of peripheral blood lymphocytes are atypical (Sézary) cells
B0a	*Clone negative*
B0b	*Clone positive*
B1	Low blood tumor burden: >5% of peripheral blood lymphocytes are atypical (Sézary) cells, but do not meet the criteria of B2
B1a	*Clone negative*
B1b	*Clone positive*
B2	High blood tumor burden: >1,000/μL Sézary cells *with positive clone*

often nonspecific and overlap with a number of otherwise benign dermatoses, such that a definitive diagnosis may be particularly difficult in some patients [3, 6]. Furthermore, there is significant interobserver variability among pathologists in the biopsy interpretation of early CTCL [6].

The increasing availability of antibodies applicable to formalin-fixed paraffin-embedded (FFPE) tissue has resulted in the widespread use of supplementary immunohistochemistry (IHC) in the diagnosis of human diseases. IHC has been used as an adjunctive tool to differentiate malignant from benign T-cells in cutaneous lymphocytic infiltrates. The restriction of T-cell subset antigens (i.e., CD4 *vs.* CD8) and/or the loss of one or more pan-T-cell antigens (i.e., CD2, CD3, CD5, and CD7) have been used as immunophenotypic criteria for the diagnosis of malignant T-cell infiltrates [6]. However, MF cells are immunophenotypically heterogeneous, and many benign dermatoses show overlapping immunophenotypic findings with CTCL, thus reducing the sensitivity and specificity of IHC in distinguishing benign from malignant infiltrates [6, 13, 14]. Rarely, flow cytometric analysis of T-cells recovered from skin biopsies has been used to evaluate immunophenotypic aberrations in CTCL [13]. Unlike cutaneous B-cell lymphomas and plasma cell disorders (i.e., kappa *vs.* lambda light chain restriction), there is no reliable protein marker for malignancy in CTCL.

Ancillary molecular testing, which is based on the detection of genetic changes within tumor cells using available technologies, has been proposed as a means to achieve a more accurate diagnosis of CTCL. Importantly, a holistic approach to the diagnosis of CTCL is required, and molecular results should be interpreted in the context of available clinical, histopathological, and immunophenotypic data. These technologies include karyotyping, loss of heterozygosity (LOH) analysis, cDNA microarrays, and most commonly, T-cell clonality studies that are based on the detection of T-cell receptor gene rearrangements (TCR-GRs) by either Southern blot analysis (SBA) or polymerase chain reaction (PCR) [6]. Of note, the costs for PCR-based clonality studies and IHC in suspected MF lesions are comparable [15]. An evaluation of a skin biopsy specimen for MF typically requires a minimum of four IHC-based stains (i.e., CD3, CD4, CD7, CD8). Thurber et al. [15] point out that the technical and professional Medicare reimbursement of four IHC stains (current procedural terminology 88342) is similar to that for PCR-based analysis of TCR-GRs (current procedural terminology 83891, 83909 × 3, 83900, 83901 × 6, 83912).

T-Cell Receptor and T-Cell Clonality

During early T-cell differentiation, genes encoding the T-cell receptor (TCR) are formed by stepwise rearrangement of variable (V), diversity (D), and joining (J) gene segments, referred to as V(D)J recombination (Fig. 10.1) [16–18]. The many different combinations of V, D, and J gene segments (combinatorial repertoire) can result in ~3×10^6 molecules for TCR-αβ and ~5×10^3 molecules for TCR-γδ. In addition, deletion and random insertion of nucleotides occur at the junction sites of the V, D, and J gene segments during the rearrangement process, resulting in highly diverse junctional regions, which significantly contribute to the total repertoire of TCR molecules, estimated to be $>10^{12}$. As TCR-GRs occur sequentially in the earliest stages of lymphoid differentiation, they are present in almost all immature and mature lymphoid cells. Functionally rearranged TCR genes result in surface membrane expression of TCR-αβ (95% of mature T-cells) or TCR-γδ molecules [16–18].

Since lymphomas and leukemias are theoretically derived from a single transformed cell, the tumor cells of a T-lymphoid malignancy contain, in principle, identical (clonal) TCR-GRs, reflecting a monoclonal T-cell population [16–18]. The presence of polyclonally-activated reactive T-cells, in the setting of a benign inflammatory process, would be reflected in heterogeneity of TCR-GRs (i.e., the rearranged genes will vary in both size and nucleotide sequence, as a function of differences in the gene segments used and variable nucleotide deletion/insertion at junctional regions) [16–18].

Evaluation of T-Cell Clonality in Cutaneous Lymphocytic Infiltrates

The determination of T-cell clonality (monoclonal *vs.* polyclonal), based on the presence or absence of clonally rearranged TCR genes, is now established as an important method to determine if a lymphocytic infiltrate is benign (inflammatory dermatosis or pseudolymphoma) or malignant (CTCL) [15, 19–46].

SBA is the gold standard for the detection of T-cell clonality, because of the low risk of false-negative and/or false-positive results if the optimal restriction enzyme(s) and probe(s) are utilized. However, SBA is rarely used to investigate T-cell clonality status in cutaneous lymphocytic infiltrates [19–22]. It has several disadvantages compared to PCR-based techniques, including the need for fresh or frozen tissue (due to its requirement for larger amounts of higher quality DNA), high cost, prolonged turnaround time, technical difficulty including labor-intensive hybridization

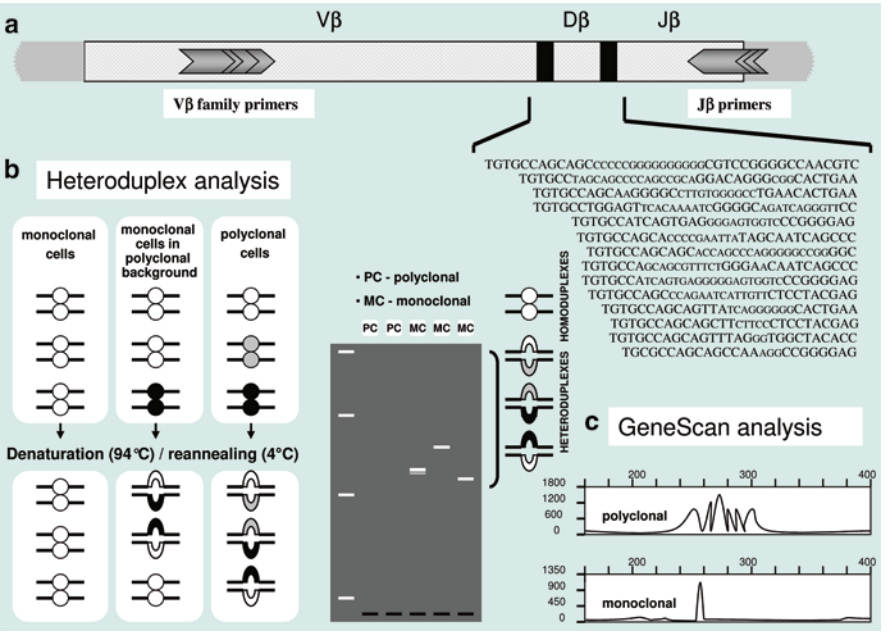

Fig. 10.1 Heteroduplex analysis (*HDA)* and GeneScan (*GS*) fragment analysis of PCR products from TCR gene rearrangement clonality testing. (**a**) Rearranged TCR-β genes show heterogeneous junctional regions that differ in size and nucleotide composition, with V, D, and J germline nucleotides (*large capital*) and randomly inserted nucleotides (*small capital*). This junctional heterogeneity can be used to differentiate between polyclonal and monoclonal PCR products based on differences in size and composition (HDA) or size only (GS). (**b**) In HDA, PCR products are denatured and allowed to rapidly reanneal. Monoclonal PCR products result in homoduplexes (distinct bands), whereas polyclonal PCR products form predominantly heteroduplexes (smear of differentially migrating fragments). (**c**) In GS, fluorochrome-labeled PCR products are denatured for high-resolution fragment analysis of the single-stranded fragments. Monoclonal PCR products of identical size result in a peak, whereas polyclonal PCR products demonstrate a Gaussian size distribution (Courtesy of Dr. Zendee Elaba, Department of Pathology, Hartford Hospital, Hartford, CT, USA: Modified from Groenen et al. [16])

with radioactive probes, and a lower level of sensitivity (i.e., it requires that the clonal population represents at least 5% of the total T-cell infiltrate) [19–22]. While SBA readily detects T-cell monoclonality in advanced stage CTCL, the lower sensitivity of SBA in detecting T-cell clones is most evident in early patch and limited plaque stage disease. Of note, skin biopsy specimens with dense lymphoid infiltrates and marked nuclear atypia of T-cells are more likely to demonstrate T-cell monoclonality by SBA [21].

PCR-based methods are more widely utilized, as a result of increased sensitivity (i.e., detection of clonal cells when they represent between 0.001% and 1% of the infiltrate), speed, reduced labor costs, lower amounts of DNA required, no requirement for radioactive substances, and applicability to archival FFPE tissue (i.e., lower quality DNA) [16–18, 46]. For PCR-based assays, rearrangements of the TCR genes can be detected by amplifying extracted DNA using primers directed against the β-, δ- and/or γ-chains (Fig. 10.1) [17]. Of note, the TCR-γ gene is most commonly utilized, in view of: (1) the limited number of V and J region gene segments and lack of D segments, thereby requiring fewer specific primers (i.e., $V_{\gamma 1-8}$, $V_{\gamma 9}$, $V_{\gamma 10}$, and $V_{\gamma 11}$) to detect as many TCR-γ GRs as possible; and (2) TCR-γ GRs are found not only in TCR-γδ+ malignancies (where they are functional), but also in TCR-αβ+ malignancies (where nonfunctional TCR-γ GRs are observed as a consequence of the chronological order of rearrangements of TCR genes in progenitor T-cells; of note, T-cells retain at least one intact rearranged TCR-γ allele, even though most no longer express TCR-γ

proteins on their surfaces) [17]. Molecular assays directed against the TCR-γ locus have a relatively high sensitivity for detection of T-cell clonality, particularly in pure monoclonal populations, and have demonstrated their diagnostic utility in the evaluation of cutaneous T-cell processes [15, 22–41]. It has been suggested that the presence of a monoclonal neoplastic population within a polyclonal reactive background may be more readily detected with assays directed against the TCR-β locus, because of its greater combinational diversity [41, 42]. However, an assessment of TCR-β clonality is rarely undertaken in the evaluation of cutaneous T-cell processes [41, 42]. In theory, combined TCR-γ and TCR-β gene rearrangement analysis could increase the sensitivity of clonality detection [41].

PCR products are analyzed using various electrophoretic techniques (Fig. 10.1), including agarose gel (AG) [23, 24], non-denaturing polyacrylamide gel (PAGE) [22, 23, 25–27], single strand conformational polymorphism analysis (SSCP) [28, 29], denaturing gradient gel electrophoresis (DGGE) [30–35], temperature gradient electrophoresis (TGGE) [23], multiplex/heteroduplex analysis (multiplex/HDA) [19, 36–38], and fluorescent-based PCR with automated high-resolution capillary electrophoresis (GeneScan, GS) [15, 19, 37–42]. Using gel electrophoresis methods, a single band (or in some cases, two bands due to bi-allelic rearrangements) is interpreted as evidence of monoclonality. A smear indicates a polyclonal population, while the presence of three to four distinct bands (± a smear) is regarded as evidence of oligoclonality. AG and PAGE separate the PCR products on the basis of length (size) alone and are prone to false-positive results. SSCP, DGGE, TGGE, and multiplex/HDA separate PCR products on the basis of both length and sequence (conformation) of the nucleotides, reducing this risk of false-positivity. The latter techniques take advantage of the junctional regional diversity to distinguish between monoclonal cells with identical junctional regions and polyclonal cells with highly diverse ones. GS-based techniques utilize length-dependent separation of amplicons and are increasingly employed in diagnostic laboratories, as they are faster, more accurate, more sensitive, and easier to interpret than other methodologies [19]. Patterns of monoclonal, polyclonal, and oligoclonal TCR-GRs using GS analysis are outlined in Fig. 10.2. Other techniques, such as tumor-specific PCR-based testing

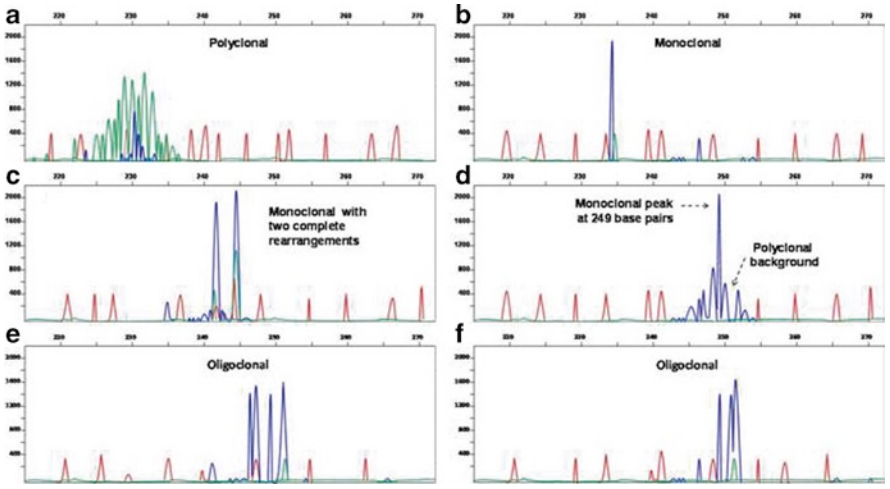

Fig. 10.2 GeneScan analysis of PCR products demonstrating patterns of T-cell receptor gene rearrangements (*TCR-GRs*). (**a**) Polyclonal. (**b**) Monoclonal. (**c**) Monoclonal with biallelic complete clonal rearrangements. (**d**) Monoclonality with polyclonal background. (**e**, **f**) Oligoclonality, defined as the presence of three to four dominant peaks, in the absence of a polyclonal background (Courtesy of Dr. Zendee Elaba, Department of Pathology, Hartford Hospital, Hartford, CT, USA)

[43, 44, 46], PCR-based RNA protection assay (PCR/RPA) [31], in situ PCR [45], oligonucleotide microchip analysis [47], and cloning and sequencing of PCR products [27, 38, 39, 43, 44], have also been used as tools to evaluate T-cell clonality. Sequencing is the gold standard, but it is too expensive and time-consuming for routine diagnostic testing. In situ PCR for TCR-β can be performed on FFPE biopsies and allows direct correlation of molecular data with microscopic findings [45]. Tumor-specific PCR-based clonality assays depend on the isolation and sequencing of a monoclonal TCR-GR from a diagnostic CTCL sample [43, 44, 46]. Using this dominant sequence, tumor-specific probes or PCR primers can then be generated and used to detect tumor involvement in additional tissue samples of the same patient ("molecular fingerprint") [43, 44, 46]. PCR/RPA is another clone-specific molecular technique, which has been advocated as a means to accurately stage, monitor response to therapy, and/or detect early recurrence of disease in individual patients with diagnosed CTCL [31, 44, 46]. It does not require analysis of nucleotide sequences or synthesis of tumor clone-specific DNA primers or probes. However, with an ability to detect one malignant cell within a background on 10^5 non-tumorous cells (0.001%), it may be too sensitive for routine clinically relevant testing [6, 31]. Currently, a clonal detection threshold of ~1% is widely regarded as the most appropriate sensitivity level for studies of cutaneous T-cell infiltrates [6]. In addition, laser capture microdissection (LCM) has been used to increase the sensitivity of subsequent clonality studies on skin biopsies [26]. This technique allows for selective sampling of operator-determined cell populations. It is particularly applicable to dermatopathology, given the small size of specimens, heterogeneous cell populations present in the skin, and difficulty of culturing cutaneous tumors. However, it is important to note that the amplification of a restricted number of LCM-extracted TCR-encoding DNA molecules from a skin biopsy could potentially lead to a monoclonal/restricted oligoclonal result, even in the presence of reactive inflammatory cells (see Section on Pseudomonoclonality).

The detection of a dominant T-cell clone has been reported in 76–100% of patients with advanced-stage MF, including tumor stage and erythrodermic disease [22, 26, 30, 36]. In this group, the correlation of clinical findings with histopathological features and immunophenotypic data is usually diagnostic for CTCL, and molecular studies for T-cell clonality are generally less important in confirming the disease. Nonetheless, there is some evidence that T-cell clonality assessment may have a role in the staging and prognostication of these patients (see later discussions). PCR-based methods are particularly useful adjuncts to the diagnosis of cases with inconclusive or borderline histopathology and/or IHC findings (i.e., early CTCL). T-cell monoclonality can be identified in early-stage MF, with detection rates ranging from 40% to 88% reported in various studies [15, 22, 23, 25, 26, 28, 30, 32, 34, 37]. Of note, the sensitivity of molecular testing in early-stage lesions varies with the method used to analyze the PCR amplicons (i.e., 60% monoclonal with multiplex/HDA *vs.* 79% for GS) [37]. Interestingly, oligoclonality is a common finding in skin lesions of early MF, and does not exclude the diagnosis of CTCL (Fig. 10.2) [19, 37]. In some cases, oligoclonality may result from associated reactive antitumor T-cell infiltration, so that the ratio of clonal tumor cells to polyclonal reactive cells is below the detection threshold of the PCR-based method used [19, 37].

PCR-based studies show variability in both their sensitivities (detected positives relative to true positives) and their clonal detection thresholds (the minimum percentage of clonal cells detectable). These variations may be due to: (1) lack of uniformity in sample selection (i.e., differences in stage and/or duration of lesions, inclusion of nonspecific lesions, interobserver variability in microscopic interpretation among pathologists, and classification system used [i.e., Kiel *vs.* EORTC]); (2) technical factors (use of fresh or frozen tissue, DNA extraction methods, TCR locus analyzed [i.e., TCR-γ *vs.* TCR-β], numbers of PCR primers used for that particular TCR locus, and method of PCR product analysis); and (3) biological factors (absence, mutation, trans-rearrangement or deletion of TCR-GRs in some lymphomas, or low absolute or relative numbers of malignant clonal T-cells [the latter found in cases with high numbers of background polyclonal reactive T-cells], and therefore

below the detection threshold for the PCR assay used). The overall sensitivity and specificity of PCR for the diagnosis of MF is ~70% and ~97%, respectively [13]. However, the lack of consistency and standardization, in addition to differences in TCR-GR profiles and detection rates (i.e., percentage of positive cases), between different protocols has resulted in poor consensus regarding the utility of clonality assays in the diagnosis of CTCL. To address this concern, the European BIOMED-2 Concerted Action BMH4-CT98-3936 was established in 2003, in order to develop standardized PCR primers sets and protocols to detect TCR-GRs in lymphomas [17]. The introduction of standardized PCR analysis should allow for comparison of T-cell clonality studies performed at different centers.

T-cell monoclonality has also been reported in MF variants, including follicular MF [48], granulomatous MF [49], hypopigmented MF [27], and the palmaris et plantaris subtype [50], in addition to other primary and secondary T-cell lymphoproliferative disorders [19, 30, 33, 42]. It is important to note that some mature extranodal peripheral T-cell lymphomas, in addition to precursor T-lymphoblastic lymphomas/leukemias, natural killer (NK)-cell and NK/T-cell lymphomas, do not show rearrangements of the TCR genes (i.e., TCR loci remain in germline configuration) [17, 19]. In addition, many of the previously cited clonality-based studies have included both MF and SS samples, based on the assumption that they are variants of the same disease [19, 30, 33, 36–38]. However, the recent demonstration of different genomic/transcriptomic profiles in MF and SS suggest that they may in fact be separate diseases with distinct clinical features and behavior [51–53].

Correlation of Histopathologic Parameters with Results of TCR-GR Analysis

As stated previously, detection rates for T-cell clonality in CTCL generally increase with advancing stage. Higher sensitivities are found for tumor and erythrodermic disease compared with early patch and plaque lesions (i.e., clonality rates generally correlate with tumor load in biopsy specimens, with higher rates of detection in biopsies with heavier/denser infiltrates). A number of studies have investigated whether there is a correlation between results of molecular studies and histopathologic diagnoses (i.e., such as "diagnostic of," "consistent with," "suggestive of," or "nondiagnostic of") in early MF lesions [25, 26, 34, 36]. Similar to results from SBA [21], Ponti et al. [36] have demonstrated that the extent and density of the lymphocytic infiltrate and presence of cytologic atypia, in addition to the degree of epidermotropism, were predictive of PCR-detected T-cell clonality in early CTCL. However, a study by Hsiao et al. [26] found that TCR-γ PCR-negative results were more likely to be associated with denser dermal infiltrates, in addition to the degree of cytologic atypia and presence of Pautrier's microabscesses, in early MF lesions. Similarly, Liebmann et al. [25] found that a lower percentage of plaque stage MF lesions demonstrated clonal TCR-GRs compared to patch stage MF lesions. The reason for these contradictory results is unclear, but may be related to different detection methods used in these studies (multiplex/HDA [36] *vs.* PAGE [25, 26]). In addition, a recruited antitumoral inflammatory response would increase the levels of reactive T-cells compared with neoplastic T-cells, potentially diluting the "clonal" signal and masking it within a polyclonal smear. Therefore, caution is required in the interpretation of negative PCR results in subsets of early MF lesions, particularly if the ratio of malignant clonal T-cells to background reactive polyclonal T-cells is low. Indeed, it has been suggested that PCR-based assays to detect clonal TCR-GRs may be most informative in lymphocyte-poor lesions of early MF, which represent perhaps the greatest diagnostic difficulty [25, 26]. In such cases, there would theoretically be less dilution of any putative clonal signal by background polyclonal events. However, it is also important to consider other issues, such as pseudomonoclonality, as outlined below.

Algorithmic Approaches to the Diagnosis of MF

A number of algorithms to aid in the diagnosis of early MF lesions have been published [3, 10–12]. These include a grading system based on microscopic criteria alone [10], and other systems incorporating clinical and histopathological features with the results of ancillary diagnostic tests, such as IHC and TCR-GR analysis [3, 11, 12]. Points are awarded for certain criteria, referred to as "basic or additional" [11] or "major and minor" [10] features, depending on the algorithm used. A defined number of total points are then required for the diagnosis of MF, based on a combination of points from the different criteria [3, 10–12]. In the algorithm proposed by Pimpinelli et al. [11], IHC and molecular criteria always require additional clinical and/or histopathological points to establish the diagnosis of MF. However, if sufficient clinical and histopathological criteria are present, then incorporation of ancillary test results is regarded as unnecessary [11]. These efforts have been undertaken in order to develop standardized criteria for diagnosis and prognostication, the selection of stage-appropriate treatment, and the design of clinical trials in patients with CTCL. However, these algorithms have yet to be clinically validated or widely adopted, and are associated with both false-positive and false-negative results [12].

Clonal Dermatoses and Cutaneous T-Cell Lymphoid Dyscrasia

It has become increasingly evident that while monoclonality is a feature of CTCL, it is not pathognomonic of malignancy. In this regard, the detection of a monoclonal TCR-GR is not reliable evidence of CTCL, as a growing body of evidence points to the presence of T-cell clones in a variety of cutaneous disorders, which in many cases are biologically and prognostically distinct from CTCL. The terms "clonal dermatitis" and "abortive/latent lymphoma" were originally coined to describe a group of apparently benign dermatoses that demonstrated T-cell clonality, despite the absence of diagnostic histopathological features of CTCL [6, 11]. It was postulated that these lesions were either precursors of CTCL or nondiagnosable CTCL, with 20–25% of cases progressing to overt CTCL within a 5-year period [6, 11].

TCR-GR studies have subsequently demonstrated monoclonal and/or restricted oligoclonal T-cell profiles in: (a) eczematous/contact dermatitis [6, 33], lymphocytic infiltrate of Jessner [33], bullous pemphigoid [33], lichenoid eruptions including lichen planus and lichen sclerosus et atrophicus [15], psoriasis [15], and erythema nodosum [15]; (b) lesions of lymphomatoid lupus profundus/discoid lupus erythematosus [42]; (c) up to 50% of cutaneous drug-associated lymphomatoid hypersensitivity reactions (reversible on discontinuation of the implicated agent) [42]; and (d) up to 30% of cases in a miscellaneous group that includes hypopigmented interface-type lesions [54], syringolymphoid hyperplasia with alopecia [54], idiopathic follicular mucinosis [42], pityriasis lichenoides chronica [42], pityriasis lichenoides et varioliformis acuta (PLEVA) [42], atypical lymphocytic lobular panniculitis [42], idiopathic and drug-induced pigmented purpuric dermatosis [42], and large plaque parapsoriasis [42, 54]. In addition, oligoclonal molecular profiles have been detected in biopsies of morphea and HIV-associated T-cell-rich pseudolymphoma (unrelated to drug therapy) [42].

These cases have now been categorized as either: (1) reactive benign T-cell infiltrates in the context of autoimmune disease or endogenous immune dysregulation (i.e., lupus erythematosus); (2) reversible drug-induced lymphomatoid hypersensitivity reactions (iatrogenic immune dysregulation); or (3) the recently described entity, cutaneous T-cell lymphoid dyscrasia (CTLD) [54]. Guitart and Magro [54] have proposed this latter term to describe a group of idiopathic chronic dermatoses, with persistent cutaneous infiltrates of monoclonal or restricted oligoclonal T-cells that

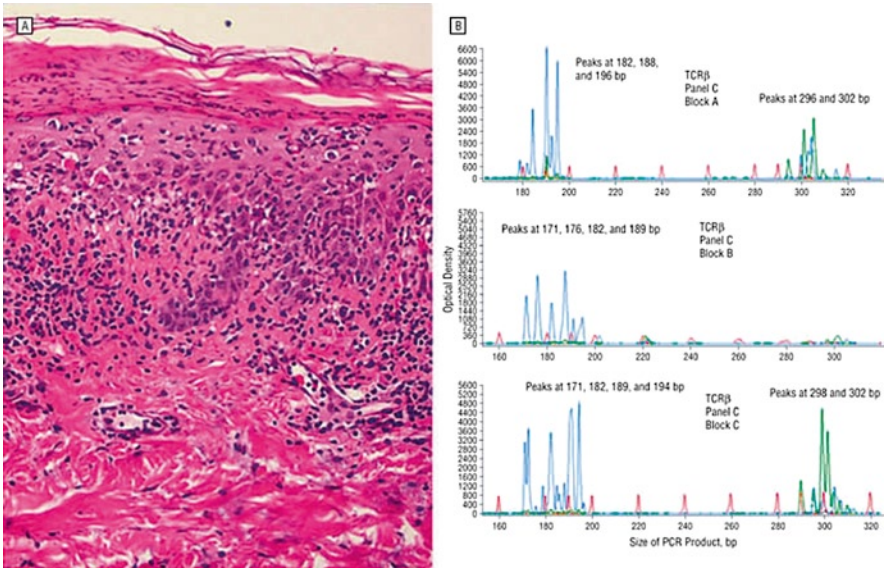

Fig. 10.3 Cutaneous T-cell lymphoid dyscrasia (*CTLD*). (**a**) Classic changes of pityriasis lichenoides as defined by epithelial hyperplasia and striking migration of lymphocytes to involve all layers of the epidermis. Extensive red blood cell extravasation is seen amid a superficial interstitial and perivascular lymphocytic infiltrate. (**b**) Molecular studies of samples from a 56-year-old woman with long-standing pityriasis lichenoides chronica obtained at different times showing an oligoclonal process with similar molecular profiles. *bp* base pairs, *PCR* polymerase chain reaction, *TCR-β* T-cell receptor β (From Guitart et al. [54]. Reprinted with permission from the American Medical Association, Copyright © 2007)

fail to fulfill clinical and/or histopathological criteria for a definitive diagnosis of CTCL (Fig. 10.3) [10, 11]. CTLD includes a hypopigmented interface variant, pigmented purpuric dermatosis, atypical lymphocytic lobular panniculitis, syringolymphoid hyperplasia with alopecia, idiopathic follicular mucinosis, pityriasis lichenoides, large plaque parapsoriasis, and clonal erythroderma [54]. All these conditions are characterized by a persistent chronic course, in the absence of a known antigenic trigger, resistance to/relapse following topical therapy, a lack of definitive architectural or cytomorphological evidence of malignancy, and reduced antigen expression (i.e., CD7 and CD62L) by T-cells, but with detectable T-cell monoclonality (or oligoclonality with a few restricted T-cell clones), and a potential, albeit low, for progression to CTCL [54]. It has been suggested that CTLD patients with identical molecular clonal profiles at different biopsy sites, and/or in whom stable repetitive dominant T-cell clones persist over time, could benefit from therapeutic modalities similar to those administered to patients with "diagnostic" early CTCL [42]. Long-term follow-up of these patients for evidence of progression to overt CTCL, including molecular analyses of multiple concurrent or sequential skin biopsy specimens, is required. This will help to determine the significance of dominant T-cell clones in "dermatoses" without initial clinical, histopathological, or immunophenotypic evidence of lymphoma, and/or without an "inciting" factor such as prior drug therapy or known history of an autoimmune disorder. In addition, these findings reaffirm the necessity to correlate the results of molecular studies with available clinical, histopathological, and immunophenotypic data.

The development of frank CTCL within the pretext of "clonal dermatitis"/CTLD is not surprising. In fact, the concept of progression from clonal precursor to overt lymphoma has been described in other settings, including lymphoid hyperplasia to lymphoma, lymphomatoid papulosis (LyP) to T-cell lymphoma (both MF and large cell lymphoma), angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) to AILD-like T-cell lymphoma, and monoclonal gammopathy of uncertain

significance to myeloma [6]. For example, a common clonal origin has been demonstrated for LyP, Hodgkin lymphoma, CD30+ large T-cell lymphoma and MF developing in individual patients [46, 55, 56]. It is also interesting to note that recent studies suggest an increase risk of CTCL in patients with certain chronic inflammatory dermatoses, including psoriasis [57] and atopic dermatitis [58]. An association between these inflammatory conditions and CTCL may be related to chronic lymphoproliferation which eventually evolves to overt lymphoma in some patients. Alternatively, certain psoriasis/atopic dermatitis therapies, misdiagnosis, or a combination of these factors may explain any association [57, 58]. Nonetheless, research does suggest that CTCL can develop within the context of chronic inflammatory reaction(s) to antigenic stimuli, possibly arising as subclones at different cutaneous sites, with subsequent emergence of more aggressive clones, and gradual selection of one dominant clone that becomes increasingly malignant over time, probably as a result of sequential somatic mutations [54].

Pseudomonoclonality

It is important to consider the possibility of pseudomonoclonality when evaluating the results of TCR-GR analyses. Cutaneous infiltrates may be quite sparse, particularly in inflammatory T-cell proliferations (reactive dermatoses) and early-stage MF lesions. In these settings, the presence of a monoclonal band or oligoclonal pattern in a single analyzed sample is not an uncommon finding [37]. An "apparent" monoclonal result may represent a true monoclonal neoplastic T-cell population, but could also be due to the amplification of a few TCR-GRs derived from a limited number of T-cells in a patient sample. For example, PCR amplification of a restricted number of extracted DNA molecules encoding the TCR-γ chain from either a small skin biopsy [16] or limited microdissected areas [59] can produce a monoclonal/restricted oligoclonal banding pattern, even in the presence of reactive inflammatory cells. Repeated analyses using the same DNA template, a second independent DNA extraction from the same sample and/or DNA from synchronous/metachronous samples are necessary to evaluate the consistency of results. In reactive conditions, dominant PCR amplicons typically vary in repeated PCR analyses of the same sample (i.e., pseudomonoclonality), whereas dominant TCR-GRs are usually reproducible (i.e., true monoclonality) in neoplastic T-cell proliferations [60]. Therefore, overinterpretation of pseudomonoclonality and overdiagnosis of CTCL can be avoided by repeated independent PCR analyses of the same sample, and should be part of any standard protocol.

Stable Clones Versus Clonal Heterogeneity

In an effort to increase the specificity of PCR analysis in the diagnosis of early MF lesions, some authors have analyzed TCR-GRs in skin biopsy specimens from two or more anatomically distinct sites (in some cases at different times) in the same patient [15, 22, 23, 25, 28, 32, 34, 39, 42]. By comparing banding patterns, these studies concluded that an identical T-cell clone [15, 22, 23, 25, 28, 32, 34, 39] or a restricted molecular profile (including two or more dominant clones) [42] can be identified at different cutaneous sites in 46–100% of patients with MF studied over the course of their disease (irrespective of disease stage or time interval between biopsies). The presence of concordant positive results (i.e., "stable clonal pattern" [37]) in two or more synchronous or sequential skin biopsies is consistent with a genetic imprint that is stable and specific for an individual patient, and useful in confirming the diagnosis of MF (Fig. 10.4). The presence of an identical T-cell clone at anatomically distinct sites using PCR is reported to be associated with a specificity of >95% in

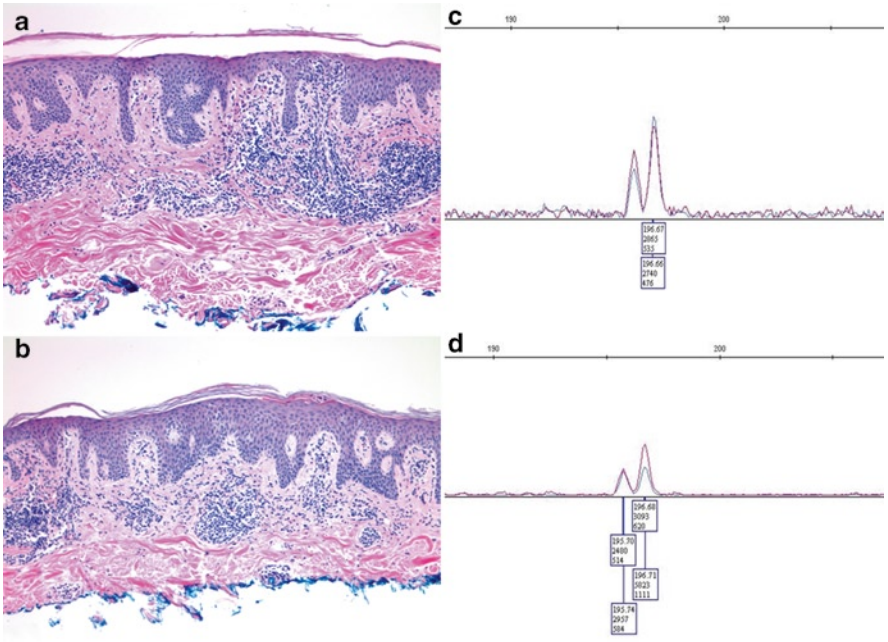

Fig. 10.4 Stable T-cell clone. (**a**, **b**) Sequential skin biopsies in the same patient that were reported as concerning for involvement by cutaneous T-cell lymphoma (*CTCL*), with demonstration of a stable clonal T-cell receptor gene rearrangement (*TCR-GR*) pattern in both biopsies (**c**, **d**) (Courtesy of Drs. Jennifer M. McNiff and Pei Hui, Yale University School of Medicine, New Haven, CT, USA)

discriminating between MF and inflammatory dermatoses [15]. It has also been reported that TCR-GR studies on multiple samples are reproducible (with identical banding patterns) in patients whose skin biopsies are interpreted as "consistent with" or "diagnostic of" CTCL [34]. However, discordant results may be found in patients whose sequential biopsies are only "suggestive of" or "nondiagnostic of" CTCL [34]. In addition, lesions of CTLD (defined in a previous section) may also show identical TCR-GR results in skin samples taken at different times and from different anatomic sites in the same patient (Fig. 10.3) [42, 54]. Interestingly, drug-induced lymphomatoid hypersensitivity reactions [42], a case of interstitial granulomatous dermatosis (which did not progress to CTCL) [15], and an unspecified benign dermatosis [19] have shown similar findings. Therefore, the identification of a constant and stable T-cell clone at different biopsy sites cannot be held as indicative of CTCL. However, close long-term follow-up for possible progression to CTCL would be judicious in any patient with nonspecific/nondiagnostic histopathology and a stable T-cell clonal pattern.

Molecular studies on human malignancies have demonstrated the presence of genetically unstable subclones during tumor pathogenesis and progression [59]. By SBA and PCR, 22–48% of patients with MF (up to 80% of patients with SS) show clonal heterogeneity in lesions from different anatomic sites [20, 37, 39]. Depending on the study, this has been defined as the presence of (1) different monoclonal TCR-GRs ("unstable clonal pattern") or (2) a common clone ("stable pathological clone"), but with additional reproducible clonal TCR-GRs, in distinct samples from the same patient (Fig. 10.5) [20, 37, 39]. Indeed, mutational analyses of microsatellite DNA from multiple microdissected areas of FFPE skin biopsy specimens suggest that CTCL may evolve by multilineage progression, tumor subclones can be detected in early-stage disease [59]. Clonal heterogeneity has been demonstrated in both early and advanced MF lesions, in addition to other cutaneous T-cell lymphoproliferative disorders (i.e., LyP) and B-cell lymphomas [20, 37]. It has been proposed that

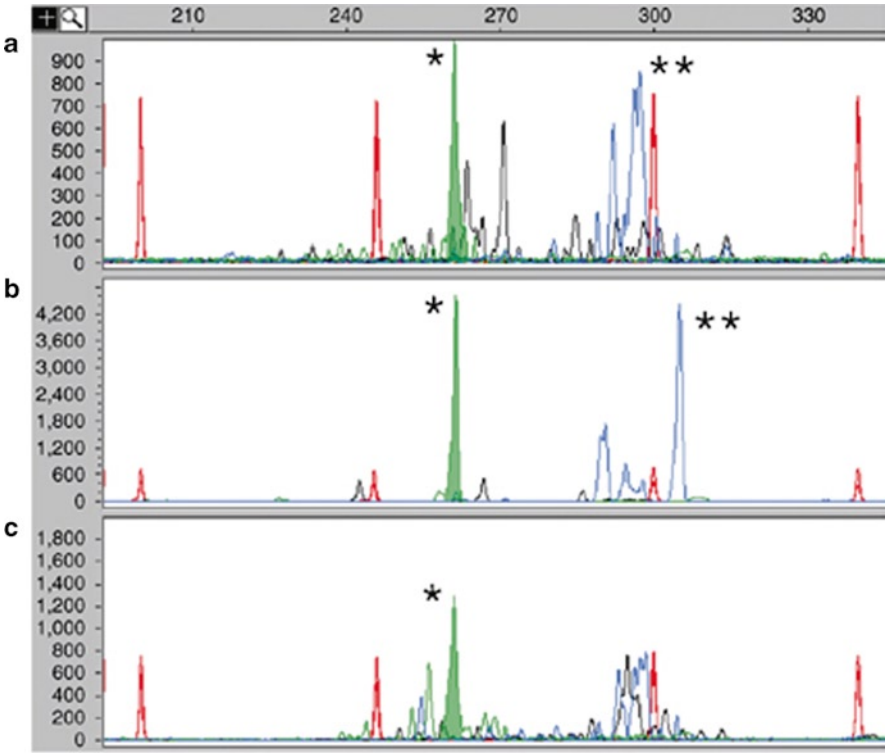

Fig. 10.5 Clonal heterogeneity in multiple cutaneous T-cell lymphoma (*CTCL*) samples from the same patient. (**a**–**c**) GeneScan (*GS*) analysis of PCR products demonstrating patterns of T-cell receptor gene rearrangements (*TCR-GRs*). (**a**) Ambiguous GS pattern in skin biopsy at diagnosis; TCRVγII GR (*) confirmed by comparison with blood pattern; (**b**) GS pattern in blood sample at diagnosis showing a pathological GR (*) and a concomitant transient GR (**); (**c**) TCRVγII clonal GR (*) in the second skin sample (From Ponti et al. [37]. Reprinted with permission from Macmillan, Copyright © 2007)

early lesions of MF may arise from polyclonal or oligoclonal activation of T-cells, possibly under the proliferative influence of super-antigens (such as bacterial antigens) [37]. In early MF, the phenomenon of clonal heterogeneity could be explained by: (1) a mixture of neoplastic and reactive T-cell clones; (2) the independent and simultaneous malignant transformation of two or more T-cells following long-term antigen stimulation, with evolution of several distinct clones at different sites; or (3) the evolution of subclones from a single dominant T-cell clone by subsequent rearrangements or deletions at TCR loci [37, 39]. Theoretically, a multiple subclonal pattern would be followed by the emergence of a stable dominant T-cell clone in advanced MF lesions, whose persistence would depend on further mutagenic events and/or therapeutic intervention. Nonetheless, some CTCL patients show persistence of clonal instability with different clonal TCR-GRs detected in sequential skin and peripheral blood (PB) samples [37]. This suggests that repetitive cycles of alternating dominant-subclone-dominant-subclone formation may occur in some individuals [37]. One question is: does the distinction of clonal homogeneity from clonal heterogeneity in multiple samples from the same patient provide prognostic information related to disease progression? Vega et al. [39] noted that patients with a common T-cell clone identified in multiple *concurrent* biopsies at the time of diagnosis were more likely to have progressive disease than those who had different clonal TCR-GRs. Their findings were noted to be independent of the clinical stage of disease at the time of diagnosis [39]. Interestingly, the same study found no correlation between TCR-GR clonal patterns in *sequential* skin biopsies and clinical progression [39]. Bignon et al. [20] and Ponti et al. [37] also

found no significant correlation between TCR-γ clonal heterogeneity and progressive disease. Therefore, the prognostic significance of clonally heterogenous TCR-GR patterns in MF patients remains unclear and warrants further study.

Composite Lymphomas, Dual Lineage Rearrangements and Lineage Infidelity

CTCL is associated with a high incidence of both synchronous and metachronous lymphoproliferative disorders of different lineage (such as B-cell lymphomas and Hodgkin lymphoma) [61–63]. A number of theories have been proposed for the simultaneous occurrence of lymphomas of different lineages, including an inherited genetic predisposition, an oncogenic molecular defect in a common progenitor cell, the presence of a common risk factor (i.e., carcinogen or oncogenic virus exposure), prior chemotherapy and/or immunosuppression [61–63]. Problems in clinical and histopathological interpretation can obviously arise, particularly if both lesions develop concurrently and show skin involvement. It is important to note that composite T- and B-cell lymphomas in the skin can show monoclonal rearrangements of both the TCR and immunoglobulin heavy chain (IgH) genes (Fig. 10.6) [61–63].

Other studies have discussed the finding of dual lineage rearrangements in cutaneous lymphoproliferative disorders, which occur in ~1% of cases [20, 64]. Kazakov et al. [64] have suggested that the detection of monoclonal TCR-GRs in some cases of cutaneous B-cell lymphoma could be explained by either (1) monoclonal or oligoclonal expansion of exuberant reactive T-cells (or reactive B-cells in the case of T-cell lymphoproliferative disorders with monoclonal IgH-GRs) or (2) lineage infidelity. Clonality studies based on rearrangements of surface receptor genes (TCR and IgH) can be regarded as lineage-specific in most cases. Genotypic lineage infidelity is rare in mature lymphoid malignancies [64]. However, immature B-cell and T-cell neoplasms may demonstrate monoclonal lineage crossover (i.e., have both monoclonal TCR- and IgH-GRs) [61, 65]. In addition, a small percentage of myeloid leukemias can show monoclonal TCR- and/or IgH-GRs [65].

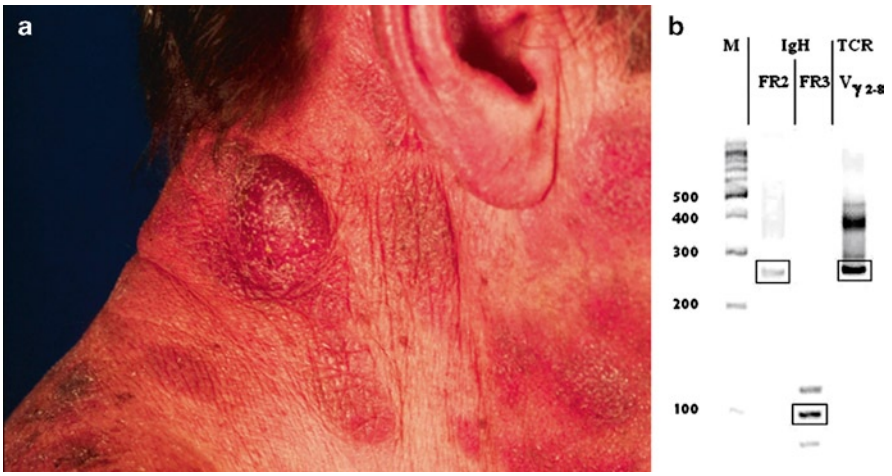

Fig. 10.6 Patient with composite lymphoma of mycosis fungoides (*MF*) and chronic lymphocytic leukemia (*CLL*). (**a**) There are patches and plaques on the face, neck, and upper back, with a tumoral lesion on the neck. Skin biopsies of patches/plaques showed histopathological and immunohistochemical findings of early MF (not shown). The tumor showed composite findings of both early MF in the epidermis and upper dermis, with CLL in the deep dermis (not shown). (**b**) Monoclonal rearrangements of both the immunoglobulin heavy chain (*IgH*) and T-cell receptor (*TCR*) genes were identified (Courtesy of Dr. Emmilia Hodak, Tel Aviv University, Tel Aviv, Israel)

Therefore, the integration of clinical, morphological and immunophenotypic data, with testing for both TCR- and IgH-GRs in some cases, is required for correct interpretation of molecular clonality studies with regard to accurate lineage determination and/or the possible presence of a synchronous or metachronous lymphoproliferative disorder.

Cytogenetics and Other Molecular Techniques

Compared to other neoplastic disorders, there is a relative paucity of information on genetic aberrations in CTCL, and a consistent pattern or diagnostic signature has not been identified in most cases. Contributing factors include the phenotypic variability of CTCL, the difficulties in obtaining substantial amounts of tumor cells from skin biopsies, and the absence or rarity of tumor cells in the PB [66]. Nonetheless, progress has been made in defining the cytogenetic aberrations and molecular/immunopathogenesis of this disease using karyotyping, ISH, CGH, array-CGH, LOH, and microarray techniques [2, 7]. CTCL is defined by a large spectrum of chromosomal abnormalities; any chromosome may be numerically or structurally altered [7]. In addition, mutations and/or epigenetic alterations of a number of tumor suppressor genes (TSGs), oncogenes and apoptosis pathway genes have been described [7, 67]. The relationship between cytogenetic abnormalities and the pathobiology of CTCL is beyond the scope of this chapter, and reviews are available elsewhere [7–9]. However, the evaluation of genomic changes as an aid to the diagnosis of CTCL will be discussed further.

Conventional cytogenetics and other molecular studies can be combined with TCR-GR analysis for CTCL diagnosis and follow-up [7]. Interestingly, the coexistence of a dominant T-cell clone and a clonal chromosomal aberration may be a more specific indicator of malignancy [68]. Muche et al. [68] have found concordant results for both features in the majority (88%) of CTCL specimens, including skin, PB, and lymph node (LN) samples.

Using a cDNA microarray-based approach, Tracey et al. [69] identified 27 genes that were differentially expressed between MF and inflammatory dermatoses, including tumor necrosis factor receptor (TNFR)-dependent apoptosis regulators, STAT4, CD40L, and other oncogenes and apoptosis inhibitors. A 6-gene (FJX1, STAT4, SYNE1, TRAF1, BIRC3, Hs.127160) prediction model for differentiation of MF from inflammatory dermatoses was constructed. This model correctly assigned 97% of cases in a blind test validation using 24 MF patients with low clinical stages [69]. Gene expression signatures associated with abnormal immunophenotype (11 genes) and tumor stage disease (5 genes: IKBKAP, FZD7, PDIA2, hAC2387, hAD7824) were also identified [69].

cDNA microarray [70] and quantitative real-time PCR [71] expression analyses of PB samples, using a panel of five to eight genes (including STAT4, GATA-3, PLS3, CD1D, and TRAIL), are reported to identify CTCL patients (who have at least 5% circulating tumor cells) with an accuracy of 90%.

Tumor-specific chromosomal translocations and/or infections by oncogenic viruses may play a role in the pathogenesis of a subset of CTCL – the presence of which may also provide avenues for diagnostic testing in some tumors. For example, the t(2;5)(p23;q35) chromosomal translocation is found in ~10% of cases of LyP and 20% of cases of CD30+ primary cutaneous large cell lymphoma, in addition to ~40% of lymph node-based CD30+ anaplastic large cell lymphomas of T-cell or null-cell lineage [72, 73]. This translocation results in inappropriate expression of NPM-ALK/p80 tyrosine kinase, which is believed to play a central role in the pathogenesis of these tumors. Karyotyping, SBA, RNA-based RT-PCR, DNA-based PCR, ISH, and IHC for the p80 fusion protein have all been employed to identify the chimeric t(2;5) transcripts [72, 73]. In addition, adult T-cell leukemia/lymphoma (ATLL) is associated with retroviral infection by *human T-cell*

lymphotropic virus 1 (HTLV-1), exhibiting clonal integration of the viral genome (Fig. 11.6) [74]. No signature chromosomal translocation or demonstrable viral etiologic factor has been identified for MF.

Staging of CTCL

Historically, the staging of patients with CTCL has been based on an evaluation of clinical and histopathological features (i.e., TNMB), including tumor, LN, metastasis, and PB parameters [3, 4]. More recently, the ISCL/EORTC has recommended revisions to this classification system, in an effort to incorporate advances related to tumor biology and diagnostic technologies (Table 10.2) [3].

Lymph Nodes

Peripheral LNs are the most common site of extracutaneous disease in MF and SS [75]. Patients with more extensive or advanced skin involvement are at increased risk for progression to LN disease, which often presents as clinically palpable lymphadenopathy [75]. The histopathological assessment of clinically palpable LNs in MF/SS patients is essential for: (1) accurate staging; (2) prognostication; and (3) selection of appropriate treatment strategies (i.e., extracutaneous involvement typically requires systemic therapies) [3, 19, 20, 22, 41, 75–79]. In patients with CTCL, clinically abnormal LNs (palpable and/or >1.5 cm in diameter) can be evaluated by surgical excision (preferred method) or fine-needle aspiration biopsy, with the integration of microscopic features, immunophenotypic (IHC and flow cytometry) findings, and molecular data often required for a definitive evaluation [3, 75]. The histopathological features in LNs of CTCL patients can be reported as normal, reactive (dermatopathic), or demonstrating varying degrees of involvement by lymphoma [3, 77]. Several classification systems that assess the histopathological extent of LN disease in patients with CTCL have been proposed, and are generally found to be reproducible and to correlate with disease involvement and outcome [3, 77, 80]. However, atypical lymphocytes may be seen in the LNs of patients with benign inflammatory dermatoses, and a subset of CTCL patients exists who have a poor prognosis despite histopathologically "normal" LNs or early/minimal LN disease [77]. It has been suggested that molecular analysis may provide more reliable diagnostic and prognostic information [41, 76, 77, 79]. Accordingly, the revised ISCL/EORTC guidelines incorporate supplementary molecular testing for LN staging in patients with MF/SS [3]. For example, N1 and N2 stages can be subdivided (i.e., N1a *vs.* N1b and N2a *vs.* N2b) based on T-cell clonality status (Table 10.2).

Clonal TCR-GRs have been detected by SBA in LNs of patients with CTCL, predicting a poor clinical outcome and reduced probability of survival [3, 19, 20, 22, 76, 78]. The frequency of monoclonal T-cell detection in LNs by SBA increases with skin stage [76]. Of note, up to 12% of CTCL patients can show monoclonal TCR-GRs in clinically normal LNs [76]. Furthermore, SBA for TCR-GRs can stratify CTCL patients with clinically normal, histopathologically indeterminate LNs into good prognosis (polyclonal) and poor prognosis (monoclonal) groups [76]. In addition, identical TCR-GRs have been found in LNs, skin lesions, and PB samples of individual patients using SBA [22].

PCR-based strategies have also been used to detect monoclonal TCR-GRs in LNs of patients with CTCL [3, 39, 41, 75, 77–79]. PCR detection of monoclonal T-cells in LNs increases with advancing skin stage, overall clinical stage and degree of histopathological LN involvement, and is associated with a poorer prognosis (Fig. 10.7) [41, 77, 78]. PCR can demonstrate identical T-cell clones in LNs and samples from other sites (i.e., skin, PB and bone marrow [BM]) in an individual

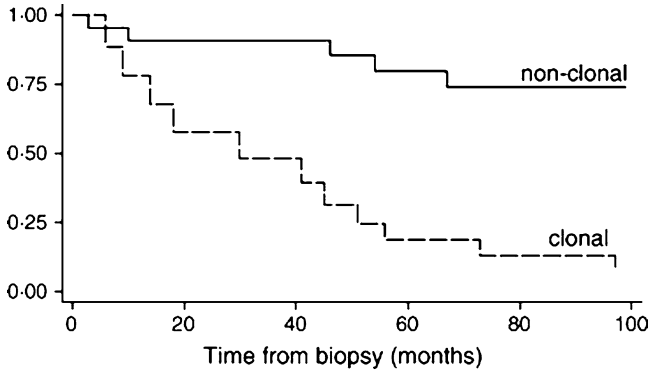

Fig. 10.7 Survival curves based on clonal and non-clonal lymph node (*LN*) samples in mycosis fungoides (*MF*) patients, corrected for age and sex (From Fraser-Andrews et al. [77]. Reprinted with permission from Wiley-Blackwell, Copyright © 2006)

patient [39, 41, 77]. However, as previously discussed for skin biopsies, different clones (i.e., clonal heterogeneity) may be detected in the LN compared with synchronous/metachronous specimens from other sites [20, 39, 41].

PCR/RPA, a highly sensitive tumor-specific receptor gene rearrangement assay, has demonstrated T-cell monoclonality in microscopically normal LNs, in addition to PB and BM samples, in patients with limited patch stage MF and SS [6, 31, 43, 44, 46]. However, many patients with early-stage MF have a normal life expectancy and do not develop extracutaneous disease [6, 31]. Therefore, PCR/RPA would obviously appear to be too sensitive for diagnosis, staging, or monitoring of disease activity in some patients. An important objective of any molecular staging strategy should be the determination of a clinically relevant sensitivity threshold for detection of occult CTCL. Prospective molecular staging of individuals with early CTCL might help identify (1) subgroups of patients at risk for progression who would benefit from aggressive systemic therapy or (2) those in whom such potentially toxic therapy would not significantly alter prognosis.

Bone Marrow, Peripheral Blood, and Other Biological Fluids

The diagnosis of PB involvement by MF and SS has historically been based on a subjective assessment of the percentage of total lymphocytes that exhibit an atypical convoluted/cerebriform morphology (Sézary cells) by light microscopy [3]. In addition, flow cytometry can be used to identify circulating neoplastic T-cells through the demonstration of: (1) altered CD4/CD8 ratios; (2) absent, reduced, or increased expression of surface antigens (i.e., CD3, CD4, CD7, and CD26); (3) expression of CD10; and/or (4) restricted TCR V-β gene usage [3, 13]. However, surface antigen deletion has also been found in the PB of patients with benign dermatoses [3]. Molecular testing for T-cell clonality offers an alternative objective means of identifying PB involvement by CTCL, and has been incorporated into the revised ISCL/EORTC staging and classification system for MF and SS (Table 10.2) [3].

Some studies have found different monoclonal TCR-GRs between PB samples and those from other sites (skin and LN) in individual patients [20, 37, 40]. These discordant results may be explained by the concept of clonal heterogeneity in CTCL (as previously discussed). However, it is important to note that a monoclonal T-cell population in the PB could be related not only to (1) a circulating neoplastic T-cell clone, but also to (2) a reactive antitumor population, (3) the well-described phenomenon of age-related monoclonal T-cell expansion, and/or (4) the development

of circulating T-cell clones secondary to long-term immunostimulatory states [37, 54]. It is known that elderly patients (>60 years old) can show monoclonal T-cell expansions (both CD4+ and CD8+ subsets) in the PB [33]. Autoreactive T-cell clones may also develop in patients with multiple sclerosis, sarcoidosis, graft-versus-host disease and autoimmune diseases, such as rheumatoid arthritis and lupus erythematosus [54]. Therefore, a definitive diagnosis of PB involvement by MF and SS would require the demonstration of the same clonal TCR-GR as that detected at other sites (i.e., skin or LNs), and exclusion of other potential causes of T-cell monoclonality.

Both SBA and PCR-based studies have found that patients with CTCL can show monoclonal TCR-GRs in the PB, BM, and other biological fluids (ascites and synovial fluid) that are identical to those detected in the skin and LNs (Fig. 10.8) [19, 20, 22, 30, 33, 37, 38, 40, 77, 81, 82]. In general, PCR-positivity at extracutaneous sites correlates with disease stage; monoclonality is either not detected or infrequently found in early-stage (stages I–II) MF, and more commonly identified in erythrodermic and/or late-stage (stages III–IV) MF [19, 20, 22, 30, 33, 37, 38, 40, 77, 81, 82]. In some cases of early-stage MF, monoclonal TCR-GRs may be found in the PB, in the absence of a significant population of morphologically or immunophenotypically abnormal T-cells [3, 81].

PCR results may become negative in PB samples following therapy; however, some patients with clinical and histopathological (skin) remission continue to demonstrate clonal T-cell populations in their PB [37, 40]. Similar to such findings in the skin (see Section on Minimal Residual Disease), the persistence of a dominant T-cell clone in the PB may be a risk factor for disease recurrence and requires further investigation. Fraser-Andrews et al. [81] have shown statistically significant differences in patient outcome based on the presence or absence of a T-cell clone in the PB (i.e., 30% *vs.* 50% 10-year survival, respectively), independent of skin stage. Furthermore, Beylot-Barry et al. [30] reported that the

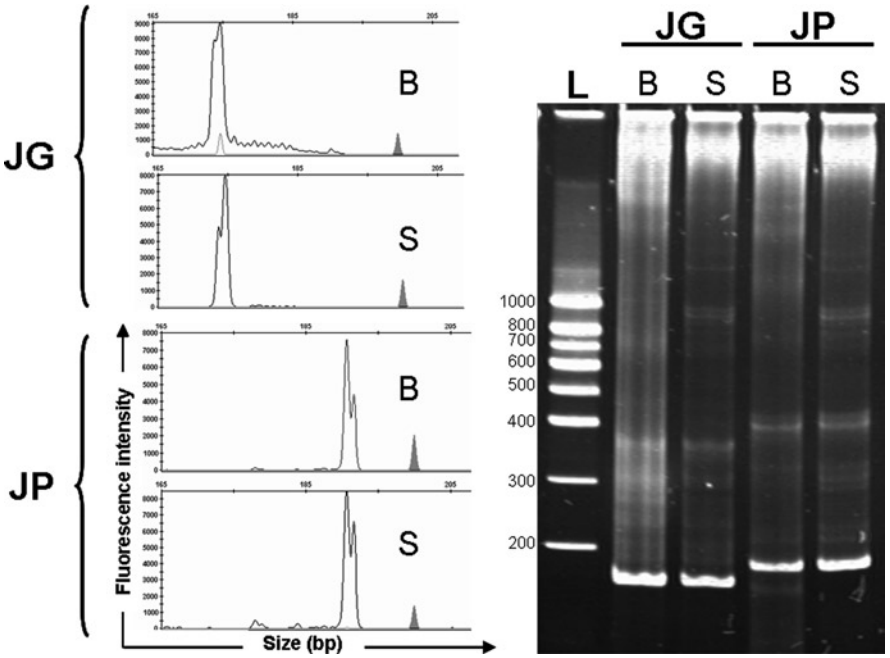

Fig. 10.8 Rearranged T-cell receptor γ (*TCR-γ*) gene PCR products, amplified from the skin (S) and blood (B) of a patient with Sézary syndrome, were subjected to capillary electrophoresis (*left panel*) and heteroduplex analysis (*HDA*) (*right panel*). TCR-γ V-JG1/JG2 and V-JP1/JP2 primers showed identical monoclonal results in skin and blood samples, indicated in the two corresponding graphs as JG (size 175 bp) and JP (size 191 bp), respectively. Internal size standards are shown in opaque peaks. For HDA, the sizes (bp) of bands in the molecular weight ladder (L) are noted (Courtesy of Dr. Sophie Marty-Grès, Prof. Jean-François Eliaou, and Prof. Olivier Dereure, CHU Montpellier, Montpellier, France)

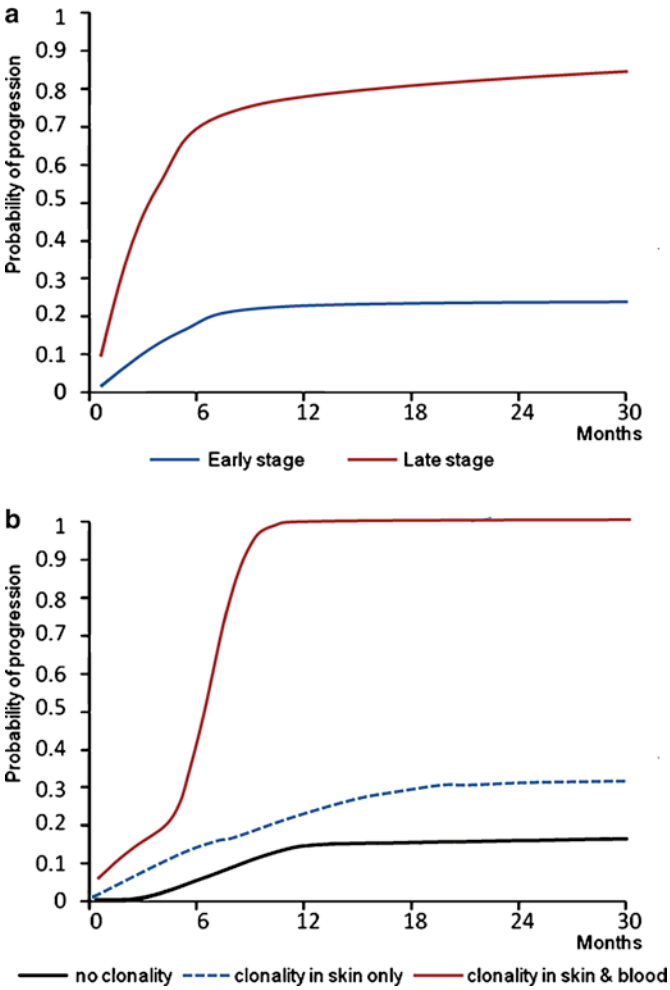

Fig. 10.9 (**a**) Probability of progression of patients with MF/SS according to the initial clinical stage (early stage *vs.* late stage). (**b**) Probability of progression of patients with MF/SS according to clonality results in skin and blood (no T-cell clone in skin, T-cell clone in skin only, and identical T-cell clone in skin and blood) (Courtesy of Dr. Zendee Elaba, Department of Pathology, Hartford Hospital, Hartford, CT, USA: Modified from Beylot-Barry et al. [30]

presence of an identical T-cell clone in the PB and skin is an independent prognostic factor for disease progression in patients with MF and SS (Fig. 10.9).

Importantly, otherwise healthy elderly individuals, and patients with both non-CTCL malignant disease (including cutaneous B-cell lymphomas) and benign dermatoses, can also show clonal TCR-GRs in their PB [3, 19, 33, 37], with identical T-cell clones found in both PB and skin samples in up to 3% of the latter cases [33]. Clinical–histopathological correlation, repeat/serial molecular studies, and/or long-term follow-up would be required for a definitive diagnosis in such instances.

Internal Organs

PCR-based analysis can also be used to confirm tumor involvement by internal organs (i.e., myocardium, liver, spleen, and lung) in patients with CTCL [83].

Prognosis of CTCL

The type and extent of skin involvement (T parameter), and presence or absence of extracutaneous disease (NMB parameters), which determine overall clinical stage, are the most important prognostic parameters in patients with CTCL [4]. Patients with limited patch/plaque disease (<10% of total-skin surface, stage IA) have an excellent prognosis with a long-term life expectancy similar to age-, sex-, and ethnicity-matched control populations [4]. However, ~9% of these patients will show disease progression. Patients with generalized patch/plaque disease (≥10% of total-skin surface, stage IB) have a 24% risk of progression and a median survival of 11 years [4]. At the other end of the spectrum are individuals with cutaneous tumors (stage IIB), generalized erythroderma (stage III), and extracutaneous disease at presentation (LNs [stage IVA] and viscera [stage IVB]) who show median survivals of 3, 4.5, and <1.5 years, respectively [4]. Patients with MF can also show transformation to a clonally-related large cell lymphoma, associated with rapid disease progression and less favorable prognosis [4, 55, 56]. SS has a 5-year survival of only 11% [5]. Survival probability can be accurately predicted in most cases using a formula that evaluates involvement of skin, LNs, PB, and visceral organs (CTCL-Severity-Index) [84]. Measurable serological biomarkers (i.e., LDH, sIL-2R, neopterin) may also show some association with disease stage and progression [84]. The prognostic utility of immunophenotyping, T-cell clonality studies, and other molecular tests has also been evaluated.

Phenotypic variations of the tumor cells (i.e., T-helper *vs.* T-cytotoxic; TCR-α/β+ *vs.* TCR-γ/δ+; and CD4 *vs.* CD8 expression) have shown no correlation with prognosis in patients with early-stage MF [24].

As stated previously, the detection of clonal T-cell populations in LNs [3, 41, 76–78] and/or PB samples [30, 81] is associated with poor outcomes in patients with MF. Very few studies have investigated if T-cell clonality status in skin biopsies of early MF influences prognosis in these patients. Massone et al. [24] reported that PCR-detected monoclonality did not have any prognostic significance in early lesions of MF. However, Ponti et al. [36] found that PCR-negativity was associated with prolonged stable disease, with only a minority (14%) of PCR-positive patients showing disease progression. In an SBA-based study by Guitart et al. [21], the presence of T-cell monoclonality negatively influenced short-term survival compared with a polyclonal result (i.e., 5-year survival 67% *vs.* 87%), but no significant differences in long-term survival were noted between both groups. Furthermore, a number of studies have suggested that the risk of clinical progression in an individual patient is significantly correlated with the presence of an identical monoclonal TCR-GR in: (1) multiple concurrent skin biopsies at diagnosis [39]; (2) in both LN and skin samples [41]; and (3) in both PB and skin samples (Fig. 10.9) [30, 82]. The prognostic significance of monoclonal TCR-GRs in the BM is unclear [3, 82]. It has been proposed that the presence of cytologically atypical lymphoid aggregates in the BM correlates with shortened survival of patients with MF, but multivariate analysis has not confirmed BM involvement as an independent prognostic parameter [3]. Prospective molecular testing of BM samples in these patients may help to clarify this point.

Karyotyping and ISH-based studies have shown that the rate of chromosomal aberrations is associated with disease activity and has prognostic significance in patients with CTCL [85]. Aberrations of chromosomes 1, 6, and 11, although increasing in prevalence with activity of the disease, are also detectable in remission (i.e., are hallmarks of existing disease). Aberrations of chromosomes 8 and 17 are found to correlate with active or progressive disease [85].

cDNA/oligonucleotide microarray technologies also hold great promise for the identification of prognostic biomarkers, in addition to pathogenic mechanisms, in CTCL. cDNA microarrays have been used for classification and prediction of survival in patients with leukemic CTCL [70]. Kari et al. [70] demonstrated that analysis of PB samples using a panel of ten genes identifies patients with poor prognosis, independent of tumor burden (Fig. 10.10) [70]. Gene expression profiling has also been accomplished in lesional skin samples of CTCL patients [5, 7]. Clinically relevant signatures have been shown to correlate with disease stage and outcome (i.e., poorest survival seen with

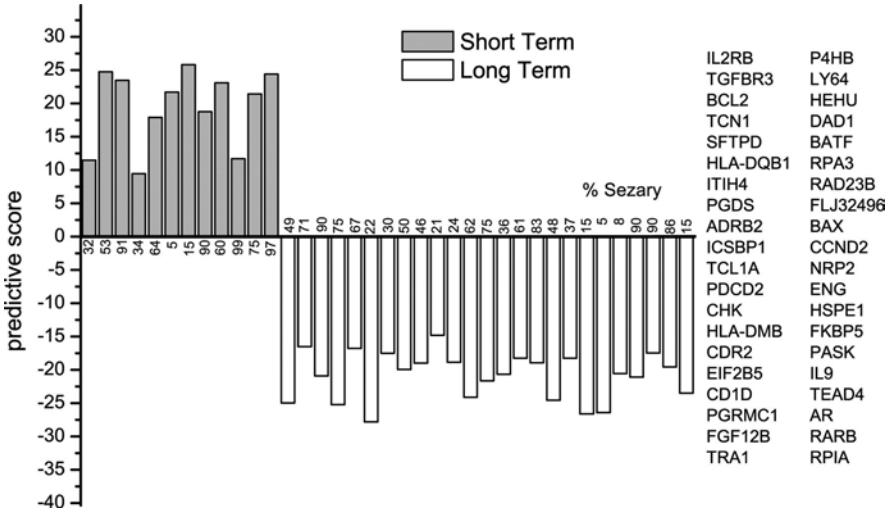

Fig. 10.10 cDNA microarray analysis of a small number of genes can be used to predict survival in patients with leukemic CTCL. *Left:* Results of cross-validation using the 40 most informative genes (20 most positive and 20 most negative). Positive scores indicate short-term (*ST*) survivors. The first 12 bars represent the ST survivors. *Right*: The genes used for classification. *Left column*, overexpressed in long-term (*LT*) survivors; *right column*, overexpressed in ST survivors. The top ten genes, five from each column, are sufficient for 100% accurate cross-validation (From Kari et al. [70]. Reprinted with permission from Rockefeller University Press, Copyright © 2003)

activated lymphocyte cluster) [5]. It has also been demonstrated that a subset of patients with cutaneous anaplastic large cell lymphoma (i.e., those with extensive limb involvement) have an unfavorable prognosis associated with a distinct gene expression profile [86]. The possible prognostic utility of microarray analysis in skin biopsies of early MF remains to be fully explored. However, its application in this scenario may be confounded by the paucity of malignant cells and/or the presence of reactive T-cells in skin biopsies of early-stage lesions [7].

Using CGH, Fischer et al. [66] found that genomic aberrations in skin biopsy samples correlated with outcome in CTCL patients (Figs. 10.11 and 10.12). The presence of greater than or equal to five aberrations, gain of 8q, and loss of 6q, 10q and 13q, were associated with a significantly shorter survival [66]. However, gain of 7 and loss of 17p (the most frequently observed chromosomal aberrations) did not influence patient prognosis [66].

Array-CGH-based studies have also identified genomic subgroups and prognostic markers for tumor stage MF [52, 53]. The presence of greater than five DNA aberrations (genomic unstable group), in addition to deletion of 9p21 and 10q26, and gain of 8q24 and 1q21-q22, is associated with poor outcome in these patients [52, 53]. Additional prospective long-term studies to validate the findings of all these reports are required.

Response to Treatment, Minimal Residual Disease, and Identification of Novel Therapeutic Targets in CTCL

Molecular-based strategies may be employed to determine response to standard therapies, define minimal residual disease and remission, detect early relapse, and uncover novel treatment strategies in patients with CTCL.

Delfau-Larue et al. [32] showed that PCR findings at diagnosis are predictive of treatment response in MF patients. The absence of a detectable cutaneous T-cell clone was associated with a

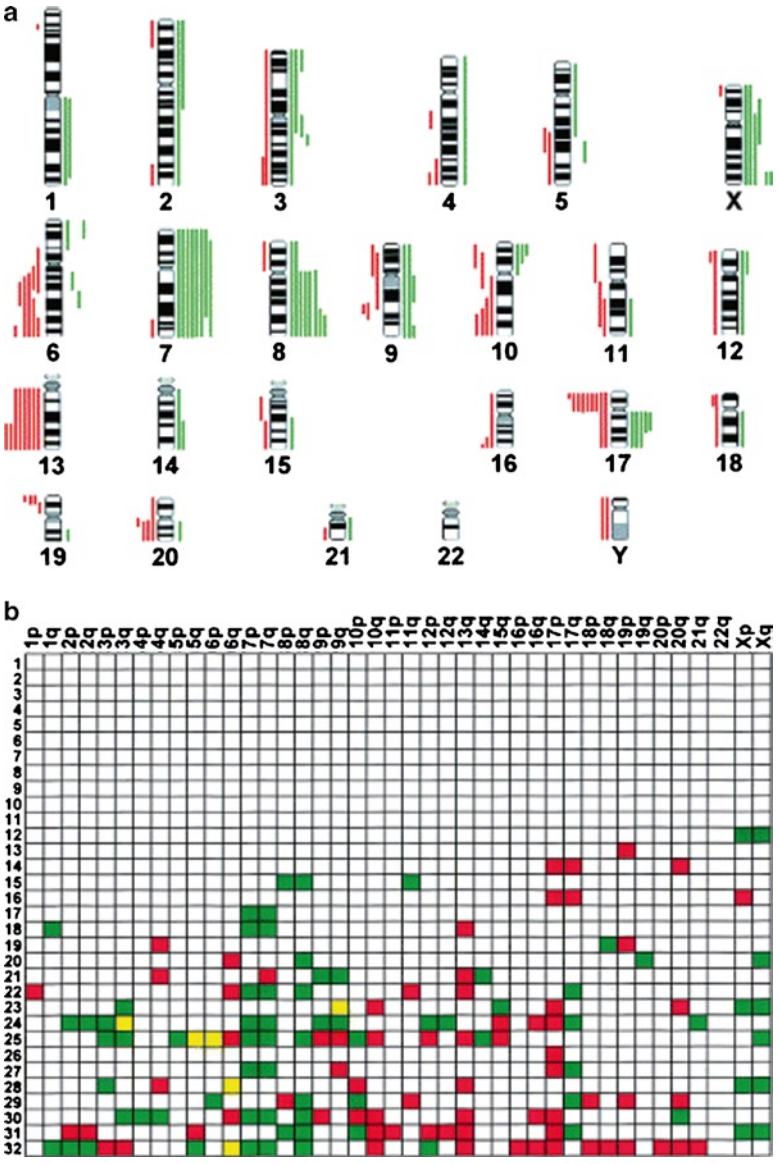

Fig. 10.11 Results of the CGH analysis of 32 patients with indolent and aggressive CTCL. (**a**) Lines on the left of the chromosome ideograms indicate loss of a chromosomal region in a patient (diminished chromatin), and lines on the right side represent gains (enhanced chromatin). (**b**) Box chart of aberrations for the long and short chromosomal arms in the 32 patients. *Red* = loss of chromatin, *green* = gain of chromatin, *yellow* = loss and gain of chromatin (From Fischer et al. [66]. Reprinted with permission from Macmillan, Copyright © 2004)

higher rate of complete remission [32]. However, complete remission may not be the therapeutic goal, as it has not been found to correlate with improved survival in patients with MF [30].

Discrepancies do exist between clinical and light microscopic responses to therapy in patients with MF, and the histopathological interpretation of post-treatment biopsies can be problematic in some cases [27]. Accordingly, the use of clonality-based testing has been proposed as a means to more objectively determine the presence of residual/recurrent skin involvement by MF [22, 27, 35, 37, 40]. Poszepczynska-Guigne et al. [35] have defined minimal residual disease (MRD) in MF as the persistence of a T-cell clone in treated MF skin lesions, despite evidence of complete clinical remission.

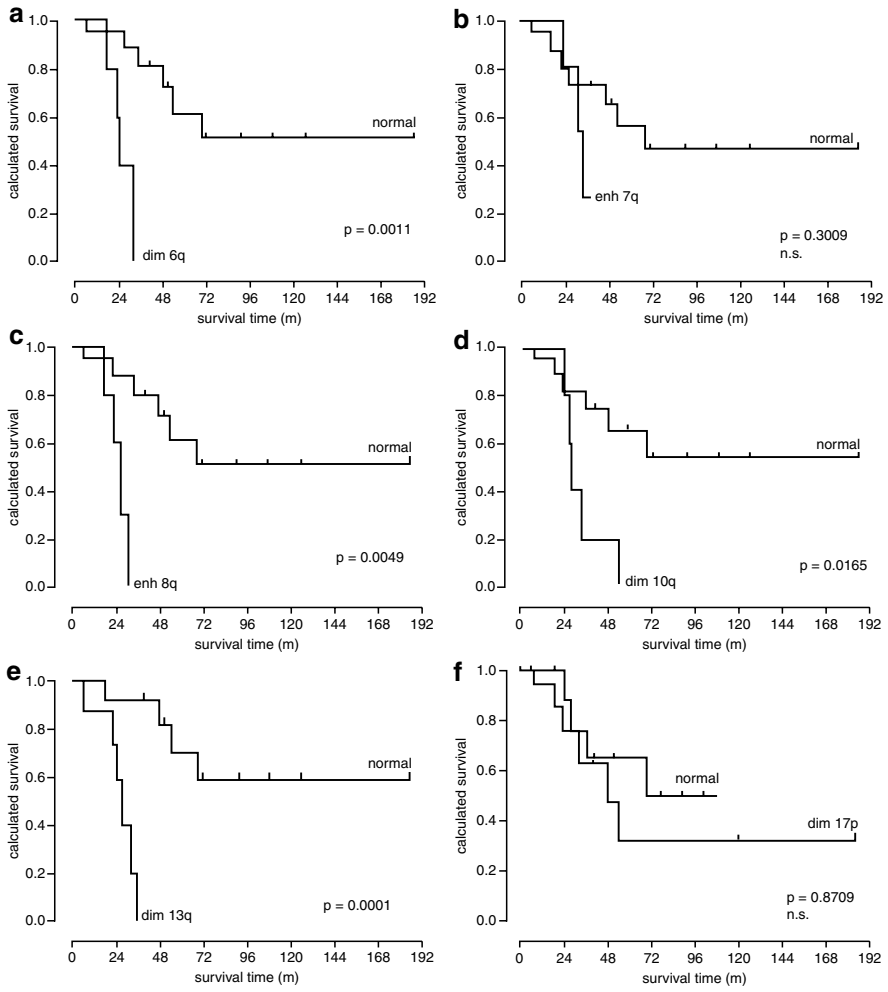

Fig. 10.12 Correlation of chromosomal imbalances with cumulative survival rates in patients with CTCL. Gain of chromatin in 8q, and loss of chromatin in 6q, 10q, and 13q, correlated with a significantly shorter survival. Loss in 17p and gain in 7 did not influence the prognosis of the disease (From Fischer et al. [66]. Reprinted with permission from Macmillan, Copyright © 2004)

Of note, PCR-based detection of MRD has been reported in 31–75% of skin and/or PB samples in such patients [22, 35, 40]. These findings might explain the occurrence of frequent relapses when clinical and/or histopathological remission appeared to have been achieved [40]. In fact, the skin may serve as a reservoir for clinically occult residual disease in CTCL [46]. However, unlike other hematopoietic tumors, the prognostic value of MRD detection in MF patients is at present unclear and requires large-scale prospective study [35]. In patients with identical TCR-GRs at different sites/times, the clonospecific imprint has been reported as a patient-dependent marker of MF [35]. In such cases, sensitivity and specificity can be increased by cloning and sequencing PCR products, with subsequent development of patient-specific primers directed against "their" clonally rearranged TCR gene(s) (i.e., "molecular fingerprint") [31, 43, 44, 46]. It has also been proposed that quantitative analysis of PCR products (i.e., real-time PCR) may have a role in defining the maximal tumor clone density that would allow the discontinuation of treatment, in order to balance the risk of relapse following treatment cessation with the risk of side effects from prolonged therapy [87].

The increasing use of proteomic and genomic technologies has led not only to a better understanding of the pathogenesis and tumor biology of CTCL, but also to the discovery of targets for therapeutic intervention and the development of novel drugs. Currently, targeted therapy for CTCL is largely based on the blockade/inhibition of specific receptors/proteins (i.e., IL-2R/CD25, CD4, CD30, CD52), whose expression by tumor cells can be determined by techniques as simple as IHC [8, 88]. Molecular technologies may reveal gene signatures/clusters that correlate with response to treatments and also provide new options for therapeutic intervention, which are more effective and/or less toxic than those currently available. An in-depth discussion of the clinical management of CTCL is beyond the scope of this chapter, and comprehensive reviews are available elsewhere [4, 8, 88]. However, a number of potential therapeutic biomarkers in MF, which have been uncovered by molecular strategies, will be discussed.

Using oligonucleotide microarray-based analysis of lesional skin, Shin et al. [5] identified differentially expressed genes significantly associated with lower-stage/treatment-responsive CTCL versus higher-stage/treatment-resistant CTCL. In the poor treatment response group, downregulated genes were involved in extracellular matrix pathway, WNT signaling pathway, epidermal development, and frizzled signaling, whereas upregulated genes included those involved in mitosis, immune response, response to virus, apoptosis, and T-cell activation [5].

In addition, the molecular (and immunologic) characterization of apoptosis pathway (STAT3, bcl-2, CD95 receptor) and cytokine (IL-2, IL-5, IL-10, CTLA-4) transcripts/proteins expressed in different stages of CTCL (i.e., cancer immunoediting principal) helps to explain the variable effectiveness of current therapies, and may be used for the development of new therapeutic strategies [8, 9].

As discussed previously, array-CGH has identified deletion of 9p21 and 10q26, and gain of 8q24 and 1q21-q22, as negative prognostic biomarkers in tumor stage MF [52, 53]. These loci may contain genes that modify the biological behavior or treatment responses of MF (i.e., CDKN2A, CDKN2B, MTAP, MGMT, EBF3, MYC, MCL1). Van Doorn et al. [53] have proposed that gain of chromosome 1q21-q22 may be associated with increased expression of the MCL1 gene and glucocorticosteroid resistance in CTCL. The demonstration of 1q21-q22 gain in treatment-refractory MF patients may identify a cohort that could potentially benefit from the addition of rapamycin (a modulator of MCL1 activity) to the chemotherapeutic regimen and restoration of glucocorticosteroid sensitivity in neoplastic T-cells [53].

An epigenetic profiling study by van Doorn et al. [89] found a high frequency of promoter hypermethylation of putative TSGs involved in DNA repair, cell cycle, and apoptosis signaling pathways (i.e., BCL7A, PTPRG, THBS4, TP73, CDKN2A, CDKN2B, CHFR, PYCARD) in CTCL. The concept that epigenetic dysregulation may be a target for therapy in CTCL is supported by the observed favorable response to treatment with epigenetic modulators (i.e., histone deacetylase inhibitors) in these tumors [8, 88, 89]. Using cDNA microarray-based gene expression profiling and quantitative real-time PCR, Ellis et al. [90] reported the activation of 3 genes and repression of 20 genes in skin biopsies of CTCL patients following oral therapy with panobinostat, a histone deacetylase inhibitor. These genes mediate biological responses such as apoptosis, immune regulation, and angiogenesis. However, the low number of patients (n = 6) precluded a correlation between altered expression of an individual gene or gene set and clinical outcome. Nonetheless, these genes are potential molecular biomarkers for panobinostat response in patients with CTCL, and further assessment of their possible role(s) in mediating the antitumor effects of this drug are warranted [90].

Transcription factor profiling of MF and SS has revealed constitutively active NF-κB, STAT, and their respective signal transduction pathways as possible therapeutic targets [91]. Such testing has also identified several prototypic drugs as inhibitors of these targets and altered pathway components [91].

Tracey et al. [92, 93] have performed comparative gene expression profiling studies on interferon alpha (IFN-α)-sensitive and IFN-α-resistant variants of a CTCL cell line. Their experiments

revealed that IFN-α is responsible for the regulation of hundreds of genes in both variants, including regulators of signal transduction, cell cycle control, apoptosis, and transcription [92, 93]. In CTCL cells, response to IFN-α was due to a combination of apoptosis induction (involving TNFSF10 and HSXIAPAF1) and cell cycle arrest (via CDK4 and CCNG2 downregulation, and CDKN2C and TSG upregulation). Resistance to IFN-α was associated with an inability to induce IRF1 and IRF7, and deregulation of HSXIAPAF1, TRADD, BAD, and BNIP3 apoptotic signals. Upregulation of RELB and LTB suggested a critical role for NF-κB in promoting cell survival in IFN-α-resistant CTCL cells [93]. Importantly, resistance to IFN-α was consistently associated with changes in the expression of a set of 39 genes, and in particular upregulation of MAL, a T-cell differentiation antigen [92]. MAL was also found to be expressed by tumor cells in a series of CTCL patients treated with IFN-α and/or photochemotherapy. MAL expression correlated with delayed response to treatment in this study (i.e., 80% of slow-responders were MAL+ *vs.* 70% of rapid-responders were MAL–) [92].

In the future, molecular techniques could be routinely used to subgroup MF patients based on predicted survival and putative drug targets, and thereby influence the selection of treatment strategies.

In summary, monoclonality is not synonymous with malignancy, and results of molecular-based T-cell clonality studies must be correlated with available clinical, histopathological, and immunophenotypic data in any individual patient. In patients with either suspected or diagnostic CTCL, the molecular confirmation of T-cell clonality status in the skin and other sites can be of diagnostic, staging, and/or prognostic value. The presence of an oligoclonal/polyclonal T-cell population in skin specimens, particularly from individuals without definitive clinical signs of malignancy, supports a benign reactive process. Importantly, the detection of a monoclonal T-cell population in a skin sample, in the absence of an inciting factor, may be an early sign of malignancy or identify those lesions with a risk, albeit low, for malignant transformation. The analysis of additional specimens (both skin-derived and from other sites) and close clinical follow-up is warranted in these latter individuals. With increased sensitivity and sophistication of molecular assays for clonality, it is likely that the quantitative level at which a clonal T-cell population is detected will become a more clinically relevant variable than a purely qualitative result (i.e., presence or absence of a T-cell clone). In addition to TCR-GR analyses, other cytogenetic and molecular techniques may have a role in the diagnosis and prognostication of patients with CTCL, and selection of appropriate therapy. As additional clinical, histopathological, immunophenotypic, and genetic information is uncovered, it is envisioned that there will be further revisions to the classification and staging guidelines for CTCL.

References

1. Willemze R, Jaffe ES, Burg G, et al. WHO-EORTC classification for cutaneous lymphomas. Blood. 2005;105:3768–85.
2. Smoller BR, Santucci M, Wood GS, et al. Histopathology and genetics of cutaneous T-cell lymphoma. Hematol Oncol Clin North Am. 2003;17:1277–311.
3. Olsen E, Vonderheid E, Pimpinelli N, et al. ISCL/EORTC. Revisions to the staging and classification of mycosis fungoides and Sézary syndrome: a proposal of the International Society for Cutaneous Lymphomas (ISCL) and the cutaneous lymphoma task force of the European Organization of Research and Treatment of Cancer (EORTC). Blood. 2007;110:1713–22. Erratum in: Blood. 2008;111:4830.
4. Zinzani PL, Ferreri AJ, Cerroni L. Mycosis fungoides. Crit Rev Oncol Hematol. 2008;65:172–82.
5. Shin J, Monti S, Aires DJ, et al. Lesional gene expression profiling in cutaneous T-cell lymphoma reveals natural clusters associated with disease outcome. Blood. 2007;110:3015–27. Erratum in: Blood. 2008;111:49.
6. Wood GS, Greenberg HL. Diagnosis, staging, and monitoring of cutaneous T-cell lymphoma. Dermatol Ther. 2003;16:269–75.
7. Karenko L, Hahtola S, Ranki A. Molecular cytogenetics in the study of cutaneous T-cell lymphomas (CTCL). Cytogenet Genome Res. 2007;118:353–61.

8. Klemke CD, Goerdt S. Molecular biology and targeted therapy of cutaneous T-cell lymphomas. G Ital Dermatol Venereol. 2008;143:365–74.
9. Wong HK. Immunopathogenesis of mycosis fungoides/Sézary syndrome (cutaneous T-cell lymphoma). G Ital Dermatol Venereol. 2008;143:375–83.
10. Guitart J, Kennedy J, Ronan S, et al. Histologic criteria for the diagnosis of mycosis fungoides: proposal for a grading system to standardize pathology reporting. J Cutan Pathol. 2001;28:174–83.
11. Pimpinelli N, Olsen EA, Santucci M, et al. International Society for Cutaneous Lymphoma. Defining early mycosis fungoides. J Am Acad Dermatol. 2005;53:1053–63.
12. Furmanczyk PS, Wolgamot GM, Kussick SJ, et al. Diagnosis of mycosis fungoides with different algorithmic approaches. J Cutan Pathol. 2010;37:8–14.
13. Meyerson HJ. Flow cytometry for the diagnosis of mycosis fungoides. G Ital Dermatol Venereol. 2008;143:21–41.
14. Murphy M, Fullen D, Carlson JA. Low CD7 expression in benign and malignant cutaneous lymphocytic infiltrates: experience with an antibody reactive with paraffin-embedded tissue. Am J Dermatopathol. 2002;24:6–16.
15. Thurber SE, Zhang B, Kim YH, et al. T-cell clonality analysis in biopsy specimens from two different skin sites shows high specificity in the diagnosis of patients with suggested mycosis fungoides. J Am Acad Dermatol. 2007;57:782–90.
16. Groenen PJ, Langerak AW, van Dongen JJ, et al. Pitfalls in TCR gene clonality testing: teaching cases. J Hematop. 2008;1:97–109.
17. van Dongen JJ, Langerak AW, Brüggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17:2257–317.
18. Langerak AW, Groenen P, van Krieken JH, et al. Immunoglobulin/T-cell receptor clonality diagnostics. Exp Opin Med Diagn. 2007;1:451–61.
19. Sandberg Y, Heule F, Lam K, et al. Molecular immunoglobulin/T- cell receptor clonality analysis in cutaneous lymphoproliferations. Experience with the BIOMED-2 standardized polymerase chain reaction protocol. Haematologica. 2003;88:659–70.
20. Bignon YJ, Souteyrand P, Roger H, et al. Clonotypic heterogeneity in cutaneous T-cell lymphomas. Cancer Res. 1990;50:6620–5.
21. Guitart J, Camisa C, Ehrlich M, et al. Long-term implications of T-cell receptor gene rearrangement analysis by Southern blot in patients with cutaneous T-cell lymphoma. J Am Acad Dermatol. 2003;48:775–9.
22. Curcó N, Servitje O, Llucià M, et al. Genotypic analysis of cutaneous T-cell lymphoma: a comparative study of Southern blot analysis with polymerase chain reaction amplification of the T-cell receptor-gamma gene. Br J Dermatol. 1997;137:673–9.
23. Menke MA, Tiemann M, Vogelsang D, et al. Temperature gradient gel electrophoresis for analysis of a polymerase chain reaction-based diagnostic clonality assay in the early stages of cutaneous T-cell lymphomas. Electrophoresis. 1995;16:733–8.
24. Massone C, Crisman G, Kerl H, et al. The prognosis of early mycosis fungoides is not influenced by phenotype and T-cell clonality. Br J Dermatol. 2008;159:881–6.
25. Liebmann RD, Anderson B, McCarthy KP, et al. The polymerase chain reaction in the diagnosis of early mycosis fungoides. J Pathol. 1997;182:282–7.
26. Hsiao PF, Hsiao CH, Lin YC, et al. Histopathologic-molecular correlation in early mycosis fungoides using T-cell receptor gamma gene rearrangement by polymerase chain reaction with laser capture microdissection. J Formos Med Assoc. 2007;106:265–72.
27. Hsiao PF, Hsiao CH, Tsai TF, et al. Minimal residual disease in hypopigmented mycosis fungoides. J Am Acad Dermatol. 2006;54(5 Suppl):S198–201.
28. Murphy M, Signoretti S, Kadin ME, et al. Detection of TCR-gamma gene rearrangements in early mycosis fungoides by non-radioactive PCR-SSCP. J Cutan Pathol. 2000;27:228–34.
29. Signoretti S, Murphy M, Cangi MG, et al. Detection of clonal T-cell receptor gamma gene rearrangements in paraffin-embedded tissue by polymerase chain reaction and nonradioactive single-strand conformational polymorphism analysis. Am J Pathol. 1999;154:67–75.
30. Beylot-Barry M, Sibaud V, Thiebaut R, et al. Evidence that an identical T cell clone in skin and peripheral blood lymphocytes is an independent prognostic factor in primary cutaneous T cell lymphomas. J Invest Dermatol. 2001;117:920–6.
31. Veelken H, Wood GS, Sklar J. Molecular staging of cutaneous T-cell lymphoma: evidence for systemic involvement in early disease. J Invest Dermatol. 1995;104:889–94.
32. Delfau-Larue MH, Dalac S, Lepage E, et al. Prognostic significance of a polymerase chain reaction-detectable dominant T-lymphocyte clone in cutaneous lesions of patients with mycosis fungoides. Blood. 1998;92:3376–80.
33. Delfau-Larue MH, Laroche L, Wechsler J, et al. Diagnostic value of dominant T-cell clones in peripheral blood in 363 patients presenting consecutively with a clinical suspicion of cutaneous lymphoma. Blood. 2000;96:2987–92.

34. Li N, Bhawan J. New insights into the applicability of T-cell receptor gamma gene rearrangement analysis in cutaneous T-cell lymphoma. J Cutan Pathol. 2001;28:412–8.
35. Poszepczynska-Guigne E, Bagot M, Wechsler J, et al. Minimal residual disease in mycosis fungoides follow-up can be assessed by polymerase chain reaction. Br J Dermatol. 2003;148:265–71.
36. Ponti R, Quaglino P, Novelli M, et al. T-cell receptor gamma gene rearrangement by multiplex polymerase chain reaction/heteroduplex analysis in patients with cutaneous T-cell lymphoma (mycosis fungoides/Sézary syndrome) and benign inflammatory disease: correlation with clinical, histological and immunophenotypical findings. Br J Dermatol. 2005;153:565–73.
37. Ponti R, Fierro MT, Quaglino P, et al. TCRgamma-chain gene rearrangement by PCR-based GeneScan: diagnostic accuracy improvement and clonal heterogeneity analysis in multiple cutaneous T-cell lymphoma samples. J Invest Dermatol. 2008;128:1030–8.
38. Gubler B, Marty-Grès S, Guillot B, et al. Molecular identity of skin and blood T-cell clones in cutaneous T-cell lymphoma patients as determined from the migration pattern of the T-cell receptor-gamma gene by capillary electrophoresis. Electrophoresis. 2009;30:999–1007.
39. Vega F, Luthra R, Medeiros LJ, et al. Clonal heterogeneity in mycosis fungoides and its relationship to clinical course. Blood. 2002;100:3369–73.
40. Dereure O, Balavoine M, Salles MT, et al. Correlations between clinical, histologic, blood, and skin polymerase chain reaction outcome in patients treated for mycosis fungoides. J Invest Dermatol. 2003;121:614–7.
41. Assaf C, Hummel M, Steinhoff M, et al. Early TCR-beta and TCR-gamma PCR detection of T-cell clonality indicates minimal tumor disease in lymph nodes of cutaneous T-cell lymphoma: diagnostic and prognostic implications. Blood. 2005;105:503–10.
42. Plaza JA, Morrison C, Magro CM. Assessment of TCR-beta clonality in a diverse group of cutaneous T-Cell infiltrates. J Cutan Pathol. 2008;35:358–65.
43. Lessin SR, Rook AH, Rovera G. Molecular diagnosis of cutaneous T-cell lymphoma: polymerase chain reaction amplification of T-cell antigen receptor beta-chain gene rearrangements. J Invest Dermatol. 1991;96:299–302.
44. Kono DH, Baccala R, Balderas RS, et al. Application of a multiprobe RNase protection assay and junctional sequences to define V beta gene diversity in Sézary syndrome. Am J Pathol. 1992;140:823–30.
45. Magro CM, Nuovo GJ, Crowson AN. The utility of the in situ detection of T-cell receptor Beta rearrangements in cutaneous T-cell-dominant infiltrates. Diagn Mol Pathol. 2003;12:133–41.
46. Wood GS, Haeffner A, Dummer R, et al. Molecular biology techniques for the diagnosis of cutaneous T-cell lymphoma. Dermatol Clin. 1994;12:231–41.
47. Gra OA, Sidorova JV, Nikitin EA, et al. Analysis of T-cell receptor-gamma gene rearrangements using oligonucleotide microchip: a novel approach for the determination of T-cell clonality. J Mol Diagn. 2007;9:249–57.
48. Boone SL, Guitart J, Gerami P. Follicular mycosis fungoides: a histopathologic, immunohistochemical, and genotypic review. G Ital Dermatol Venereol. 2008;143:409–14.
49. Gallardo F, García-Muret MP, Servitje O, et al. Cutaneous lymphomas showing prominent granulomatous component: clinicopathological features in a series of 16 cases. J Eur Acad Dermatol Venereol. 2009;23:639–47.
50. Kim ST, Jeon YS, Sim HJ, et al. Clinicopathologic features and T-cell receptor gene rearrangement findings of mycosis fungoides palmaris et plantaris. J Am Acad Dermatol. 2006;54:466–71.
51. Booken N, Gratchev A, Utikal J, et al. Sézary syndrome is a unique cutaneous T-cell lymphoma as identified by an expanded gene signature including diagnostic marker molecules CDO1 and DNM3. Leukemia. 2008;22:393–9.
52. Salgado R, Servitje O, Gallardo F, et al. Oligonucleotide array-CGH identifies genomic subgroups and prognostic markers for tumor stage mycosis fungoides. J Invest Dermatol. 2009;130:1126–35.
53. van Doorn R, van Kester MS, Dijkman R, et al. Oncogenomic analysis of mycosis fungoides reveals major differences with Sézary syndrome. Blood. 2009;113:127–36.
54. Guitart J, Magro C. Cutaneous T-cell lymphoid dyscrasia: a unifying term for idiopathic chronic dermatoses with persistent T-cell clones. Arch Dermatol. 2007;143:921–32.
55. Wood GS, Bahler DW, Hoppe RT, et al. Transformation of mycosis fungoides: T-cell receptor beta gene analysis demonstrates a common clonal origin for plaque-type mycosis fungoides and CD30+ large-cell lymphoma. J Invest Dermatol. 1993;101:296–300.
56. Wood GS, Crooks CF, Uluer AZ. Lymphomatoid papulosis and associated cutaneous lymphoproliferative disorders exhibit a common clonal origin. J Invest Dermatol. 1995;105:51–5.
57. Gelfand JM, Shin DB, Neimann AL, et al. The risk of lymphoma in patients with psoriasis. J Invest Dermatol. 2006;126:2194–201.
58. Mehrany K, El-Azhary RA, Bouwhuis SA, et al. Cutaneous T-cell lymphoma and atopy: is there an association? Br J Dermatol. 2003;149:1013–7.
59. Rübben A, Kempf W, Kadin ME, et al. Multilineage progression of genetically unstable tumor subclones in cutaneous T-cell lymphoma. Exp Dermatol. 2004;13:472–83.
60. Klemke CD, Poenitz N, Dippel E, et al. T-cell clonality of undetermined significance. Arch Dermatol. 2006;142:393–4.

61. Hallermann C, Kaune KM, Tiemann M, et al. High frequency of primary cutaneous lymphomas associated with lymphoproliferative disorders of different lineage. Ann Hematol. 2007;86:509–15. Erratum in: Ann Hematol. 2007;86:517. Kaune, Matthias Kjell [corrected to Kaune, Kjell Matthias].
62. Karsai S, Hou JS, Telang G, et al. Sézary syndrome coexisting with B-cell chronic lymphocytic leukemia: case report and review of the literature. Dermatology. 2008;216:68–75.
63. Barzilai A, Trau H, David M, et al. Mycosis fungoides associated with B-cell malignancies. Br J Dermatol. 2006;155:379–86.
64. Kazakov DV, Kutzner H, Palmedo G, et al. Primary cutaneous lymphoproliferative disorders with dual lineage rearrangement. Am J Dermatopathol. 2006;28:399–409.
65. Olsen RJ, Chang CC, Herrick JL, et al. Acute leukemia immunohistochemistry: a systematic diagnostic approach. Arch Pathol Lab Med. 2008;132:462–75.
66. Fischer TC, Gellrich S, Muche JM, et al. Genomic aberrations and survival in cutaneous T cell lymphomas. J Invest Dermatol. 2004;122:579–86.
67. Hwang ST, Janik JE, Jaffe ES, et al. Mycosis fungoides and Sézary syndrome. Lancet. 2008;371:945–57.
68. Muche JM, Karenko L, Gellrich S, et al. Cellular coincidence of clonal T cell receptor rearrangements and complex clonal chromosomal aberrations-a hallmark of malignancy in cutaneous T cell lymphoma. J Invest Dermatol. 2004;122:574–8.
69. Tracey L, Villuendas R, Dotor AM, et al. Mycosis fungoides shows concurrent deregulation of multiple genes involved in the TNF signaling pathway: an expression profile study. Blood. 2003;102:1042–50.
70. Kari L, Loboda A, Nebozhyn M, et al. Classification and prediction of survival in patients with the leukemic phase of cutaneous T cell lymphoma. J Exp Med. 2003;197:1477–88.
71. Nebozhyn M, Loboda A, Kari L, et al. Quantitative PCR on 5 genes reliably identifies CTCL patients with 5% to 99% circulating tumor cells with 90% accuracy. Blood. 2006;107:3189–96.
72. Wood GS. Analysis of the t(2;5)(p23;q35) translocation in CD30+ primary cutaneous lymphoproliferative disorders and Hodgkin's disease. Leuk Lymphoma. 1998;29:93–101.
73. Kadin ME, Morris SW. The t(2;5) in human lymphomas. Leuk Lymphoma. 1998;29:249–56.
74. Sugita K, Shimauchi T, Kabashima R, et al. Loss of tumor cell CCR4 expression upon leukemic change in adult T-cell leukemia/lymphoma. J Am Acad Dermatol. 2009;61:163–4.
75. Pai RK, Mullins FM, Kim YH, et al. Cytologic evaluation of lymphadenopathy associated with mycosis fungoides and Sézary syndrome: role of immunophenotypic and molecular ancillary studies. Cancer. 2008;114:323–32.
76. Kern DE, Kidd PG, Moe R, et al. Analysis of T-cell receptor gene rearrangement in lymph nodes of patients with mycosis fungoides. Prognostic implications. Arch Dermatol. 1998;134:158–64.
77. Fraser-Andrews EA, Mitchell T, Ferreira S, et al. Molecular staging of lymph nodes from 60 patients with mycosis fungoides and Sézary syndrome: correlation with histopathology and outcome suggests prognostic relevance in mycosis fungoides. Br J Dermatol. 2006;155:756–62.
78. Juarez T, Isenhath SN, Polissar NL, et al. Analysis of T-cell receptor gene rearrangement for predicting clinical outcome in patients with cutaneous T-cell lymphoma: a comparison of Southern blot and polymerase chain reaction methods. Arch Dermatol. 2005;141:1107–13. Erratum in: Arch Dermatol. 2006;142:210.
79. Galindo LM, Garcia FU, Hanau CA, et al. Fine-needle aspiration biopsy in the evaluation of lymphadenopathy associated with cutaneous T-cell lymphoma (mycosis fungoides/Sézary syndrome). Am J Clin Pathol. 2000;113:865–71.
80. Vonderheid EC, Diamond LW, van Vloten WA, et al. Lymph node classification systems in cutaneous T-cell lymphoma. Evidence for the utility of the Working Formulation of Non-Hodgkin's Lymphomas for Clinical Usage. Cancer. 1994;73:207–18.
81. Fraser-Andrews EA, Woolford AJ, Russell-Jones R, et al. Detection of a peripheral blood T cell clone is an independent prognostic marker in mycosis fungoides. J Invest Dermatol. 2000;114:117–21.
82. Sibaud V, Beylot-Barry M, Thiébaut R, et al. Bone marrow histopathologic and molecular staging in epidermotropic T-cell lymphomas. Am J Clin Pathol. 2003;119:414–23.
83. Servitje O, Limón A, Blanco A, et al. Cardiac involvement and molecular staging in a fatal case of mycosis fungoides. Br J Dermatol. 1999;141:531–5.
84. Utikal J, Schadendorf D, Ugurel S. Serologic and immunohistochemical prognostic biomarkers of cutaneous malignancies. Arch Dermatol Res. 2007;298:469–77.
85. Karenko L, Sarna S, Kähkönen M, et al. Chromosomal abnormalities in relation to clinical disease in patients with cutaneous T-cell lymphoma: a 5-year follow-up study. Br J Dermatol. 2003;148:55–64.
86. Woo DK, Jones CR, Vanoli-Storz MN, et al. Prognostic factors in primary cutaneous anaplastic large cell lymphoma: characterization of clinical subset with worse outcome. Arch Dermatol. 2009;145:667–74.
87. Wood GS. Analysis of clonality in cutaneous T cell lymphoma and associated diseases. Ann NY Acad Sci. 2001;941:26–30.
88. Lansigan F, Choi J, Foss FM. Cutaneous T-cell lymphoma. Hematol Oncol Clin North Am. 2008;22:979–96.

89. van Doorn R, Zoutman WH, Dijkman R, et al. Epigenetic profiling of cutaneous T-cell lymphoma: promoter hypermethylation of multiple tumor suppressor genes including BCL7a, PTPRG, and p73. J Clin Oncol. 2005;23:3886–96.
90. Ellis L, Pan Y, Smyth GK, et al. Histone deacetylase inhibitor panobinostat induces clinical responses with associated alterations in gene expression profiles in cutaneous T-cell lymphoma. Clin Cancer Res. 2008;14:4500–10.
91. Döbbeling U. Transcription factor profiling shows new ways towards new treatment options of cutaneous T cell lymphomas. Curr Drug Discov Technol. 2007;4:24–30.
92. Tracey L, Villuendas R, Ortiz P, et al. Identification of genes involved in resistance to interferon-alpha in cutaneous T-cell lymphoma. Am J Pathol. 2002;161:1825–37.
93. Tracey L, Spiteri I, Ortiz P, et al. Transcriptional response of T cells to IFN-alpha: changes induced in IFN-alpha-sensitive and resistant cutaneous T cell lymphoma. J Interferon Cytokine Res. 2004;24:185–95.

Chapter 11
Cutaneous Non-MF T-Cell and NK-Cell Lymphoproliferative Disorders

Shih-Sung Chuang

Southern blot analysis (SBA) and polymerase chain reaction (PCR)-based testing for monoclonal T-cell receptor gene rearrangements (TCR-GRs), fluorescence in situ hybridization (FISH) for t(2;5)(p23;q35), in situ hybridization for Epstein-Barr virus-encoded mRNA (EBER-ISH), and SBA for human T-cell lymphotropic virus type I (HTLV-I) provirus integration are the most common molecular techniques used for the diagnosis of primary cutaneous non-mycosis fungoides (MF) T-cell and natural killer (NK)-cell lymphoproliferative disorders.

SBA is the gold standard for the study of T-cell clonality. However, this technology requires fresh/frozen tissue as substrate, is labor-intensive, and is not widely available in pathology laboratories. It has been largely replaced by PCR-based methods using DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissue as template. PCR analysis of TCR-GRs employs either conventional or BIOMED-2 primers [1–5]. The latter were developed by a consortium of European laboratories in an effort to provide standardized primers/protocols and allow for comparison of T-cell clonality studies performed at different centers [2]. In our recent investigation of a series of primary intestinal T-cell lymphomas, we reported that the standardized BIOMED-2 protocols were more sensitive in detecting monoclonal T-cell proliferations than the conventional primer sets [6]. Nonetheless, as conventional primers are less expensive and require fewer analysis tubes than the BIOMED-2 protocols, our strategy has been to use the former for initial screening. Those samples showing polyclonal results with these conventional primer sets are then further evaluated using the BIOMED-2 protocols.

FISH is frequently used in the diagnosis of B-cell non-Hodgkin's lymphomas (NHL). For example, the *c-MYC* rearrangement in Burkitt lymphoma and t(14;18) translocation in follicular lymphoma can be detected by this method. However, due to the lack of specific chromosomal aberrations in the majority of T-cell NHL, with the possible exception of the t(2;5)(p23;q35) chromosomal translocation in anaplastic large cell lymphoma (ALCL), FISH is rarely used in the routine diagnosis of T-cell lymphoproliferative disorders. The t(2;5)(p23;q35) translocation results in inappropriate expression of NPM-ALK/p80 tyrosine kinase, which is believed to play a central role in the pathogenesis of ALCL.

EBER-ISH is necessary for the diagnosis of extranodal NK/T-cell lymphomas. The majority of tumor cells are EBER-positive in almost all cases. Owing to improvements in automation, many laboratories now currently perform EBER-ISH using autostainers developed for immunohistochemistry, albeit with modified staining protocols.

S.-S. Chuang (✉)
Department of Pathology, Chi-Mei Medical Center, Tainan and Taipei Medical University,
901, Chuang Hwa Road, Yung Kang City, Tainan 710, Taiwan
e-mail: cmh5301@mail.chimei.org.tw

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_11,

SBA can also be used to demonstrate the integration of human T-cell lymphotropic virus type I (HTLV-I) provirus in tumor cells of adult T-cell leukemia/lymphoma (ATLL) [7].

MF is the prototypic primary cutaneous T-cell lymphoma (CTCL) and accounts for nearly half of all CTCL cases in Western populations [8]. However, non-MF CTCL subtypes are more frequent among Asians [9–13]. Importantly, the prognosis for patients with non-MF CTCL subtypes is generally poorer than for those with MF. Table 10.1 summarizes the 2005 WHO-EORTC classification of cutaneous non-MF T-cell and NK-cell neoplasms [8, 14, 15]. In this chapter, the application of molecular techniques to the diagnosis of these lymphoproliferative disorders is discussed.

Primary Cutaneous CD30-Positive Lymphoproliferative Disorders

Primary cutaneous CD30-positive lymphoproliferative disorders (CD30-positive LPD) are the second most common group of CTCL and include lymphomatoid papulosis (LyP), primary cutaneous anaplastic large cell lymphoma (PC-ALCL), and borderline lesions [8, 16, 17]. These diseases form a continuum with overlapping clinical and histopathological features. LyP is at the clinically benign end of this spectrum and is characterized by a recurrent, self-healing, papulonodular skin eruption. In contrast, most cases of PC-ALCL present as a solitary skin nodule or tumor, often with ulceration and without the waxing and waning features of LyP. Borderline lesions are those in which a definitive distinction between LyP and PC-ALCL cannot be made, despite careful clinical–histopathological correlation.

Lymphomatoid Papulosis (LyP)

Despite its clinically benign nature, LyP was first shown to be a monoclonal T-cell disorder in 1986 by Weiss et al. [18]. Using either whole tissue sections or single CD30-positive cells, identical dominant T-cell clones can be found in both (1) multiple regressing skin lesions of LyP and (2) LyP lesions and any "associated" primary CTCL (such as MF or PC-ALCL) that develops, in an individual patient [19–22]. The latter indicates related T-cell lymphoproliferative disorders in such patients, despite distinct clinical and histopathological characteristics. Three microscopic subtypes of LyP (A, B and C) have been described and represent a continuum with overlapping features [8, 16, 23, 24]. In type A lesions, scattered or small clusters of CD30-positive large lymphocytes are admixed with numerous inflammatory cells. Type B lesions are uncommon (less than 10% of cases), and are characterized by an epidermotropic infiltration of small atypical CD30-negative cells with cerebriform nuclei similar to MF. Type C lesions are comprised of monotonous or large sheets of CD30-positive large lymphocytes with relatively few inflammatory cells.

A diagnosis of LyP is typically made on the basis of its distinct clinical and histopathological features, and molecular studies add little value. Greisser et al. [25] used a highly sensitive PCR-based automated high-resolution fragment analysis on FFPE tissues, but did not detect monoclonal TCR-GRs in any of the ten type A and B LyP lesions studied. The negative results of their study probably reflected the presence of small numbers of neoplastic cells in both types of lesions that were below the detection threshold of their method. In contrast, using PCR-heteroduplex analysis for TCR-γ GRs in FFPE tissues, Zackheim et al. [21] identified monoclonal T-cells in 100% (7/7) of type A LyP cases tested [21]. Evidence of T-cell clonality has been found in up to 50% of type C LyP lesions [25].

Primary Cutaneous Anaplastic Large Cell Lymphoma (PC-ALCL)

PC-ALCL is composed of large anaplastic, pleomorphic or immunoblastic lymphocytes, with the majority of cells (>75%) demonstrating CD30 expression. Patients should not have clinical evidence or a history of MF, thereby excluding large cell transformation in a pre-existing disorder. Systemic ALCL with secondary cutaneous involvement should also be excluded. Studies have shown monoclonal TCR-GRs in ~50–100% of PC-ALCL [25–27]. However, in routine daily practice, T-cell clonality studies are usually not required to definitively diagnose this clinical entity. Figure 11.1 demonstrates a situation where TCR-GR analysis may be useful in certain cases of PC-ALCL. This patient presented with four concurrent cutaneous lesions on his right big toe and bilateral heels without lymphadenopathy. The tumor cells in the right big toe and left heel lesions shared the same immunophenotype, including CD30-positivity. Interestingly, the cells in the right big toe tumor

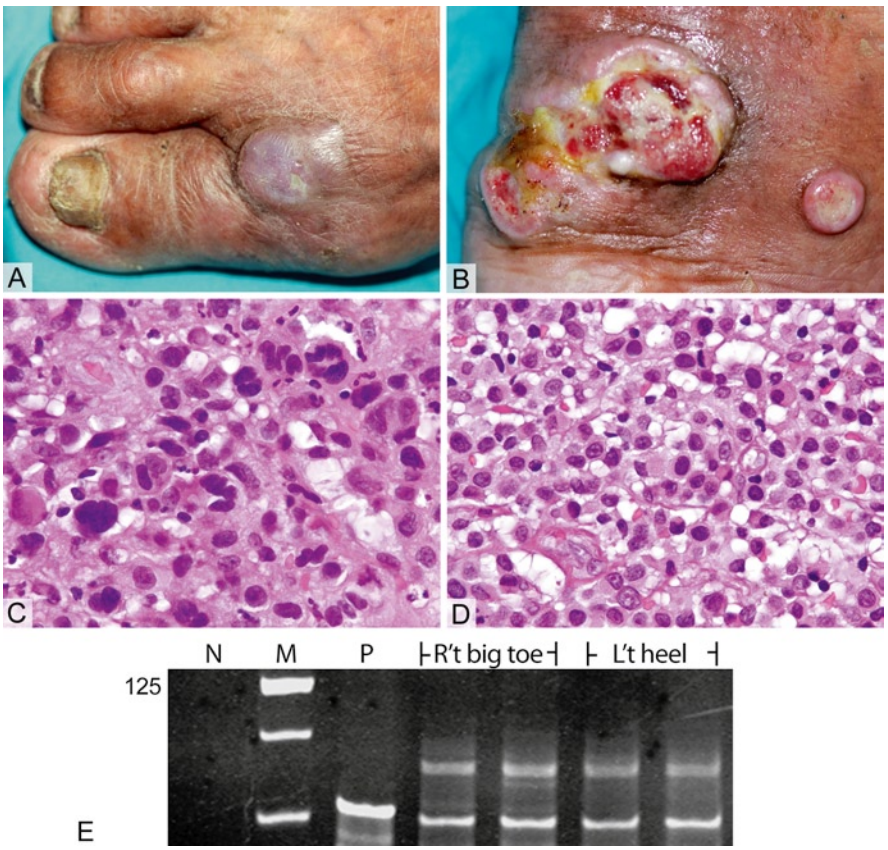

Fig. 11.1 Primary cutaneous anaplastic large cell lymphoma (*PC-ALCL*) in an 84-year-old male. The patient presented with four concurrent skin lesions on both lower legs without evidence of lymphadenopathy. (**a, c**) Indurated, nonulcerated tumor on the right big toe, showing marked nuclear pleomorphism with formation of multinucleated giant cells. (**b, d**) Two ulcerated nodules on the left heel, with relatively monomorphous tumor cells. Tumor cells in both specimens showed identical phenotypes by immunohistochemistry (coexpression of CD4 and CD30, with loss of CD7 [data not shown]). (**e**) Gel electrophoresis of PCR amplicons using conventional TCR-γ gene primers demonstrated the same monoclonal T-cell pattern in both tumors. This supports the same clonal origin, despite the variable histopathological features. *N* negative control, *M* 25 bp size marker with the most intense band at 125 bp, *P* positive control. The patient underwent local radiotherapy and achieved complete resolution of all the skin lesions within 5 months

were highly pleomorphic, while those in the left heel lesion were relatively monomorphous in appearance. TCR-GR analysis confirmed that both tumors were of the same monoclonal T-cell origin, despite different histopathological features.

Systemic ALCL may occasionally involve the skin and should be differentiated from primary cutaneous CD30-positive LPD. The clonal origin of synchronous or metachronous nodal and cutaneous tumors may be resolved by TCR-GR-based studies, and if necessary, cloning and sequencing of PCR products [28–30]. If both tumors are of the same clonal origin, the resultant amplicons should be of the same size and sequence. TCR-GR analysis may be a challenge if the systemic ALCL is an unusual microscopic variant, such as the lymphohistiocytic subtype, in which the tumor cells are scant and inadequate for accurate T-cell clonality analysis [31]. Figure 11.2 illustrates such a case in which a young woman developed concurrent lymph node and cutaneous lesions. The skin findings included multiple, nontender, nonitching, erythematous papules on the neck, chest, and abdomen. Using a dual-color, break-apart probe directed at the *ALK* gene for locus-specific FISH, we demonstrated *ALK* gene rearrangements in both the nodal and cutaneous lesions. Our results confirmed that the skin findings represented secondary cutaneous involvement by systemic ALCL rather than a primary CTCL. Unlike their systemic counterparts, PC-ALCL does not carry translocations involving the *ALK* gene on chromosome 2.

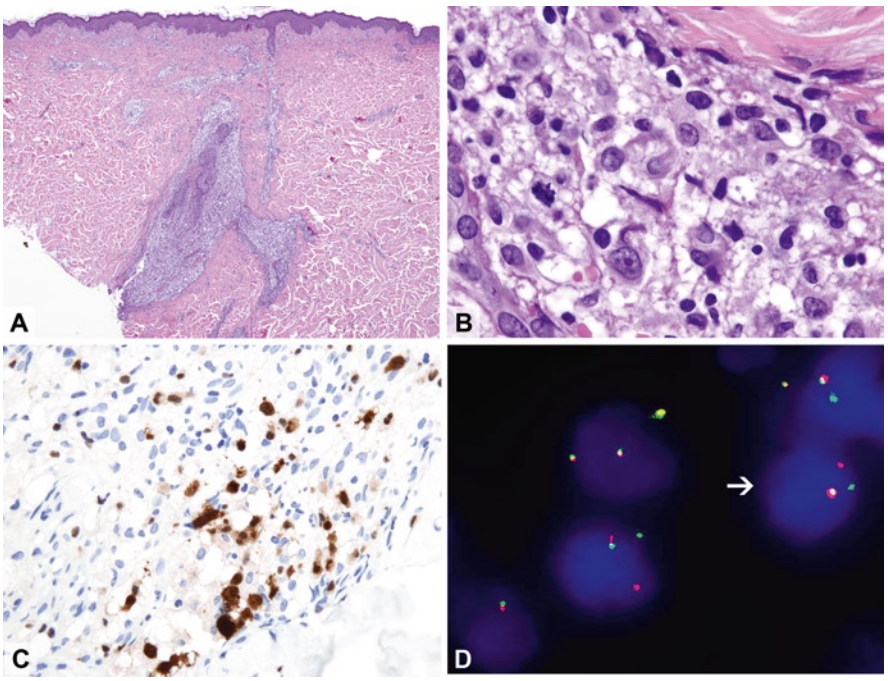

Fig. 11.2 Secondary cutaneous involvement by nodal lymphohistiocytic variant of systemic anaplastic large cell lymphoma (*ALCL*) in a 30-year-old woman. The lymph node was diffusely infiltrated by numerous histiocytes and a lesser population of medium-sized atypical lymphocytes expressing CD30 and ALK protein (not shown). (**a, b**) The skin biopsy showed a perivascular and perifollicular infiltrate of numerous histiocytes and scant tumor cells. (**c**) Immunohistochemistry demonstrated scattered ALK-positive tumor cells. (**d**) FISH using dual-color, break-apart rearrangement probes for the *ALK* gene identified one fused (normal) signal and two split (one *green* and one *orange*) signals, indicating translocation involving one *ALK* gene (*arrow*) (From Chuang et al. [31]. Reprinted with permission from Elsevier, Copyright © 2009)

Primary Cutaneous Extranodal NK/T-Cell Lymphoma, Nasal Type

Primary cutaneous extranodal NK/T-cell lymphoma, nasal type (PC-NKTCL) is a very aggressive hematopoietic neoplasm. While most cases are of natural killer (NK)-cell origin, rare examples show T-cell lineage. The tumor cells usually express cytoplasmic CD3, cytotoxic markers [T-cell intracellular antigen-1 (TIA-1), granzyme B, and perforin], and the NK-cell marker CD56. Most importantly, the tumor cells are positive for Epstein-Barr virus-encoded mRNA (EBER). The majority of cases show germline TCR genes indicating a true NK-cell origin. Rarely, monoclonal TCR-GRs are found as a function of T-cell lineage. Hemophagocytosis and/or hemophagocytic syndrome/hemophagocytic lymphohistiocytosis (HLH) may occur at presentation or during disease course, as shown in Fig. 11.3. In a recent study, we found that PC-NKTCL is one of the most common forms of primary CTCL in Taiwan, and is associated with a poorer prognosis compared to other subtypes [13].

Subcutaneous Panniculitis-like α/β (Alpha/Beta) T-Cell Lymphoma

Subcutaneous panniculitis-like α/β T-cell lymphoma (SPTCL) was first reported in 1991 by Gonzalez et al. [32]. In their initial description, tumors were composed of small and large atypical lymphocytes that rimmed subcutaneous adipocytes, simulating a panniculitis. An association with HLH and an aggressive clinical course were seen [32]. Monoclonal T-cells were detected in one of three cases studied for TCR-β GRs [32]. More recent studies have shown that SPTCL is comprised of two distinct entities: (1) cases with a TCR-α/β phenotype, a lower rate of HLH, and a much better prognosis; and (2) cases with a TCR-γ/δ phenotype, a higher frequency of HLH, and a poorer

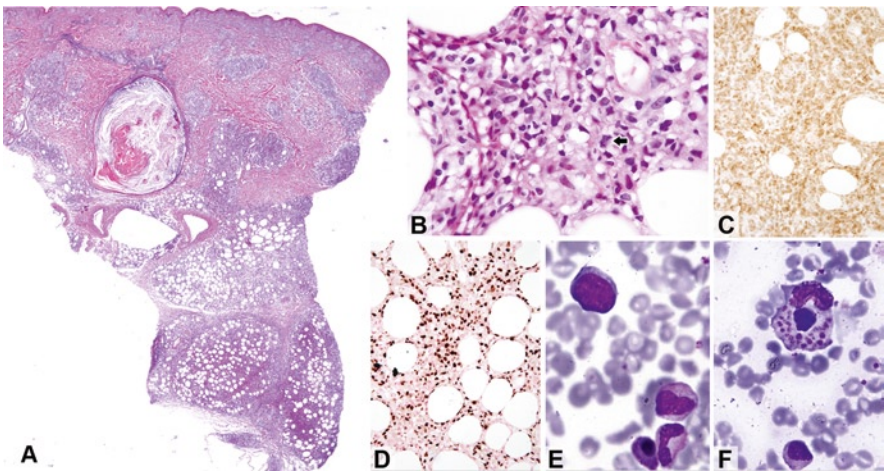

Fig. 11.3 Primary cutaneous extranodal NK/T-cell lymphoma, nasal type, on the left lower leg of a 26-year-old woman. (**a**) Skin biopsy showed a diffuse lymphocytic infiltration involving dermis and subcutaneous fat with sparing of the overlying epidermis, and an incidental epidermal inclusion cyst. No angioinvasion or tumor necrosis was identified. (**b**) Subcutaneous infiltration by atypical small- to medium-sized lymphocytes with irregular nuclear contours and a mitotic figure was noted (*arrow*). (**c**) Tumor cells universally expressed CD56. (**d**) In situ hybridization for Epstein-Barr virus-encoded mRNA (EBER-ISH) was positive in the majority of tumor cells. Polyclonal TCR-γ GRs were detected using conventional primers (data not shown). The patient's skin disease was refractory to chemotherapy, and she developed a relapse on her lip and bone marrow involvement with hemophagocytic lymphohistiocytosis. (**e**) A neoplastic lymphocyte and (**f**) a benign histiocyte showing hemophagocytosis in the bone marrow aspirate

prognosis [33–35]. In the 2005 WHO-EORTC classification, SPTCL is reserved for those examples with the α/β phenotype, while tumors with the γ/δ phenotype are reclassified as primary cutaneous γ/δ T-cell lymphoma (PC-GDTCL) [8].

Rimming of adipocytes by lymphocytes is not specific for SPTCL or indeed malignancy. This histopathological feature may be seen in other CTCL subtypes, in addition to lesions of lupus erythematosus panniculitis (lupus profundus) and cytophagic histiocytic panniculitis. Therefore, the latter reactive entities can be microscopic mimics of SPTCL and a distinction requires the integration of clinical features, serological data, and TCR-GR clonality studies [36–38]. Monoclonal TCR-GRs are detected in the majority of SPTCL cases [34, 35, 39], in contrast to lupus erythematosus panniculitis and cytophagic histiocytic panniculitis which show polyclonal results [36–38].

Primary Cutaneous Peripheral T-Cell Lymphomas, Rare Subtypes

Peripheral T-cell lymphomas (PTCL) involve the skin either as primary lesions or secondary manifestations of systemic disease. Three provisional entities of primary cutaneous non-MF T-cell lymphomas are defined in the 2005 WHO-EORTC classification: (1) primary cutaneous γδ T-cell lymphoma (PC-GDTCL); (2) primary cutaneous CD8-positive aggressive epidermotropic cytotoxic T-cell lymphoma (PC-CD8TCL); and (3) primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma (PC-CD4TCL) [8]. The remaining rare cases of non-MF CTCL that do not fit neatly into the above categories are classified as peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS).

There are only a few clonality-based studies on these uncommon entities and T-cell monoclonality detection rates seem to differ among subtypes. Due to the rarity of these tumors, authors of early studies usually combined cases of each entity together for analysis, with only very recent publications dealing with each specific subtype.

Primary Cutaneous γ/δ T-Cell Lymphoma

PC-GDTCL is a group of primary CTCL of γ/δ T-cells with a cytotoxic phenotype, and now includes the γ/δ variant of SPTCL [40]. Three histopathological patterns (epidermotropic, dermal, and subcutaneous) may be present within a single biopsy specimen or different specimens from the same patient. The vast majority of cases analyzed show monoclonal TCR-GRs, except in one series where monoclonal T-cell expansion was detected in only 50% (4/8) of cases studied [34, 41, 42].

Primary Cutaneous CD8-Positive Aggressive Epidermotropic Cytotoxic T-Cell Lymphoma

PC-CD8TCL is a very rare subtype of CTCL, usually characterized by generalized skin lesions composed of a proliferation of small/medium or large pleomorphic cytotoxic T-cells showing marked epidermotropism. An aggressive clinical course is typical (median survival of 32 months). To date, there are only approximately twenty cases reported in the scientific literature. In most studies, almost all cases tested for TCR-GRs were found to be monoclonal and had poor outcomes [43–47]. However, in one study, only one of five cases showed T-cell monoclonality [34]. The reason for the discrepancy in monoclonal TCR-GR detection rates between studies is unknown.

One possible cause may be a lower sensitivity of the conventional TCR-γ gene primers used in the latter study, rather than the more sensitive and extensive BIOMED-2 primer sets. In another report, all five epidermotropic CD8-positive primary CTCL analyzed were polyclonal for TCR-GRs [48]. Four of these patients showed spontaneous regression of their lesions, while the remaining patient, who initially also had lymph node involvement, died of disease dissemination within 20 months of diagnosis [48]. The high rate of spontaneous regression and good prognosis in this Japanese study by Hagiwara et al. [48] are at variance with the poor prognosis of previously reported PC-CD8TCL cases [43–47]. This suggests that either the former represent a subtype of CTCL distinct from PC-CD8TCL or that a wider spectrum of clinical behavior exists for PC-CD8TCL.

Primary Cutaneous CD4-Positive Small/Medium-Sized Pleomorphic T-Cell Lymphoma

PC-CD4TCL are comprised of predominantly small- to medium-sized neoplastic T-cells with a minor population of large lymphocytes (<30%), usually admixed with numerous reactive cells, including B-cells, histiocytes, plasma cells, and sometimes eosinophils [49, 50]. Figure 11.4 illustrates a typical case with a demonstrable monoclonal TCR-γ GR. Detection rates of T-cell monoclonality in PC-CD4TCL are high. In 1995, Friedmann et al. [51] reported a series of 11 primary

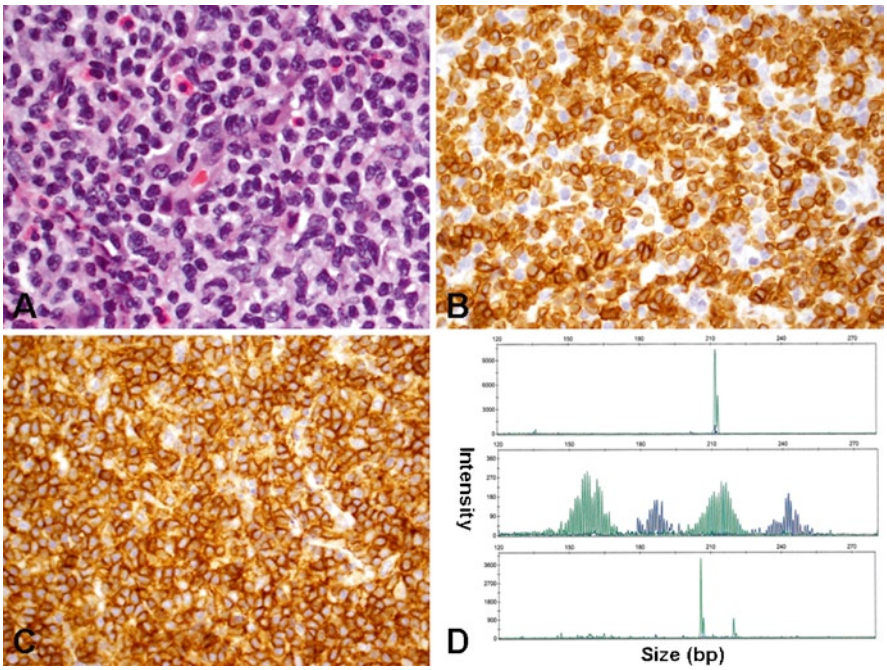

Fig. 11.4 Indolent but recurrent primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma in a 43-year-old man. (**a**) Biopsy from a recurrent preauricular skin lesion showed a diffuse proliferation of small lymphoid cells with slightly irregular nuclei. The lymphoid cells expressed CD3 (**b**) and CD4 (**c**), but were negative for CD8 (not shown). (**d**) Monoclonal result with polymerase chain reaction-based analysis of TCR-γ GRs using BIOMED-2 Vγfl and Vγ10 primers. *Top panel*, monoclonal control; *middle panel*, polyclonal control; and *lower panel*, patient sample (Courtesy of Dr. Siok-Bian Ng, Department of Pathology, National University Health System, Singapore)

cutaneous pleomorphic small T-cell lymphomas. Most of these cases were CD4-positive, and all nine cases tested for TCR-γ GRs were found to be monoclonal. In a separate Mayo Clinic study, all 15 cases of PC-CD4TCL showed T-cell monoclonality [52]. In two recent Spanish studies, a dominant T-cell clone was detected in 14 of 16 and 15 of 17 cases, using the BIOMED-2 protocols for TCR-β and TCR-γ GRs, respectively [50, 53]. Grogg et al. [52] noted that the tumor cells in these lesions are often small with only mild-to-moderate cytologic atypia. Furthermore, immunophenotypic aberrancy is rare [52], although a high frequency of CD7 loss was reported by Garcia-Herrera et al. [50]. Therefore, the differentiation of this entity from lesions with reactive T-cell infiltrates (i.e., T-cell pseudolymphomas) relies heavily on the demonstration of T-cell monoclonality. However, it is important to note that results of T-cell clonality studies should be interpreted in the context of clinical, histopathological, and immunophenotypic features, as reactive conditions may, on occasion, show monoclonal TCR-GR results [8, 14, 50, 52, 54, 55].

Primary Cutaneous Peripheral T-Cell Lymphoma, Not Otherwise Specified

Rare examples of primary CTCL that do not fit into the above subgroups are classified as PTCL, NOS. This is a poorly defined and rare category, and more cases are needed for a better understanding of these lesions. Figure 11.5 illustrates a case of primary cutaneous PTCL, NOS. The lesion was initially diagnosed as PC-NKTCL based on the expression of CD2, CD3, granzyme B, and diffuse EBER-positivity, although CD56 was negative. Additional immunohistochemical stains revealed that the tumor cells expressed other T-cell lineage markers. Furthermore, a monoclonal T-cell population was identified by TCR-γ GR analysis. According to the 2005 WHO-EORTC classification of cutaneous lymphomas [8], this case might be classified either as a PC-NKTCL (of T-cell lineage) or a primary cutaneous (Epstein-Barr virus-positive cytotoxic) PTCL, NOS, which was favored.

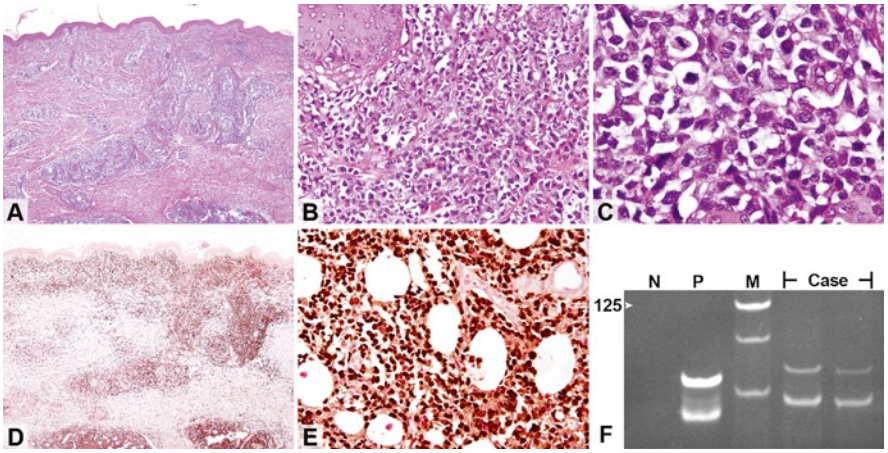

Fig. 11.5 Primary cutaneous peripheral T-cell lymphoma, not otherwise specified (PTCL, NOS) on the right thigh of a 78-year-old woman. (**a**) Lymphocytic infiltration of the dermis and superficial subcutaneous tissue with a predilection for skin adnexae. (**b**, **c**) The atypical lymphocytes were medium-sized with marked nuclear pleomorphism and frequent mitotic figures, but without epidermotropism. The tumor phenotype was $CD2^+$ $CD3^+$ $CD4^-$ $CD5^+$ $CD7^+$ $CD8^+$ $CD16^+$ $CD20^-$ $CD30^-$ $CD56^-$ $CD57^-$ TIA-1^+ granzyme B^+ and beta $F1^-$ (not shown). (**d**, **e**) Tumor cells were diffusely positive for Epstein-Barr virus-encoded mRNA by in situ hybridization (EBER-ISH). (**f**) T-cell lineage was indicated by monoclonal result on gel electrophoresis of PCR amplicons, generated using conventional TCR-γ gene primers. *N* negative control, *P* positive control, *M* 25 bp size marker with the most intense band at 125 bp. The patient showed progressive disease, despite combination chemotherapy, and died within 4 months

Adult T-Cell Leukemia/Lymphoma

Adult T-cell leukemia/lymphoma (ATLL) is a hematopoietic neoplasm caused by human T-cell lymphotropic virus type I (HTLV-I) infection, which is endemic in southwestern Japan, the Caribbean Basin, and parts of central Africa [56]. Most HTLV-I-infected individuals remain asymptomatic, but 1–5% of carriers have a lifelong risk of ATLL development. ATLL is characterized by a broad spectrum of cytologic features varying from small, medium, and large tumors cells, to most commonly, anaplastic cells with polylobated nuclei (so-called "floral cells" in the peripheral blood). Skin lesions are seen in more than 50% of patients with ATLL. Epidermal infiltration with Pautrier-like microabscesses is common. Dermal infiltration typically shows a perivascular distribution, with extension into the subcutaneous fat in some cases. Figure 11.6 illustrates a case of ATLL with cutaneous involvement. The Japanese Lymphoma Study Group has defined four clinical variants of this disease: (1) acute, (2) lymphomatous, (3) chronic, and (4) smoldering [57]. Patients in the smoldering phase usually present with skin lesions, but without lymphocytosis, hypercalcemia, lymphadenopathy, or hepatosplenomegaly. In contrast, patients with chronic phase disease show

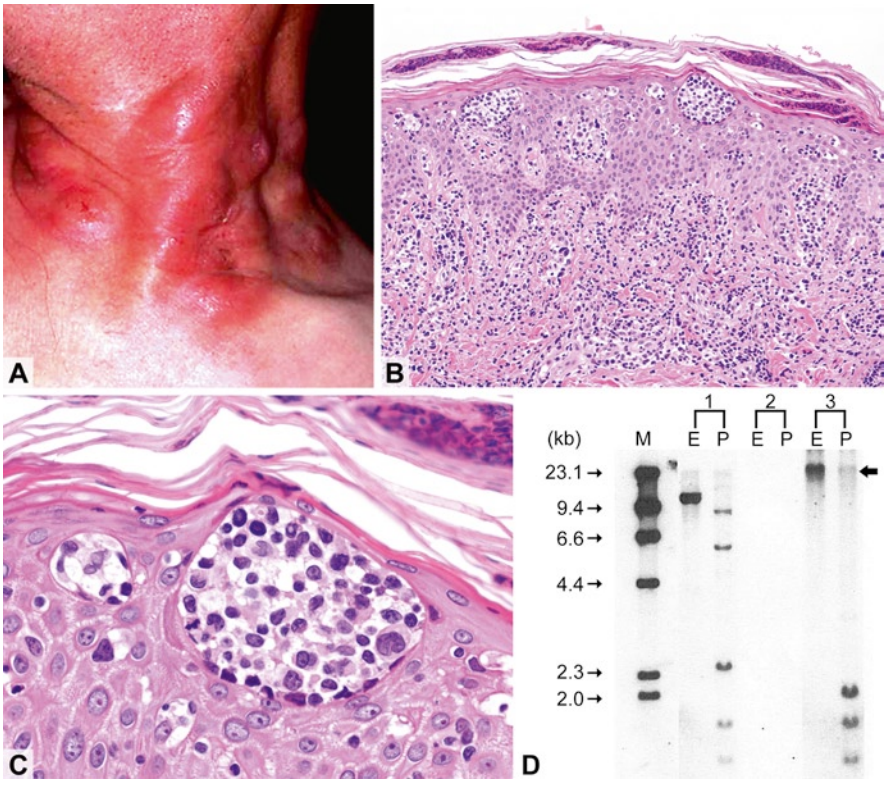

Fig. 11.6 Adult T-cell leukemia/lymphoma (ATLL) in a 68-year-old Japanese man. (**a**) Multiple cutaneous nodules, some with central ulceration, on the neck. (**b**) Extensive lymphomatous infiltration in the epidermis and dermis. (**c**) Pleomorphic tumor cells forming Pautrier-like microabscesses in the epidermis. (**d**) Southern blot analysis, using HTLV-I probe and digestion with *Eco*RI (E) and *Pst*I (P) restriction enzymes, demonstrates monoclonal integration pattern for HTLV-I provirus (one distinct band >9 kb) in the patient's tumor cells (*arrow*). *M* size marker, *Lane 1* positive control, *Lane 2* negative control, *Lane 3* patient sample (Courtesy of Prof. Koichi Ohshima, Department of Pathology, School of Medicine, Kurume University, Kurume, Japan)

lymphocytosis with mild lymphadenopathy and/or hepatosplenomegaly without hypercalcemia. In patients with ATLL and cutaneous lesions, a poorer prognosis is associated with clinical evidence of papulonodular disease than with generalized erythroderma. The prognosis is also worse for patients with histopathological evidence of nodular or diffuse infiltration of medium- to large-sized tumor cells in the skin, compared with those showing perivascular infiltration of small- to medium-sized cells [58]. ATLL may initially present as a skin-limited disorder. Cutaneous-type ATLL without the development of leukemic change or visceral organ involvement for many years has been described [58–61].

For the majority of individuals infected with HTLV-I, the clonality of T-cells with respect to proviral DNA integration varies from undetectable to polyclonal. In a small proportion of patients, monoclonal integration is associated with malignant transformation, namely, ATLL. The diagnosis of ATLL is based on seropositivity for HTLV-I infection, and histopathologically and/or cytologically proven PTCL of helper T-cell phenotype. A recent international consensus meeting on ATLL recommended molecular analysis for HTLV-I integration in all suspected ATLL cases [62]. In all types of ATLL, the tumor cells show monoclonal integration of HTLV-I proviral DNA. Using SBA for the detection of HTLV-I proviral integration, the cellular DNA is digested with both *Eco*RI and *Pst*I endonucleases (restriction enzymes). There is no cleavage site in the "normal" HTLV-I proviral genome for *Eco*RI. Therefore, when the DNA is digested by *Eco*RI, most cases (93%) of ATLL with monoclonal HTLV-I integration show one distinct band >9 kb (i.e., ordinary/complete pattern), as illustrated in Fig. 11.6. Digestion with *Pst*I shows one or more clear bands containing viral-cellular DNA, in addition to the three internal fragments of 2.5, 1.8, and 1.2 kb. Importantly, extraordinary integration patterns (i.e., (1) defective type: one or two bands <9 kb and (2) multiple type: two or more bands >9 kb), following DNA digestion with *Eco*RI, can be observed in a minority of cases [63–65]. The detection of bands that are <9 kb results from the presence of an *Eco*RI cleavage site within a "defective" HTLV-I proviral genome. Of note, the presence of extraordinary integration patterns in patients with ATLL is associated with distinct clinical–histopathological subtypes and prognosis [63–65].

For patients from endemic areas, it is important to note that HTLV-I carriers (those with seropositivity for anti-HTLV antibody) may also develop PTCL unrelated to this virus. SBA for tumoral HTLV-I proviral integration can help to differentiate these PTCL subtypes from true ATLL. This distinction is of important clinical significance. The outcome for patients with ATLL is poor, while individuals with HTLV-I-unrelated PTCL show intermediate prognosis [7]. In nonendemic areas (such as Taiwan), rare patients with PTCL can also have seropositivity for anti-HTLV antibody, again raising the differential of either HTLV-I-related ATLL or PTCL developing in incidental HTLV-I carriers. SBA for integrated provirus is mandatory for such patients. For example, the author has experience with a case of angioimmunoblastic T-cell lymphoma (AITL) in a Taiwanese lady who was seropositive for HTLV-I. Interestingly, there is a rare morphologic variant of ATLL with features of AITL, which includes proliferation of high-endothelial venules, a polymorphous lymphoid infiltrate, and medium-sized neoplastic cells with clear cytoplasm [65]. SBA showed that there was no proviral integration in the tumor cells, indicating this case was an example of AITL developing in an incidental HTLV-I carrier rather than an AITL-like ATLL [66]. As mentioned previously, the prototypic neoplastic cell in ATLL is a leukemic cell with a highly polylobated nucleus. The author has encountered a patient with a mature helper T-cell leukemia without lymphadenopathy. The leukemic cells were small but nonlobated. However, the patient's serum was positive for anti-HTLV. SBA confirmed integration of HTLV-I proviral DNA in the leukemic cells, indicating that in nonendemic areas, ATLL may present with atypical morphology (i.e., nonfloral leukemic cells). Interestingly, an extraordinary integration pattern of HTLV-I proviral DNA (i.e., single band of <9 kb) was found in this particular case [67].

References

1. McCarthy KP, Sloane JP, Kabarowski JH, et al. A simplified method of detection of clonal rearrangements of the T-cell receptor-gamma chain gene. Diagn Mol Pathol. 1992;1:173–9.
2. van Dongen JJ, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia. 2003;17:2257–317.
3. Langerak AW. Undersized, oversized? It is not one-size-fits-all in lymphoid clonality detection. Leuk Res. 2008;32:203–4.
4. Vargas RL, Felgar RE, Rothberg PG. Detection of clonality in lymphoproliferations using PCR of the antigen receptor genes: does size matter? Leuk Res. 2008;32:335–8.
5. Liao YL, Chang ST, Kuo SY, et al. Angioimmunoblastic T-cell lymphoma of cytotoxic T-cells phenotype containing a large B-cell proliferation with an undersized B-cell clonal product. Appl Immunohistochem Mol Morphol. 2010;18:185–89.
6. Chuang SS, Chang ST, Chuang WY, et al. NK-cell lineage predicts poor survival in primary intestinal NK-cell and T-cell lymphomas. Am J Surg Pathol. 2009;33:1230–40.
7. Ohshima K, Suzumiya J, Sato K, et al. Nodal T-cell lymphoma in an HTLV-I-endemic area: proviral HTLV-I DNA, histological classification and clinical evaluation. Br J Haematol. 1998;101:703–11.
8. Willemze R, Jaffe ES, Burg G, et al. WHO-EORTC classification for cutaneous lymphomas. Blood. 2005; 105:3768–85.
9. Su IJ, Wu YC, Chen YC, et al. Cutaneous manifestations of postthymic T cell malignancies: description of five clinicopathologic subtypes. J Am Acad Dermatol. 1990;23:653–62.
10. Nagasawa T, Miwa H, Nakatsuka S, et al. Characteristics of cutaneous lymphomas in Osaka, Japan (1988–1999) based on the European Organization for research and treatment of cancer classification. Am J Dermatopathol. 2000;22:510–4.
11. Lee MW. Characteristics of cutaneous lymphomas in Korea. Clin Exp Dermatol. 2003;28:639–46.
12. Yasukawa K, Kato N, Kodama K, et al. The spectrum of cutaneous lymphomas in Japan: a study of 62 cases based on the World Health Organization Classification. J Cutan Pathol. 2006;33:487–91.
13. Liao JB, Chuang SS, Chen HC, et al. Clinicopathologic analysis of cutaneous lymphoma in Taiwan: a high frequency of extranodal NK/T-cell lymphoma, nasal type, with an extremely poor prognosis. Arch Pathol Lab Med. 2010;134:996–1002.
14. Burg G, Kempf W, Cozzio A, et al. WHO/EORTC classification of cutaneous lymphomas 2005: histological and molecular aspects. J Cutan Pathol. 2005;32:647–74.
15. Burg G, Jaffe ES, Kempf W, et al. Haematolymphoid tumours. In: Leboit PE, Burg G, Weedon D, Sarasin A, editors. World Health Organization classification of tumours pathology and genetics skin tumours. Lyon: IARC; 2006. p. 165–228.
16. Bekkenk MW, Geelen FA, van Voorst Vader PC, et al. Primary and secondary cutaneous CD30(+) lymphoproliferative disorders: a report from the Dutch Cutaneous Lymphoma Group on the long-term follow-up data of 219 patients and guidelines for diagnosis and treatment. Blood. 2000;95:3653–61.
17. Ralfkiaer E, Willemze R, Pauli M, et al. Primary cutaneous CD30-positive lymphoproliferative disorders. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: IARC; 2008. p. 300–1.
18. Weiss LM, Wood GS, Trela M, et al. Clonal T-cell populations in lymphomatoid papulosis. Evidence of a lymphoproliferative origin for a clinically benign disease. N Engl J Med. 1986;315:475–9.
19. Chott A, Vonderheid EC, Olbricht S, et al. The dominant T cell clone is present in multiple regressing skin lesions and associated T cell lymphomas of patients with lymphomatoid papulosis. J Invest Dermatol. 1996;106:696–700.
20. Steinhoff M, Hummel M, Anagnostopoulos I, et al. Single-cell analysis of CD30+ cells in lymphomatoid papulosis demonstrates a common clonal T-cell origin. Blood. 2002;100:578–84.
21. Zackheim HS, Jones C, Leboit PE, et al. Lymphomatoid papulosis associated with mycosis fungoides: a study of 21 patients including analyses for clonality. J Am Acad Dermatol. 2003;49:620–3.
22. Gallardo F, Costa C, Bellosillo B, et al. Lymphomatoid papulosis associated with mycosis fungoides: clinicopathological and molecular studies of 12 cases. Acta Derm Venereol. 2004;84:463–8.
23. Liu HL, Hoppe RT, Kohler S, et al. CD30+ cutaneous lymphoproliferative disorders: the Stanford experience in lymphomatoid papulosis and primary cutaneous anaplastic large cell lymphoma. J Am Acad Dermatol. 2003;49:1049–58.
24. Willemze R, Beljaards RC. Spectrum of primary cutaneous CD30 (Ki-1)-positive lymphoproliferative disorders. A proposal for classification and guidelines for management and treatment. J Am Acad Dermatol. 1993;28:973–80.

25. Greisser J, Palmedo G, Sander C, et al. Detection of clonal rearrangement of T-cell receptor genes in the diagnosis of primary cutaneous CD30 lymphoproliferative disorders. J Cutan Pathol. 2006;33:711–5.
26. Kohler S, Jones CD, Warnke RA, et al. PCR-heteroduplex analysis of T-cell receptor gamma gene rearrangement in paraffin-embedded skin biopsies. Am J Dermatopathol. 2000;22:321–7.
27. Kong YY, Dai B, Kong JC, et al. Neutrophil/eosinophil-rich type of primary cutaneous anaplastic large cell lymphoma: a clinicopathological, immunophenotypic and molecular study of nine cases. Histopathology. 2009;55:189–96.
28. Chang ST, Chuang SS, Wang YH. Polyostotic primary bone lymphoma with progression limited in the skeleton: identical clonal origin of the initial and progressed tumors and a late relapse by VDJ rearrangement analysis. Leuk Lymphoma. 2006;47:1144–8.
29. Chuang SS, Liu H, Huang Y, et al. Primary cerebral diffuse large B-cell lymphoma relapsed solely in the skin with the same clonal origin. Appl Immunohistochem Mol Morphol. 2009;17:77–81.
30. Chen YF, Li YC, Chen LM, et al. Primary cutaneous diffuse large B-cell lymphoma relapsed solely as a huge lung tumor mimicking a primary pulmonary lymphoma. Int J Hematol. 2010;91:112–16.
31. Chuang SS, Hsieh YC, Ye H, et al. Lymphohistiocytic anaplastic large cell lymphoma involving skin: a diagnostic challenge. Pathol Res Pract. 2009;205:283–7.
32. Gonzalez CL, Medeiros LJ, Braziel RM, et al. T-cell lymphoma involving subcutaneous tissue. A clinicopathologic entity commonly associated with hemophagocytic syndrome. Am J Surg Pathol. 1991;15:17–27.
33. Toro JR, Liewehr DJ, Pabby N, et al. Gamma-delta T-cell phenotype is associated with significantly decreased survival in cutaneous T-cell lymphoma. Blood. 2003;101:3407–12.
34. Massone C, Chott A, Metze D, et al. Subcutaneous, blastic natural killer (NK), NK/T-cell, and other cytotoxic lymphomas of the skin: a morphologic, immunophenotypic, and molecular study of 50 patients. Am J Surg Pathol. 2004;28:719–35.
35. Willemze R, Jansen PM, Cerroni L, et al. Subcutaneous panniculitis-like T-cell lymphoma: definition, classification, and prognostic factors: an EORTC Cutaneous Lymphoma Group Study of 83 cases. Blood. 2008;111: 838–45.
36. Magro CM, Crowson AN, Kovatich AJ, et al. Lupus profundus, indeterminate lymphocytic lobular panniculitis and subcutaneous T-cell lymphoma: a spectrum of subcuticular T-cell lymphoid dyscrasia. J Cutan Pathol. 2001;28:235–47.
37. Massone C, Kodama K, Salmhofer W, et al. Lupus erythematosus panniculitis (lupus profundus): clinical, histopathological, and molecular analysis of nine cases. J Cutan Pathol. 2005;32:396–404.
38. Chen RL, Hsu YH, Ueda I, et al. Cytophagic histiocytic panniculitis with fatal haemophagocytic lymphohistiocytosis in a paediatric patient with perforin gene mutation. J Clin Pathol. 2007;60:1168–9.
39. Kong YY, Dai B, Kong JC, et al. Subcutaneous panniculitis-like T-cell lymphoma: a clinicopathologic, immunophenotypic, and molecular study of 22 Asian cases according to WHO-EORTC classification. Am J Surg Pathol. 2008;32:1495–502.
40. Gaulard P, Berti E, Willemze R, et al. Primary cutaneous peripheral T-cell lymphoma, rare subtypes. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: IARC; 2008. p. 302–5.
41. Toro JR, Beaty M, Sorbara L, et al. Gamma delta T-cell lymphoma of the skin: a clinical, microscopic, and molecular study. Arch Dermatol. 2000;136:1024–32.
42. Przybylski GK, Wu H, Macon WR, et al. Hepatosplenic and subcutaneous panniculitis-like gamma/delta T cell lymphomas are derived from different Vdelta subsets of gamma/delta T lymphocytes. J Mol Diagn. 2000;2:11–9.
43. Berti E, Tomasini D, Vermeer MH, et al. Primary cutaneous CD8-positive epidermotropic cytotoxic T cell lymphomas. A distinct clinicopathological entity with an aggressive clinical behavior. Am J Pathol. 1999;155:483–92.
44. Santucci M, Pimpinelli N, Massi D, et al. Cytotoxic/natural killer cell cutaneous lymphomas. Report of EORTC Cutaneous Lymphoma Task Force Workshop. Cancer. 2003;97:610–27.
45. Kim SK, Kim YC, Kang HY. Primary cutaneous aggressive epidermotropic CD8(+) cytotoxic T-cell lymphoma with atypical presentation. J Dermatol. 2006;33:632–4.
46. Fika Z, Karkos PD, Badran K, et al. Primary cutaneous aggressive epidermotropic CD8 positive cytotoxic T-cell lymphoma of the ear. J Laryngol Otol. 2007;121:503–5.
47. Introcaso CE, Kim EJ, Gardner J, et al. CD8+ epidermotropic cytotoxic T-cell lymphoma with peripheral blood and central nervous system involvement. Arch Dermatol. 2008;144:1027–9.
48. Hagiwara M, Takata K, Shimoyama Y, et al. Primary cutaneous T-cell lymphoma of unspecified type with cytotoxic phenotype: clinicopathological analysis of 27 patients. Cancer Sci. 2009;100:33–41.
49. Beljaards RC, Meijer CJ, Van der Putte SC, et al. Primary cutaneous T-cell lymphoma: clinicopathological features and prognostic parameters of 35 cases other than mycosis fungoides and CD30-positive large cell lymphoma. J Pathol. 1994;172:53–60.

50. Garcia-Herrera A, Colomo L, Camos M, et al. Primary cutaneous small/medium CD4+ T-cell lymphomas: a heterogeneous group of tumors with different clinicopathologic features and outcome. J Clin Oncol. 2008;26:3364–71.
51. Friedmann D, Wechsler J, Delfau MH, et al. Primary cutaneous pleomorphic small T-cell lymphoma. A review of 11 cases. The French Study Group on Cutaneous Lymphomas. Arch Dermatol. 1995;131:1009–15.
52. Grogg KL, Jung S, Erickson LA, et al. Primary cutaneous CD4-positive small/medium-sized pleomorphic T-cell lymphoma: a clonal T-cell lymphoproliferative disorder with indolent behavior. Mod Pathol. 2008;21:708–15.
53. Rodriguez Pinilla SM, Roncador G, Rodriguez-Peralto JL, et al. Primary cutaneous CD4+ small/medium-sized pleomorphic T-cell lymphoma expresses follicular T-cell markers. Am J Surg Pathol. 2009;33:81–90.
54. Cerroni L. Lymphoproliferative lesions of the skin. J Clin Pathol. 2006;59:813–26.
55. Lin MT, Chang HM, Huang CJ, et al. Massive expansion of EBV+ monoclonal T cells with CD5 down regulation in EBV-associated haemophagocytic lymphohistiocytosis. J Clin Pathol. 2007;60:101–3.
56. Ohshima K, Jaffe ES, Kikuchi M. Adult T-cell leukemia/lymphoma. In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Vardiman JW, editors. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: IARC; 2008. p. 281–4.
57. Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). Br J Haematol. 1991;79:428–37.
58. Yamaguchi T, Ohshima K, Karube K, et al. Clinicopathological features of cutaneous lesions of adult T-cell leukaemia/lymphoma. Br J Dermatol. 2005;152:76–81.
59. Takahashi K, Tanaka T, Fujita M, et al. Cutaneous-type adult T-cell leukemia/lymphoma. A unique clinical feature with monoclonal T-cell proliferation detected by Southern blot analysis. Arch Dermatol. 1988;124:399–404.
60. Yagi H, Takigawa M, Hashizume H. Cutaneous type of adult T cell leukemia/lymphoma: a new entity among cutaneous lymphomas. J Dermatol. 2003;30:641–3.
61. Amano M, Kurokawa M, Ogata K, et al. New entity, definition and diagnostic criteria of cutaneous adult T-cell leukemia/lymphoma: human T-lymphotropic virus type 1 proviral DNA load can distinguish between cutaneous and smoldering types. J Dermatol. 2008;35:270–5.
62. Tsukasaki K, Hermine O, Bazarbachi A, et al. Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. J Clin Oncol. 2009;27:453–9.
63. Shimamoto Y, Suga K, Shibata K, et al. Clinical importance of extraordinary integration patterns of human T-cell lymphotropic virus type I proviral DNA in adult T-cell leukemia/lymphoma. Blood. 1994;84:853–8.
64. Tsukasaki K, Tsushima H, Yamamura M, et al. Integration patterns of HTLV-I provirus in relation to the clinical course of ATL: frequent clonal change at crisis from indolent disease. Blood. 1997;89:948–56.
65. Karube K, Suzumiya J, Okamoto M, et al. Adult T-cell lymphoma/leukemia with angioimmunoblastic T-cell lymphoma-like features: report of 11 cases. Am J Surg Pathol. 2007;31:216–23.
66. Chuang SS, Ichinohasama R, Chu JS, Ohshima K. Differential diagnosis of angioimmunoblastic T-cell lymphoma with seropositivity for anti-HTLV antibody from adult T-cell leukemia/lymphoma. Int J Hematol 2010;91:687–91.
67. Hsieh YC, Chang ST, Huang WT, et al. Adult T-cell leukemia/lymphoma comprising non-floral leukemic cells in Taiwan, a country non-endemic for human T-cell leukemia virus type I. Leuk Lymphoma. 2009;50:1540–2.

Chapter 12
Cutaneous B-Cell Lymphomas

Antonio Subtil

Cutaneous lymphoproliferative disorders are a markedly heterogeneous group of diseases and represent one of the most challenging areas in dermatopathology. Careful correlation of clinical, histopathological, immunophenotypic, and molecular findings is essential for the accurate diagnosis and proper classification of these neoplasms [1]. There are several types of B-cell lymphoma which may show skin involvement, either primarily or as a secondary manifestation [2, 3]. While the vast majority of nodal/systemic lymphomas are of B-cell lineage [4, 5], primary cutaneous B-cell neoplasms represent only a minority of all primary cutaneous lymphomas [2, 3]. Primary cutaneous lymphomas often demonstrate different clinical behaviors, prognoses, and responses to therapy as compared with systemic/nodal lesions of similar lineage with secondary skin involvement [2, 3]. This is particularly true for cutaneous B-cell lymphomas [2, 3]. Appropriate staging at the time of diagnosis is necessary for accurate classification of a neoplastic lymphocytic infiltrate in the skin [2, 3].

Advances in immunology, immunohistochemistry, and molecular biology continually enhance our understanding of cutaneous lymphomas. As a result, classification schemes for these disorders have frequently changed over time [2–4]. Recent consensus efforts have resulted in the 2005 WHO-EORTC classification for cutaneous lymphomas (Table 10.1) [2], and the 2008 WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues [4]. Table 12.1 outlines the WHO classification of mature B-cell neoplasms.

Molecular Analysis of B-Cell Neoplasms

B-Cell Antigen Receptor Gene Rearrangements

The proper function of most lymphocytes in adaptive immunity is dependent upon the presence and interactions of surface antigen receptors, which are composed of multi-subunit glycoprotein molecules [6]. The antigen receptors in B-cells are immunoglobulins (Ig). The genes encoding Ig include a heavy chain (*IGH*) gene (14q32), kappa light chain gene (2p12), and lambda light chain gene (22q11). These genes normally rearrange in B-cells, with multiple possible combinations leading to both diversity and specificity in the recognition of a large number of different antigens [6].

A. Subtil (✉)
Departments of Dermatology and Pathology, Yale University School of Medicine,
333 Cedar Street, P.O. Box 208059, New Haven, CT 06520-8059, USA
e-mail: antonio.subtil@yale.edu

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_12,

Table 12.1 WHO 2008 classification of tumors of hematopoietic and lymphoid tissues – mature B-cell neoplasms [4]

Chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Splenic B-cell marginal zone lymphoma
Hairy cell leukemia
Splenic B-cell lymphoma/leukemia, unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukemia-variant
Lymphoplasmacytic lymphoma/Waldenström macroglobulinemia
Heavy chain diseases (Alpha heavy chain disease, Gamma heavy chain disease, Mu heavy chain disease)
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone lymphoma
Pediatric nodal marginal zone lymphoma
Follicular lymphoma
Pediatric follicular lymphoma
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma, NOS
T-cell/histiocyte-rich large B-cell lymphoma
Primary diffuse large B-cell lymphoma of the CNS
Primary cutaneous diffuse large B-cell lymphoma, leg type
EBV-positive diffuse large B-cell lymphoma of the elderly
Diffuse large B-cell lymphoma associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK-positive large B-cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Primary effusion lymphoma
Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma

During physiological B-cell maturation, the Ig genes undergo rearrangements and different segments of the genes are spliced out to produce a unique coding sequence composed of variable (V), joining (J), constant (C), and, in the case of *IGH*, diversity (D) regions (Fig. 12.1). The sequence of Ig gene rearrangement is as follows: (a) an *IGH* D segment is joined with an *IGH* J segment; (b) the DJ segment is then combined with a V segment to create a VDJ sequence (encoding the variable antigen recognition portion of the IGH molecule); (c) subsequent splicing of the C segment leads to the *IGH* gene rearrangement product VDJC; and (d) RNA transcription, mRNA processing, and translation lead to the formation of the heavy chain protein, which associates with the final product of VJC rearrangement of either kappa or lambda light chain genes to form the complete Ig. Additional Ig repertoire diversity is achieved by nucleotide losses and/or additions via terminal deoxynucleotidyl transferase (TdT) activity, somatic hypermutation, and antigen affinity maturation in follicular germinal center B-cells [7, 8].

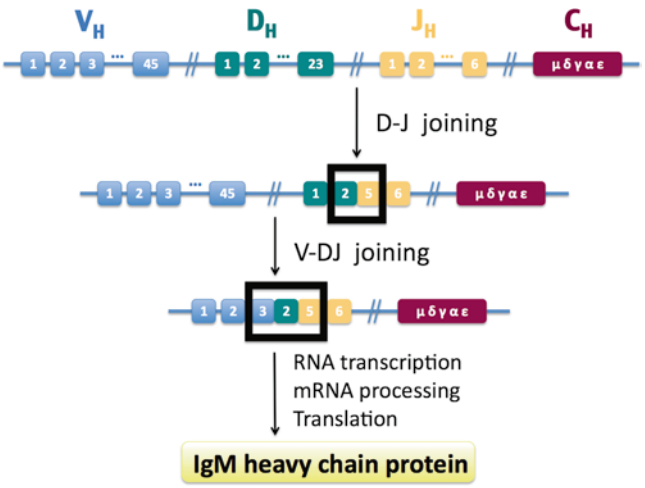

Fig. 12.1 Rearrangement of immunoglobulin heavy chain (*IGH*) gene. An *IGH* diversity (D) segment is initially combined with a joining (J) segment. The DJ segment is then joined with a variable (V) segment to create a VDJ sequence, which encodes the variable antigen recognition portion of the IGH molecule. Subsequent splicing of the C (constant) segment leads to the *IGH* gene rearrangement product VDJC. RNA transcription, mRNA processing and translation, results in the formation of the heavy chain protein, which associates with the final product of VJC rearrangement of either kappa or lambda light chain genes to form the complete Ig

The antigen receptor gene rearrangement process does not always produce a functional product. If the first *IGH* gene rearrangement is nonfunctional, a second attempt at rearrangement can occur with the other *IGH* allele. Therefore, a given B-cell may have two *IGH* gene rearrangements, only one of which is functional. After successful *IGH* rearrangement, a similar sequence occurs within the kappa light chain genes. Lambda light chain gene rearrangement takes place only if both kappa genes fail to produce a functional product. B-cells with unsuccessful rearrangement of Ig genes are usually eliminated via apoptosis [7].

Molecular Technologies

The molecular evaluation of B-cell neoplasms generally encompasses two strategies: (a) identification of homogeneity (monoclonality) versus heterogeneity (polyclonality) of Ig gene rearrangements (Fig. 12.2); and/or (b) detection of the presence or absence of certain pathological chromosomal abnormalities and translocations. These qualitative and quantitative techniques may be useful in the differential diagnosis of neoplastic versus reactive lymphocytic infiltrates, as well as in their classification through the identification of specific genetic aberrations [2, 4].

There are a number of different molecular technologies that can be utilized for the diagnosis of cutaneous B-cell infiltrates, including Southern blot analysis (SBA), polymerase chain reaction (PCR), and in situ hybridization (ISH). Each methodology is associated with distinct advantages and disadvantages, and variable degrees of applicability depending on the molecular target of interest. These technologies are discussed in more detail in Chap. 3. SBA was the first widely used method to evaluate Ig gene rearrangements [7]. Despite being considered the gold standard for the molecular detection of lymphocyte clonality, most laboratories have now discontinued SBA because it is very labor intensive, expensive, and takes several days to complete [8]. PCR-based testing is a faster, more automated and cheaper alternative, and has now become the main methodology used in

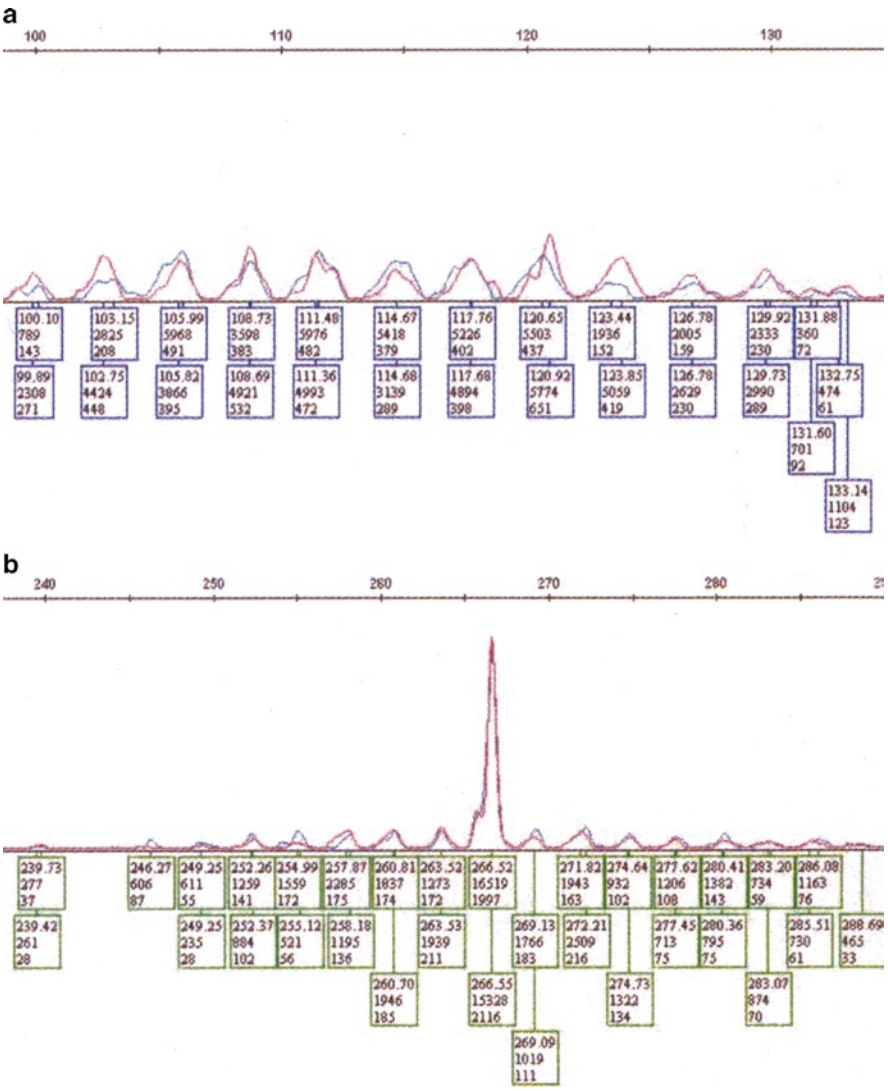

Fig. 12.2 Capillary electrophoresis of PCR amplicons of rearranged immunoglobulin heavy chain (*IGH*) gene, demonstrating polyclonal (**a**) and monoclonal (**b**) patterns

molecular laboratories. There are four conserved framework regions (FR) that provide reliable targets for *IGH* consensus primers for PCR amplification: three within VH (FR 1, 2, and 3) and one in JH. These FR are interspersed with three rearrangement-prone regions, called complementarity determining regions (CDR) [8]. FR1-JH primers generate products as large as 400 base pairs (bp), while FR2-JH products range from 200 to 300 bp, and FR3-JH products from 70 to 170 bp [8–10]. ISH is another technique that can be used to determine kappa and lambda Ig light chain expression in the cytoplasm of plasma cells on formalin-fixed paraffin-embedded (FFPE) tissue sections (Fig. 12.3) [11]. In addition, fluorescence in situ hybridization (FISH) is a very useful method for detection of specific genomic aberrations, including numerical chromosomal abnormalities and structural changes, such as translocations [7].

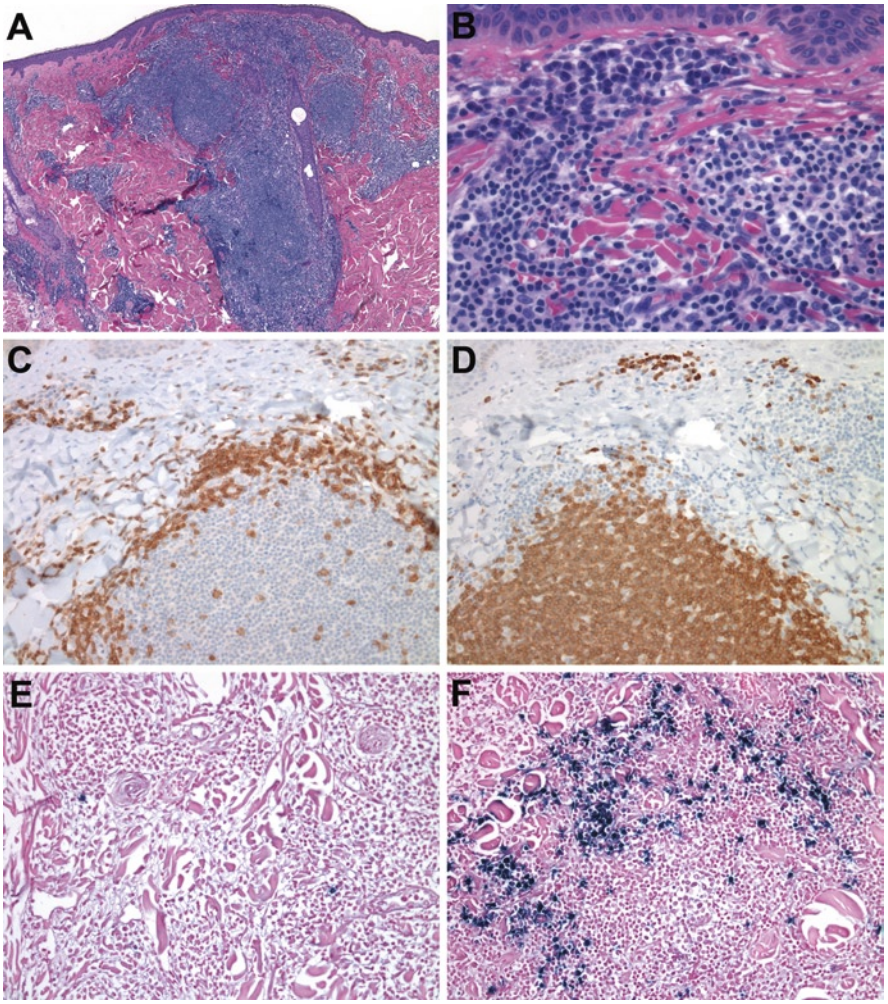

Fig. 12.3 Primary cutaneous marginal zone B-cell lymphoma. (**a**) Nodular, nonepidermotropic dermal infiltrate. (**b**) The infiltrate is composed of small lymphocytes, lymphoplasmacytoid cells, and plasma cells. (**c**) Expression of CD3 by a small subset of reactive T-cells. (**d**) Expression of CD79a by neoplastic B-cells and plasma cells. Detection of monotypic immunoglobulin (Ig) lambda light chain expression: kappa (**e**) and lambda (**f**)

Interpretation of Molecular Results

Clinical–histopathological correlation is necessary for accurate diagnosis and proper classification of cutaneous lymphoproliferative disorders [2, 3]. The results of molecular genetic studies should not be interpreted in isolation, and their significance must always be determined in relation to the other findings. Careful integration of all of the available data is critical to help prevent erroneous conclusions and adverse clinical consequences. Pitfalls do exist in the interpretation of molecular results in this setting, and include both false-positives and false-negatives [7].

The demonstration of a monoclonal B-cell population is very useful in the appropriate clinical–histopathological context. However, it is important to be aware that a monoclonal Ig gene rearrangement by itself does not establish a diagnosis of B-cell lymphoma, since it may also be

detected in benign cutaneous lymphoid hyperplasia [12]. Nihal et al. [12] identified monoclonal *IGH* gene rearrangements in 14 out of 44 cases (32%) of cutaneous reactive lymphoid hyperplasia. In addition, a monoclonal Ig gene rearrangement does not necessarily establish a B-cell lineage, since it may occasionally be identified in acute myeloid leukemia [4] and T-cell lymphoblastic lymphoma [13]. Lineage infidelity appears to be relatively uncommon, but must be considered in order to avoid an incorrect interpretation concerning the derivation of an atypical lymphocytic infiltrate [7]. Furthermore, other genetic abnormalities associated with B-cell lymphoma have been occasionally described in normal individuals without lymphoma, such as the t(14;18) *IGH*/*BCL2* translocation [14].

The sensitivity of molecular tests is not 100% [4]. The possibility of a false-negative result should always be considered, and correlated with the clinical and histopathological findings. Several factors can cause diminished sensitivity, including poor specimen quality and variable fixation protocols. Of note, the sensitivity of PCR for *IGH* gene rearrangements in FFPE tissue is reduced compared with fresh tissue, and may be as low as 40–60% [7]. Another potential cause of false-negative *IGH* PCR is failure of primer annealing. This may be due to alterations in the DNA sequence (that is amplified using the primer), as a result of somatic hypermutation in postgerminal center B-cells and/or the occurrence of unusual or complex gene rearrangements that may "escape" the most commonly used primers [7].

Cutaneous B-Cell Lymphomas

Primary cutaneous B-cell lymphomas are a heterogeneous group of disorders and are defined as neoplastic B-cell infiltrates that are present in the skin without evidence of extracutaneous disease at the time of diagnosis [2]. The 6-month time-frame previously required to confirm absence of systemic involvement for primary cutaneous classification is no longer used [2–4]. Primary cutaneous B-cell lymphomas may be subdivided, according to clinical behavior, into indolent (primary cutaneous marginal zone B-cell lymphoma and primary cutaneous follicle center lymphoma) and intermediate (primary cutaneous diffuse large B-cell lymphoma, leg type and other) groups (Table 12.2).

The main focus of this chapter is on primary cutaneous B-cell lymphomas. Of course, B-cell lymphomas of systemic origin may also involve the skin secondarily. Some of these latter entities are covered briefly at the end of the chapter.

Primary Cutaneous Marginal Zone B-Cell Lymphoma

Primary cutaneous marginal zone B-cell lymphoma (PCMZL) is a very indolent extranodal B-cell lymphoma composed of a morphologically heterogeneous mononuclear infiltrate of small lymphocytes with a variable plasma cell component [2, 15]. Most cases of apparent primary cutaneous plasmacytoma are actually examples of PCMZL with prominent plasma cell differentiation [2, 16]. In the WHO 2008 classification, PCMZL has been included in the category of extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) [4]. The prognosis of PCMZL is excellent, with a disease-specific 5-year survival of 99% (Table 12.2) [2].

The typical clinical presentation consists of red to violaceous papules, plaques and/or nodules located on the trunk or arms. In contrast with primary cutaneous follicle center lymphoma (PCFCL), the presence of multifocal skin lesions is common in PCMZL. Dissemination to extracutaneous sites is very rare, but recurrences in the skin may be observed [2]. An association with *Borrelia burgdorferi*

Table 12.2 Relative frequencies and disease-specific 5-year survivals of primary cutaneous B-cell lymphomas

Subtype	Number of cases (out of 1,905 patients)	Frequency (among all primary cutaneous lymphomas) (%)	Frequency (among primary cutaneous B-cell lymphomas) (%)	Disease-specific 5-year survival (%)
Marginal zone B-cell lymphoma	127	7	30	99
Follicle center lymphoma	207	11	48	95
Diffuse large B-cell lymphoma, leg type	85	4	20	55
Diffuse large B-cell lymphoma, other	4	<1	~1	50
Intravascular large B-cell lymphoma	6	<1	~1	65

Data source: 1,905 patients with a primary cutaneous lymphoma registered at the Dutch and Austrian Cutaneous Lymphoma Group between 1986 and 2002 [2]

infection has been reported in some European countries; however, this may be a regional phenomenon, since a similar association is not identified in other geographical locations [2, 17].

The histopathology of PCMZL consists of variably dense, nodular to diffuse, nonepidermotropic lymphocytic infiltrates centered in the dermis (Fig. 12.3). There is an admixture of small lymphocytes, marginal zone B-cells, lymphoplasmacytoid cells, plasma cells, and small reactive T-cells; the proportion of each of these components is variable. Plasma cells are often located at the periphery of the nodular aggregates and in the superficial dermis [2]. Reactive (non-neoplastic) lymphoid follicles with germinal centers are often present, and may lead to diagnostic confusion with PCFCL [18, 19]. In addition, the heterogeneity of the cellular infiltrates of PCMZL may cause diagnostic confusion with cutaneous reactive lymphoid hyperplasia [16].

The neoplastic B-cells of PCMZL demonstrate CD20, CD79a, and BCL2 protein immunoreactivity. Expression of CD5, CD10, or BCL6 by neoplastic cells is not observed, although associated reactive germinal centers are CD10/BCL6-positive and BCL2 protein-negative. Unlike other cutaneous B-cell lymphomas, monotypic Ig light chain expression is easily demonstrable on FFPE sections in the vast majority of cases (Fig. 12.3) [2]. The postulated cell of origin is a postgerminal center, marginal zone B-cell [4].

A monoclonal *IGH* gene rearrangement is detected in ~75–80% of PCMZL cases [2, 16, 20]. B-cell monoclonality can be demonstrated in FFPE specimens with significant sensitivity (85%) and high specificity (96%) using BIOMED-2 PCR protocols and primer sets [20]. The frequency at which chromosomal translocations and trisomies occur in extranodal marginal zone lymphomas of mucosa-associated lymphoid tissue (MALT lymphoma) varies significantly with the primary site (Table 12.3) [4]. Similar to tumors at other extranodal sites, PCMZL shows a generally low frequency of several abnormalities, including t(11;18)(q21;q21) *API2/MALT1*, t(14;18)(q32;q21) *IGH/MALT1*, t(3;14)(p14.1;q32) *FOXP1/IGH*, trisomy 3, and trisomy 18 [4]. The translocation t(1;14)(p22;q32) *BCL10/IGH* does not appear to be a significant abnormality in PCMZL [4]. The t(11;18) translocation involving *API2* and *MALT1* results in the production of a chimeric protein and has been associated with resistance to *Helicobacter pylori* antibiotic therapy in gastric MALT lymphomas; however, this chromosomal abnormality is present in <10% of cutaneous cases [4, 21].

Table 12.3 Anatomic site and chromosomal abnormalities (%) in extranodal marginal zone lymphomas of mucosa-associated lymphoid tissue (MALT lymphoma) [4]

	t(11;18)	t(14;18)	t(3;14)	t(1;14)		
Site	API2-MALT1	IGH-MALT1	FOXP1-IGH	BCL10-IGH	+3	+18
Skin	0–8	0–14	0–10	–	20	4
Gastric	6–26	1–5	–	–	11	6
Intestinal	12–56	–	–	0–13	75	25
Ocular	0–10	0–25	0–20	–	38	13
Salivary	0–5	0–16	–	0–2	55	19
Lung	31–53	6–10	–	2–7	20	7
Thyroid	0–17	–	0–50	–	17	–

FOXP1/IGH results in transcriptional deregulation [4]. *MALT1* and *BCL2* genes are both located within the chromosomal region 18q21 and may be translocated with *IGH* at 14q32. Despite sharing a similar karyotypic terminology of t(14;18)(q32;q21), the translocation involving *IGH* and *MALT1* is distinct from the translocation between *IGH* and *BCL2* [22]. The *IGH/MALT1* translocation has been identified in a subset of extranodal marginal zone lymphomas at various anatomic sites, including the skin [4]. However, there have also been reports of very rare cases of PCMZL with the *IGH/BCL2* translocation, which is usually seen in systemic follicular lymphoma, a subset of primary cutaneous follicle center lymphoma, and a subset of systemic diffuse large B-cell lymphoma [4, 20, 22]. Aberrant somatic hypermutation has been identified in PCMZL, involving *PIM1* and *c-MYC* genes [23]. Inactivation of the tumor suppressor genes *DAPK* (death-associated protein kinase) and *p16 (INK4a)* by promoter hypermethylation has also been shown to be a relatively frequent event in PCMZL at its initial presentation; however, any possible association with tumor progression remains to be defined [24, 25].

Primary Cutaneous Follicle Center Lymphoma

PCFCL is a cutaneous neoplasm of follicle center B-cells [2]. The clinical behavior and prognosis of PCFCL is significantly more favorable than systemic/nodal follicular lymphoma [3, 4]. PCFCL shows a disease-specific 5-year survival of 95% (Table 12.2) [2].

The clinical presentation of PCFCL is usually of solitary or localized, grouped plaques and tumors, most commonly on the scalp/forehead or trunk. Tumors may be associated with satellite erythematous papules and plaques. Unlike PCMZL, multifocal skin lesions are uncommon in PCFCL [2].

The histopathology of PCFCL consists of a moderate to dense, nonepidermotropic lymphocytic infiltrate with follicular, follicular and diffuse, or a diffuse growth pattern. There is an admixture of centrocytes (cleaved follicle center cells) and variable numbers of centroblasts (large noncleaved follicle center cells). In addition, follicles with abnormal morphologic features, such as reduced/absent mantle zone and lack of tingible-body macrophages, are commonly seen [2–4]. PCFCL should be differentiated from other forms of B-cell lymphoma, particularly primary cutaneous diffuse large B-cell lymphoma (PCLBCL), leg type, which demonstrates a diffuse growth pattern and a monotonous proliferation of centroblasts (i.e., without centrocytes) (Table 12.4) [2, 18, 19].

The neoplastic B-cells in PCFCL express CD20, CD79a, and BCL6. CD10 expression is variable and often absent in cases with a diffuse growth pattern. CD5 and CD43 are negative in the neoplastic cells. The proliferation rate, evaluated with Ki-67 (MIB-1) stain, is reduced and nonpolarized compared to the germinal centers of reactive lymphoid follicles. Unlike PCLBCL, leg type, most

Table 12.4 Characteristics of primary cutaneous follicle center lymphoma and primary cutaneous diffuse large B-cell lymphoma, leg type [2]

Characteristics	Primary cutaneous follicle center lymphoma	Primary cutaneous diffuse large B-cell lymphoma, leg type
Clinical findings	Middle-aged adults with lesions on the head or back	Elderly patients with lesions on the leg(s) (rare cases may occur elsewhere)
Histopathological features	Admixture of centrocytes and centroblasts, with variable growth pattern (follicular, follicular and diffuse, diffuse)	Confluent sheets of large lymphocytes resembling centroblasts or immunoblasts. Diffuse growth pattern
BCL2	Usually negative	Positive
IRF4/MUM1	Negative	Positive
FOX-P1	Negative	Positive

cases of PCFCL are negative for IRF4/MUM1 and FOX-P1. While the vast majority of systemic/nodal follicular lymphomas express BCL2, PCFCL are usually negative for this protein [2]. The postulated cell of origin is a mature germinal center B-cell [4].

Detection of B-cell monoclonality using the BIOMED-2 PCR method has been reported in up to 91% of cases of PCFCL [20]. Importantly, an inability to detect *IGH* gene rearrangements in some cases may result from somatic hypermutation [4]. There are significant differences in the various methodologies for BCL2 detection, and immunohistochemical and molecular methods are not interchangeable [4, 26, 27]. The immunohistochemical stain for BCL2 detects BCL2 *protein*, which has antiapoptotic properties, and is normally expressed by memory B- and T-cells, but is absent in reactive germinal center B-cells [22]. In contrast, molecular techniques for *BCL2*, such as PCR and FISH, detect the t(14;18)(q32;q21) *IGH/BCL2 translocation*, which is present in the vast majority of cases of systemic/nodal follicular lymphoma and a subset of systemic diffuse large B-cell lymphoma (Fig. 3.7) [4]. Several systemic B-cell lymphomas, including mantle cell lymphoma (MCL) and small lymphocytic lymphoma (SLL), express BCL2 protein, but lack the *IGH/BCL2* translocation [4, 28]. Most cases of PCFCL do not express BCL2 protein and do not show the t(14;18) translocation [29–31]. However, a number of studies have reported the presence of t(14;18) and/or BCL2 protein expression in a minority of PCFCL (10–40% of cases) (Fig. 12.4) [26, 27, 32, 33]. Interestingly, clinical presentation and behavior have been noted to be similar for both BCL2 and/or t(14;18)-positive and -negative cases of PCFCL with a follicular growth pattern [2]. Nonetheless, identification of BCL2 protein expression and/or t(14;18) translocation should raise suspicion of a systemic lymphoma involving the skin secondarily. In such instances, appropriate staging procedures would be necessary to determine the tumor site of origin at the time of initial presentation [2]. Inactivation of *p15 (INK4b)* and *p16 (INK4a)* tumor suppressor genes by promoter hypermethylation has been reported in ~10% and ~30% of cases of PCFCL, respectively [25]. A gene expression profile of germinal center B-cell-like lymphoma has been demonstrated in PCFCL [34].

Primary Cutaneous Diffuse Large B-Cell Lymphoma, Leg Type

PCLBCL, leg type is a rare cutaneous B-cell lymphoma composed exclusively of large neoplastic B-cells. Lesions predominantly affect elderly patients and occur characteristically on the lower legs [4, 35].

Patients usually present with rapidly growing, red or bluish-red, tumors on one or both legs. Most patients are women, with a male:female ratio of ~1:3–4 [4]. Unlike other primary cutaneous

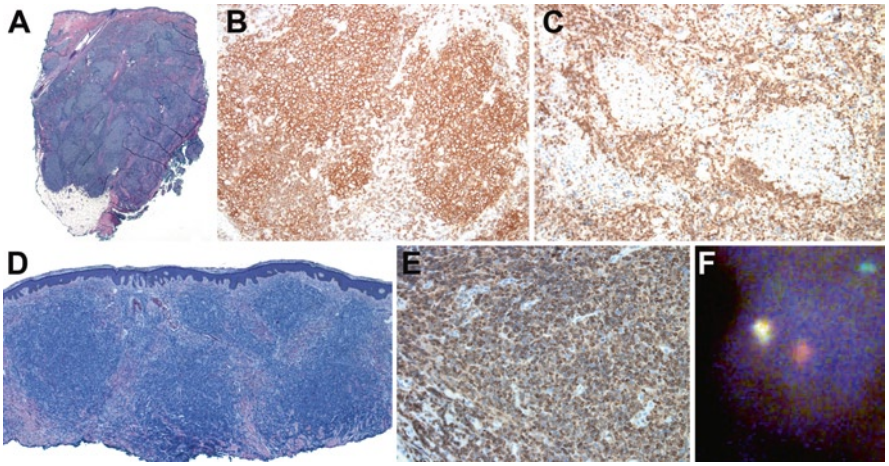

Fig. 12.4 (**a**–**c**) Primary cutaneous follicle center lymphoma. (**a**) Dense nodular, nonepidermotropic dermal infiltrate composed of crowded lymphoid follicles. (**b**) Expression of CD20 by neoplastic B-cells with a nodular/follicular arrangement. (**c**) Absence of BCL2 protein expression by immunohistochemistry within neoplastic lymphoid follicles. (**d**–**f**) Systemic follicular lymphoma with initial presentation in the skin. (**d**) Dense nodular, nonepidermotropic dermal lymphocytic infiltrate. (**e**) BCL2 protein expression by immunohistochemistry within neoplastic lymphoid follicles. (**f**) Positive detection of BCL2 translocation by FISH (dual-color, break-apart rearrangement probes showing one *green*, one *red*, and one *composite yellow* signals)

B-cell lymphomas, extracutaneous dissemination is common, and the prognosis is unfavorable with a disease-specific 5-year survival of ~55% (Table 12.2) [2, 36]. Other rare types of primary cutaneous large B-cell lymphoma, such as primary cutaneous intravascular large B-cell lymphoma and primary cutaneous plasmablastic lymphoma, show a prognosis similar to that of PCLBCL, leg type [2].

PCLBCL, leg type, typically shows a dense and diffuse infiltrate of monomorphous, confluent sheets of large atypical lymphocytes (centroblasts or immunoblasts) in the dermis, with variable extension into the panniculus. A significant component of centrocytes/cleaved cells is not present. Mitotic figures are frequently observed. The epidermis is usually not involved [2, 4].

The large neoplastic lymphocytes express CD20 and CD79a. BCL6 expression is frequent, but CD10 is usually negative. Unlike PCFCL, there is strong expression of BCL2 protein, IRF4/MUM1, and FOX-P1 in PCLBCL, leg type (Table 12.4) [2, 4]. A small subset of cases are BCL2-negative, but demonstrate comparable clinical outcomes [4]. The postulated cell of origin is a peripheral postgerminal center B-cell [4].

A monoclonal *IGH* gene rearrangement is detected in ~80% of cases and may be identified in FFPE tissue [37, 38]. Although strong BCL2 protein expression is evident in most cases, the t(14;18)(q32;q21) *IGH/BCL2* translocation is not present [31, 39]. It is postulated that BCL2 overexpression may result from amplification of the *BCL2* gene [40]. Inactivation of *p15 (INK4b)* and *p16 (INK4a)* tumor suppressor genes by promoter hypermethylation has been observed in 11% and 44% of PCLBCL, leg type, respectively [25]. Chromosomal imbalances have been detected in up to 85% of cases, with gains of 18q and 7p, and loss of 6q, as common findings [40, 41]. Translocations involving *MYC, BCL6*, and *IGH* genes have also been reported [42]. A gene expression profile of activated B-cell-like diffuse large B-cell lymphoma has been demonstrated [34]. Inactivation of *CDKN2A*, either by deletion or promoter hypermethylation, has been reported as an unfavorable prognostic sign [43].

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a systemic/nodal B-cell neoplasm that usually involves the peripheral blood, bone marrow, spleen, and lymph nodes. The infiltrate is typically composed of small monomorphous lymphocytes, admixed with a smaller subset of prolymphocytes and paraimmunoblasts within proliferation centers [4]. CLL is the most common leukemia in Western countries [44]. Nonspecific skin lesions may develop in CLL/SLL, including exaggerated cutaneous reactions to insect bites and drugs [45]. However, secondary cutaneous involvement by leukemic cells (i.e., leukemia cutis) may also occur, sometimes identified as an incidental finding in excision specimens for other cutaneous tumors [46, 47]. A CD20+, CD79a+, CD5+, CD10−, CD23+, CD43+, BCL2 protein+, and cyclin D1− immunophenotype is commonly found [4].

Ig genes are clonally rearranged in CLL/SLL, with 40–50% of cases unmutated and 50–60% of cases showing somatic hypermutation [48, 49]. Unmutated examples are associated with a more aggressive clinical course [49]. In addition, *IGH-V* gene usage is highly selective and commonly associated with autoantibody reactivity [50]. CD38 and ZAP-70 protein expression are indicators of an adverse prognosis [51]. Chromosome 11q22–q23, 17p, and 6q deletions are also associated with a poor outcome, although isolated 13q deletion indicates a more favorable prognosis [52, 53].

Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is a systemic/nodal lymphoid neoplasm composed of small- to medium-sized B-cells with a characteristic *CCND1* (cyclin D1) translocation. The prognosis of MCL is unfavorable, with a median survival of ~3 to 5 years [4]. Most cases show a monomorphous infiltrate of small- to medium-sized lymphocytes with irregular nuclear contours. However, blastoid and pleomorphic variants have also been described (Fig. 12.5) [4]. Nonspecific cutaneous lesions may occur in the setting of MCL, including exaggerated arthropod bite-like reactions [54]. Secondary cutaneous involvement by MCL may be seen, but is rare [55]. The immunophenotype is typically CD20+, CD5+, CD10−, CD23−, CD43+, BCL2 protein+, BCL6−, FMC7+, and cyclin D1 protein+ [28].

A monoclonal *IGH* gene rearrangement has been demonstrated in FFPE skin biopsies of MCL [56]. The primary genetic event in MCL is the t(11;14)(q13;q32) translocation between the *IGH* and *CCND1* genes; however, variant translocations have rarely been reported [57, 58]. In cases of suspected cutaneous involvement by MCL, detection of the t(11;14) fusion can be diagnostically useful and may be demonstrated by interphase FISH on FFPE tissue sections (Fig. 12.5) [56, 59]. Deregulated cyclin D1 protein expression appears to overcome the cell cycle suppressive effect of *RB1* and *p27 (kip1)*, resulting in uncontrolled lymphoid proliferation in MCL [60].

Burkitt Lymphoma

Burkitt lymphoma (BL) is a highly proliferative systemic B-cell neoplasm with frequent leukemic or extranodal presentation [4]. Secondary cutaneous involvement by BL is rarely described [61]. Clinical variants include endemic BL in equatorial Africa, sporadic BL in Western countries,

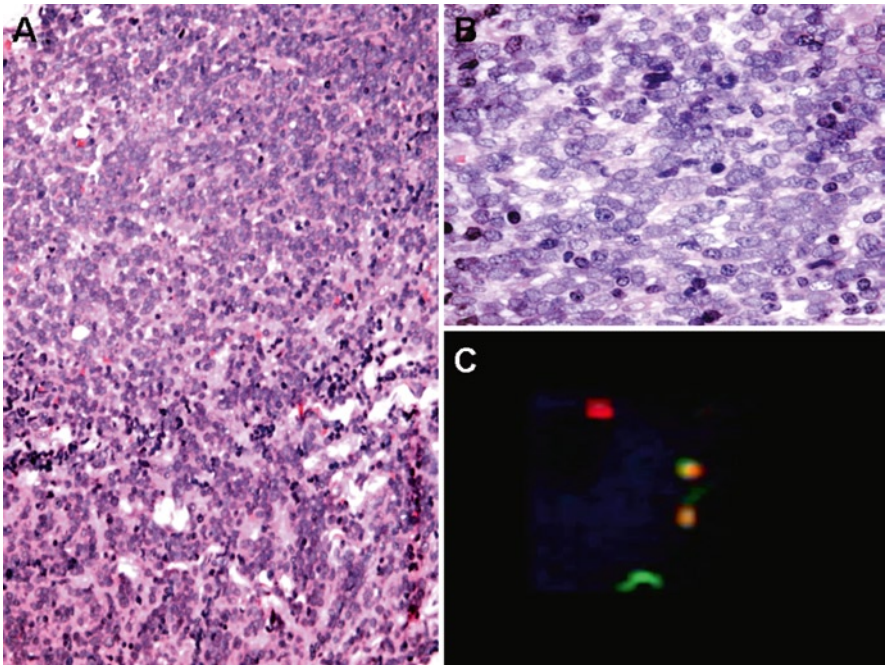

Fig. 12.5 (**a**, **b**) Blastoid mantle cell lymphoma (*MCL*) characterized by sheets of medium-sized monotonous cells, with irregular nuclei, dispersed chromatin, inconspicuous nucleoli, and scant cytoplasm. (**c**) FISH detection of t(11;14) translocation. Additional fusion and/or isolated *CCND1* (cyclin D1) signals, a complex genetic profile typically seen in the blastoid variant of MCL, were seen (Courtesy of Drs. Carlos Bacchi and Bruna Estrozi, Pathology Reference Laboratory, Botucatu, São Paulo, Brazil)

and immunodeficiency-associated BL, which may occur as the first manifestation of acquired immunodeficiency syndrome (AIDS) [4]. The infiltrate is usually composed of medium-sized lymphocytes with a diffuse growth pattern and an extremely high proliferation fraction. A "starry sky" appearance is common and results from scattered macrophages with ingested apoptotic tumor cells. The immunophenotype is usually CD20+, CD10+, BCL6+, TdT−, BCL2 protein–, and nearly 100% positivity with Ki-67 [28].

Ig genes are clonally rearranged with somatic hypermutation [4]. Translocations involving *MYC* are highly characteristic, but not specific, since they may also occur in a subset of systemic diffuse large B-cell lymphoma (Fig. 3.7) [62]. Most cases show a t(8;14) translocation between *MYC* (8q24) and *IGH* (14q32) genes; however, kappa (2p12) or lambda (22q11) Ig light chain genes may occasionally be translocated with *MYC* [4]. Other abnormalities involving *p16 (INK4a), TP53, p73, BAX, p130/RB2,* and *BCL6* have also been described, and may lead to promotion of cell growth and/or inhibition of apoptosis [4].

Lymphomatoid Granulomatosis

Lymphomatoid granulomatosis (LYG) is a rare angiocentric and angiodestructive Epstein-Barr virus (EBV)-associated extranodal B-cell lymphoproliferative disorder with a broad clinicopathologic spectrum, varying widely from a relatively indolent process to an aggressive lymphoma [4]. The skin is the extrapulmonary organ most commonly involved in LYG [63]. The infiltrate is

usually composed of EBV+ CD20+ B-cells admixed with reactive T-cells, the latter often predominating. A three-tier histopathological grading system has been developed and is based on the proportion of EBV+ B-cells relative to the reactive inflammatory background [4].

Monoclonal Ig gene rearrangements can be detected in most cases of grade 2 and grade 3 LYG, but are often not identified in grade 1 lesions, possibly secondary to the rarity of neoplastic cells in the latter [4]. SBA may also show clonal integration of EBV [64].

Hodgkin Lymphoma

Hodgkin lymphoma (HL) represents ~30% of all lymphomas [4]. Most cases arise in lymph nodes, particularly at cervical and mediastinal sites, and show a relatively small number of scattered large mononuclear and/or multinucleated tumor cells, in association with different reactive inflammatory background patterns. Cutaneous involvement by HL can rarely occur, and may be due to retrograde lymphatic spread, direct extension from underlying involved lymph nodes, or hematogenous dissemination [65, 66]. HL is subclassified into nodular lymphocyte predominant (NLPHL) and classical HL (CHL) types.

NLPHL is a B-cell neoplasm characterized by a nodular, or nodular and diffuse, proliferation of scattered large CD20+, CD79a+, PAX5+, OCT-2+, BOB.1+, BCL6+, CD45+, CD30−, and CD15− neoplastic cells (known as LP, "popcorn", or L&H cells), within large meshworks of CD21+ follicular dendritic cell processes, admixed with reactive lymphocytes and histiocytes [4]. LP cells have monoclonally rearranged Ig genes; however, the rearrangements are usually not detected in DNA extracted from whole tissue sections, but only in that isolated from individual tumor cells [67–69]. Aberrant somatic hypermutations have been identified in up to 80% of cases of NLPHL, involving *PAX5, PIM1, MYC*, and *RhoH/TTF* [70].

CHL is a monoclonal lymphoid neoplasm composed of CD30+, CD15+/−, CD45−, CD20−/+, CD79a−/+, and PAX5+ mononuclear Hodgkin cells and multinucleated Reed-Sternberg (HRS) cells, in association with a variable admixture of reactive small lymphocytes, eosinophils, histiocytes, plasma cells, neutrophils, fibroblasts, and collagen fibers [4]. There are four histologic subtypes of CHL: nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte-depleted [4]. In ~98% of cases, CHL is derived from mature B-cells at the germinal center stage of differentiation, showing functional Ig gene rearrangements, but defective Ig transcription and acquisition of B-cell inappropriate gene products [71, 72]. In very rare cases of CHL, the neoplastic cells are derived from peripheral/post-thymic T-cells and show monoclonal T-cell receptor gene rearrangements [73–75]. Similar to NLPHL, monoclonal antigen receptor gene rearrangements are usually detectable only in the DNA of isolated tumor cells, and not from whole tissue sections [67]. Cytogenetic studies have demonstrated aneuploidy and hypertetraploidy [76]. Comparative genomic hybridization has revealed recurrent gains on chromosomal arms 2p, 9p, and 12q, and distinct high-level amplifications on chromosomal bands 4p16, 4q23-q24, and 9p23–p24 [77].

Acknowledgments Our sincere gratitude to Jeffrey Sklar, M.D., for his invaluable assistance with the images of molecular tests.

References

1. Pimpinelli N, Olsen EA, Santucci M, et al. Defining early mycosis fungoides. J Am Acad Dermatol. 2005;53:1053–63.
2. Willemze R, Jaffe ES, Burg G, et al. WHO-EORTC classification for cutaneous lymphomas. Blood. 2005;105:3768–85.

3. Willemze R, Kerl H, Sterry W, et al. EORTC classification for primary cutaneous lymphomas: a proposal from the Cutaneous Lymphoma Study Group of the European Organization for Research and Treatment of Cancer (EORTC). Blood. 1997;90:354–71.
4. Swerdlow SH, Campo E, Harris NL, Jaffee ES, Pileri SA, Stein H, et al., editors. WHO Classification of Tumors of Hematopoietic and Lymphoid Tissues. Lyon: IARC; 2008.
5. Armitage JO, Weisenburger DD. New approach to classifying non-Hodgkin's lymphomas: clinical features of the major histologic subtypes. Non-Hodgkin's lymphoma classification project. J Clin Oncol. 1998;16:2780–95.
6. Pan L, Cesarman E, Knowles DM. Antigen receptor genes: structure, function, and genetic analysis of their rearrangements. In: Knowles DM, editor. Neoplastic Hematopathology. 2nd ed. Philadelphia, PA: Lippincott, Williams & Wilkins; 2001. p. 307–28.
7. Braziel RM, Fan G. B-cell lymphomas. In: Leonard DGB, editor. Molecular Pathology in Clinical Practice: Oncology. New York: Springer; 2009. p. 179–94.
8. Gulley ML. Leukemias and lymphomas. In: Leonard DGB, editor. Diagnostic Molecular Pathology. Philadelphia, PA: Elsevier Science; 2003. p. 113–27.
9. Ramasamy I, Brisco M, Morley A. Improved PCR method for detecting monoclonal immunoglobulin heavy chain rearrangement in B cell neoplasms. J Clin Pathol. 1992;45:770–5.
10. Slack DN, McCarthy KP, Wiedemann LM, et al. Evaluation of sensitivity, specificity, and reproducibility of an optimized method for detecting clonal rearrangements of immunoglobulin and T-cell receptor genes in formalin-fixed, paraffin-embedded sections. Diagn Mol Pathol. 1993;2:223–32.
11. Walker E, Robertson AG, Boorman JG, et al. Primary cutaneous plasmacytoma: the use of in situ hybridization to detect monoclonal immunoglobulin light chain mRNA. Histopathology. 1992;20:135–8.
12. Nihal M, Mikkola D, Horvath N, et al. Cutaneous Lymphoid Hyperplasia: A Lymphoproliferative Continuum With Lymphomatous Potential. Hum Pathol. 2003;34:617–22.
13. Pilozzi E, Muller-Hermelink HK, Falini B, et al. Gene rearrangements in T-cell lymphoblastic lymphoma. J Pathol. 1999;188:267–70.
14. Limpens J, Stad R, Vos C, et al. Lymphoma-associated translocation t(14;18) in blood B cells in normal individuals. Blood. 1995;85:2528–36.
15. Hoefnagel JJ, Vermeer MH, Jansen PM, et al. Primary cutaneous marginal zone B-cell lymphoma: clinical and therapeutic features in 50 cases. Arch Dermatol. 2005;141:1139–45.
16. Geyer JT, Ferry JA, Longtine JA, et al. Characteristics of cutaneous marginal zone lymphomas with marked plasmacytic differentiation and a T cell-rich background. Am J Clin Pathol. 2010;133:59–69.
17. Wood GS, Kamath NV, Guitart J, et al. Absence of Borrelia burgdorferi DNA in cutaneous B-cell lymphomas from the United States. J Cutan Pathol. 2001;28:502–7.
18. Leinweber B, Colli C, Chott A, et al. Differential diagnosis of cutaneous infiltrates of B lymphocytes with follicular growth pattern. Am J Dermatopathol. 2004;26:4–13.
19. de Leval L, Harris NL, Longtine J, et al. Cutaneous B-cell lymphomas of follicular and marginal zone types: use of Bcl-6, CD10, Bcl-2, and CD21 in differential diagnosis and classification. Am J Surg Pathol. 2001;25:732–41.
20. Morales AV, Arber DA, Seo K, et al. Evaluation of B-cell clonality using the BIOMED-2 PCR method effectively distinguishes cutaneous B-cell lymphoma from benign lymphoid infiltrates. Am J Dermatopathol. 2008;30:425–30.
21. Liu H, Ruskon-Fourmestraux A, Lavergne-Slove A, et al. Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma to Helicobacter pylori eradication therapy. Lancet. 2001;357:39–40.
22. Palmedo G, Hantschke M, Ruetten A, et al. Primary cutaneous marginal zone B-cell lymphoma may exhibit both the t(14;18)(q32;q21) IGH/BCL2 and the t(14;18)(q32;q21) IGH/MALT1 translocation: an indicator for clonal transformation towards higher-grade B-cell lymphoma? Am J Dermatopathol. 2007;29:231–6.
23. Deutsch AJ, Fruewirth M, Aigelsreiter A, et al. Primary cutaneous marginal zone B-cell lymphomas are targeted by aberrant somatic hypermutation. J Invest Dermatol. 2009;129:476–9.
24. Takino H, Li C, Hu S, et al. Primary cutaneous marginal zone B-cell lymphoma: a molecular and clinicopathological study of cases from Asia, Germany, and the United States. Mod Pathol. 2008;21:1517–26.
25. Child FJ, Scarisbrick JJ, Calonje E, et al. Inactivation of tumor suppressor genes p15(INK4b) and p16(INK4a) in primary cutaneous B cell lymphoma. J Invest Dermatol. 2002;118:941–8.
26. Mirza I, Macpherson S, Paproski S, et al. Primary cutaneous follicular lymphoma: an assessment of clinical, histopathologic, immunophenotypic, and molecular features. J Clin Oncol. 2002;20:647–55.
27. Kim BK, Surti U, Pandya A, et al. Clinicopathologic, immunophenotypic, and molecular cytogenetic fluorescence in situ hybridization analysis of primary and secondary cutaneous follicular lymphomas. Am J Surg Pathol. 2005;29:69–82.
28. Higgins RA, Blankenship JE, Kinney MC. Application of immunohistochemistry in the diagnosis of non-Hodgkin and Hodgkin lymphoma. Arch Pathol Lab Med. 2008;132:441–61.

29. Cerroni L, Volkenandt M, Rieger E, et al. Bcl-2 protein expression and correlation with the interchromosomal (14;18) translocation in cutaneous lymphomas and pseudolymphomas. J Invest Dermatol. 1994;102:231–5.
30. Child FJ, Russell-Jones R, Woolford AJ, et al. Absence of the t(14;18) chromosomal translocation in primary cutaneous B-cell lymphoma. Br J Dermatol. 2001;144:735–44.
31. Geelen FAMJ, Vermeer MH, Meijer CJLM, et al. Bcl-2 expression in primary cutaneous large B-cell lymphoma is site-related. J Clin Oncol. 1998;16:2080–5.
32. Aguilera NS, Tomaszewski MM, Moad JC, et al. Cutaneous follicle center lymphoma: a clinicopathologic study of 19 cases. Mod Pathol. 2001;14:828–35.
33. Bergman R, Kurtin PJ, Gibson LE, et al. Clinicopathologic, immunophenotypic, and molecular characterization of primary cutaneous follicular B-cell lymphoma. Arch Dermatol. 2001;137:432–9.
34. Hoefnagel JJ, Dijkman R, Basso K, et al. Distinct types of primary cutaneous large B-cell lymphoma identified by gene expression profiling. Blood. 2005;105:3671–8.
35. Vermeer MH, Geelen FA, van Haselen CW, et al. Primary cutaneous large B-cell lymphomas of the legs. A distinct type of cutaneous B-cell lymphoma with an intermediate prognosis. Dutch Cutaneous Lymphoma Working Group. Arch Dermatol. 1996;132:1304–8.
36. Grange F, Beylot-Barry M, Courville P, et al. Primary cutaneous diffuse large B-cell lymphoma, leg type: clinicopathologic features and prognostic analysis in 60 cases. Arch Dermatol. 2007;143:1144–50.
37. Belousova IE, Vanecek T, Skreg SV, et al. Unusual clinicopathological presentation of primary cutaneous diffuse large B-cell lymphoma, leg type, with multiple nodules and widespread garland-like lesions. Am J Dermatopathol. 2009;31:370–4.
38. Kodama K, Massone C, Chott A, et al. Primary cutaneous large B-cell lymphomas: clinicopathologic features, classification, and prognostic factors in a large series of patients. Blood. 2005;106:2491–7.
39. Goodlad JR, Krajewski AS, Batstone PJ, et al. Primary cutaneous diffuse large B-cell lymphoma: prognostic significance of clinicopathologic subtypes. Am J Surg Pathol. 2003;27:1538–45.
40. Mao X, Lillington D, Child FJ, et al. Comparative genomic hybridization analysis of primary cutaneous B-cell lymphomas: identification of common genomic alterations in disease pathogenesis. Genes Chromosom Cancer. 2002;35:144–55.
41. Hallermann C, Kaune K, Siebert R, et al. Cytogenetic aberration patterns differ in subtypes of primary cutaneous B-cell lymphomas. J Invest Dermatol. 2004;122:1495–502.
42. Hallermann C, Kaune KM, Gesk S, et al. Molecular cytogenetic analysis of chromosomal breakpoints in the IGH, MYC, BCL6 and MALT1 gene loci in primary cutaneous B-cell lymphomas. J Invest Dermatol. 2004;123:213–9.
43. Dijkman R, Tensen CP, Jordanova ES, et al. Array-based comparative genomic hybridization analysis reveals recurrent chromosomal alterations and prognostic parameters in primary cutaneous large B-cell lymphoma. J Clin Oncol. 2006;24:296–305.
44. Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med. 1995;333:1052–7.
45. Pedersen J, Carganello J, van der Weyden MB. Exaggerated reaction to insect bites in patients with chronic lymphocytic leukemia. Clinical and histological findings. Pathology. 1990;22:141–3.
46. Cerroni L, Zenahlik P, Hoefler G, et al. Specific cutaneous infiltrates of B-cell chronic lymphocytic leukemia: a clinicopathologic and prognostic study of 42 patients. Am J Surg Pathol. 1996;20:1000–10.
47. Walther BS, Gibbons G, Chan EF, et al. Leukemia cutis (involving chronic lymphocytic leukemia) within excisional specimens: a series of 6 cases. Am J Dermatopathol. 2009;31:162–5.
48. Damle RN, Wasil T, Fais F, et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood. 1999;94:1840–7.
49. Hamblin TJ, Davis Z, Gardiner A, et al. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood. 1999;94:1848–54.
50. Oscier DG, Thompsett A, Zhu D, et al. Differential rates of somatic hypermutation in V(H) genes among subsets of chronic lymphocytic leukemia defined by chromosomal abnormalities. Blood. 1997;89:4153–60.
51. Ghia P, Guida G, Stella S, et al. The pattern of CD38 expression defines a distinct subset of chronic lymphocytic leukemia (CLL) patients at risk of disease progression. Blood. 2003;101:1262–9.
52. Dohner H, Stilgenbauer S, Benner A, et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N Engl J Med. 2000;343:1910–6.
53. Cordone I, Masi S, Mauro FR, et al. p53 expression in B-cell chronic lymphocytic leukemia: a marker of disease progression and poor prognosis. Blood. 1998;91:4342–9.
54. Khamaysi Z, Dodiuk-Gad RP, Weltfriend S, et al. Insect bite-like reaction associated with mantle cell lymphoma: clinicopathological, immunopathological, and molecular studies. Am J Dermatopathol. 2005;27:290–5.
55. Sen F, Medeiros LJ, Lu D, et al. Mantle cell lymphoma involving skin: cutaneous lesions may be the first manifestation of disease and tumors often have blastoid cytologic features. Am J Surg Pathol. 2002;26:1312–8.
56. Dubus P, Young P, Beylot-Barry M, et al. Value of interphase FISH for the diagnosis of t(11:14)(q13;q32) on skin lesions of mantle cell lymphoma. Am J Clin Pathol. 2002;118:832–41.

57. Komatsu H, Yoshida K, Seto M, et al. Overexpression of PRAD1 in a mantle zone lymphoma patient with a t(11;22)(q13;q11) translocation. Br J Haematol. 1993;85:427–9.
58. Gesk S, Klapper W, Martín-Subero JI, et al. A chromosomal translocation in cyclin D1-negative/cyclin D2-positive mantle cell lymphoma fuses the CCND2 gene to the IGK locus. Blood. 2006;108:1109–10.
59. Estrozi B, Sanches Jr JA, Varela PC, et al. Primary cutaneous blastoid mantle cell lymphoma-case report. Am J Dermatopathol. 2009;31:398–400.
60. Jares P, Colomer D, Campo E. Genetic and molecular pathogenesis of mantle cell lymphoma: perspectives for new targeted therapeutics. Nat Rev Cancer. 2007;7:750–62.
61. Jacobson MA, Hutcheson AC, Hurray DH, et al. Cutaneous involvement by Burkitt lymphoma. J Am Acad Dermatol. 2006;54:1111–3.
62. Hummel M, Bentink S, Berger H, et al. A biologic definition of Burkitt's lymphoma from transcriptional and genomic profiling. N Engl J Med. 2006;354:2419–30.
63. Beaty MW, Toro J, Sorbara L, et al. Cutaneous lymphomatoid granulomatosis: correlation of clinical and biologic features. Am J Surg Pathol. 2001;25:1111–20.
64. Medeiros LJ, Peiper SC, Elwood L, et al. Angiocentric immunoproliferative lesions: a molecular analysis of eight cases. Hum Pathol. 1991;22:1150–7.
65. Sioutos N, Kerl H, Murphy SB, et al. Primary cutaneous Hodgkin's disease. Unique clinical, morphologic, and immunophenotypic findings. Am J Dermatopathol. 1994;16:2–8.
66. Tassies D, Sierra J, Montserrat E, et al. Specific cutaneous involvement in Hodgkin's disease. Hematol Oncol. 1992;10:75–9.
67. Braeuninger A, Küppers R, Strickler JG, et al. Hodgkin and Reed-Sternberg cells in lymphocyte predominant Hodgkin disease represent clonal populations of germinal center-derived tumor B cells. Proc Natl Acad Sci USA. 1997;94:9337–42.
68. Marafioti T, Hummel M, Anagnostopoulos I, et al. Origin of nodular lymphocyte-predominant Hodgkin's disease from a clonal expansion of highly mutated germinal-center B cells. N Engl J Med. 1997;337:453–8.
69. Ohno T, Stribley JA, Wu G, et al. Clonality in nodular lymphocyte-predominant Hodgkin's disease. N Engl J Med. 1997;337:459–65.
70. Liso A, Capello D, Marafioti T, et al. Aberrant somatic hypermutation in tumor cells of nodular-lymphocyte-predominant and classic Hodgkin lymphoma. Blood. 2006;108:1013–20.
71. Kanzler H, Kuppers R, Hansmann ML, et al. Hodgkin and Reed-Sternberg cells in Hodgkin's disease represent the outgrowth of a dominant tumor clone derived from (crippled) germinal center B cells. J Exp Med. 1996;184:1495–505.
72. Marafioti T, Hummel M, Foss HD, et al. Hodgkin and Reed-Sternberg cells represent an expansion of a single clone originating from a germinal center B-cell with functional immunoglobulin gene rearrangements but defective immunoglobulin transcription. Blood. 2000;95:1443–50.
73. Davis TH, Morton CC, Miller-Cassman R, et al. Hodgkin's disease, lymphomatoid papulosis, and cutaneous T-cell lymphoma derived from a common T-cell clone. N Engl J Med. 1992;326:1115–22.
74. Muschen M, Rajewsky K, Brauninger A, et al. Rare occurrence of classical Hodgkin's disease as a T cell lymphoma. J Exp Med. 2000;191:387–94.
75. Seitz V, Hummel M, Marafioti T, et al. Detection of clonal T-cell receptor gamma-chain gene rearrangements in Reed-Sternberg cells of classic Hodgkin disease. Blood. 2000;95:3020–4.
76. Schlegelberger B, Weber-Matthiesen K, Himmler A, et al. Cytogenetic findings and results of combined immunophenotyping and karyotyping in Hodgkin's disease. Leukemia. 1994;8:72–80.
77. Joos S, Kupper M, Ohl S, et al. Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells. Cancer Res. 2000;60:549–52.

Chapter 13
Leukemia Cutis

Michael J. Murphy

The diagnosis and classification of leukemia requires the integration of clinical features and light microscopic findings with the results of cytochemical, immunological (flow cytometry and/or immunohistochemistry), and molecular studies [1]. Immunophenotypic and genotypic technologies are commonly applied to peripheral blood (PB) and bone marrow (BM) specimens in the initial work-up and management of patients with leukemia. It is critical to definitively characterize the disease, due to differences in treatment regimens and prognosis among leukemia subtypes [1]. Skin lesions that may arise in patients with leukemia can be divided into two groups: (a) "leukemids" or nonspecific reactions, in which inflammatory lesions contain no neoplastic cells; and (b) leukemia cutis (LC) or specific lesions, in which leukemic cells (myeloid or lymphoid) infiltrate the skin [2, 3]. Commonly used terms for LC composed of myeloid blasts include chloroma, extramedullary myeloid sarcoma, granulocytic sarcoma, and monoblastic sarcoma [1, 3]. This chapter discusses some of the limitations of traditional methodologies, and potential applications of molecular technologies, in the diagnosis and follow-up of patients with LC.

General Considerations

Skin involvement has been described for almost all forms of leukemia (Tables 13.1–13.3); although, the frequency of LC varies widely, depending on the underlying disease subtype. LC most commonly arises in the context of a known pre-existing (a) acute leukemia or (b) myeloproliferative neoplasm (MPN), myelodysplastic/myeloproliferative neoplasm (MDS/MPN), or myelodysplastic syndrome (MDS), with or without blast transformation [2–6]. LC in the setting of both congenital leukemia and therapy-related leukemia has also been described. In addition, LC can be seen in individuals with no prior history of a hematological disorder (i.e., "aleukemic" LC) [2–6]. The majority of LC cases, including "aleukemic" variants, are found to represent myelomonocytic and monocytic subtypes [2–6]. The frequency of cutaneous involvement is reported in the range of 2–15% for adults with acute myeloid leukemia (AML), although up to 50% of patients with acute leukemia of "monocytic" origin may have skin disease [2–8]. Cutaneous involvement has been described in 20–70% of patients with mature T-/NK-cell leukemias and 4–20% of patients with chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), but is rare (~1% incidence) in cases of precursor B or T lymphoblastic leukemia/lymphoma (ALL/LBL) [3]. Importantly, children are reported

M.J. Murphy (✉)
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, 21 South Rd., Farmington, CT 06030, USA
e-mail: drmichaelmurphy@netscape.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_13,

Table 13.1 Examples of hematopoietic disorders associated with the development of cutaneous lesions

Myeloid disorders
Acute myeloid leukemia (AML) and related precursor neoplasms
Acute myelomonocytic leukemia
Acute monocytic leukemia
Acute promyelocytic leukemia
Less common AML subtypes
Therapy-related myeloid neoplasms
Congenital leukemia
Blastic plasmacytoid dendritic cell neoplasm
MPN, MDS/MPN, and MDS
Chronic myelogenous leukemia
Mastocytosis
Chronic myelomonocytic leukemia
Juvenile myelomonocytic leukemia
Refractory anemia
Lymphoid disorders
Precursor lymphoid neoplasms
B lymphoblastic leukemia/lymphoma
T lymphoblastic leukemia/lymphoma
Non-B/non-T lymphoblastic leukemia/lymphoma
Mature B-cell neoplasms
Chronic lymphocytic leukemia/small lymphocytic lymphoma
Plasma cell neoplasms
Mature T- and NK-cell neoplasms
Sézary syndrome
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Adult T-cell leukemia/lymphoma
Mixed phenotype acute leukemia
B/myeloid, T/myeloid, NK/myeloid types

MPN myeloproliferative neoplasms, *MDS/MPN* myelodysplastic/myeloproliferative neoplasms, *MDS* myelodysplastic syndromes

to show a higher frequency of LC compared with adults, and infants with congenital leukemia develop skin involvement in 25–30% of cases [3]. The temporal relationship between the presence of LC and acute leukemia subtypes is variable; however, cutaneous involvement typically occurs late in the course of the disease, and there is a strong correlation between LC and leukemic involvement at other extramedullary sites [6]. Of note, the risk of extramedullary relapse following therapy for leukemia is higher for patients with skin involvement [8, 9]. Cutaneous lesions may also be the first sign of relapse of acute leukemia following chemotherapy or hematopoietic stem cell transplantation, and occur in the absence of any microscopic, cytogenetic, or molecular evidence of BM disease [8–11]. Except for reports of a subset of acute promyelocytic leukemia (APL) and a number of cases arising in the congenital setting, the prognosis for patients with acute LC is generally very poor [8, 12–14]. In addition, the development of LC in patients with MPN is usually associated with impending blast phase transformation and disease acceleration (Table 13.3) [15–17]. Therefore, the finding of specific leukemic skin infiltration in this latter setting would appear to identify a subgroup of patients with MPN who require treatment as for AML [15]. Similarly, the diagnosis of LC in patients with MDS is a poor prognostic marker and associated with disease progression, but independent of transformation to AML [4]. These latter patients often succumb to BM failure, complications of treatment, infections, or other co-morbidities.

Table 13.2 Molecular studies undertaken in cases of leukemia cutis (LC) in patients with acute myeloid leukemia (AML)

Study	*n*	Diagnosis	Molecular technique in skin	Molecular findings in skin	Molecular findings in BM (methods; timing and results, in relation to skin findings)	Comments
Rubin [63]	1	Acute monoblastic leukemia	KY	47XX, +8 or 9?, del(12)(p12), t(9;11)(p21;q23), der(19)t(19;?)(p13;?)	KY Prior Concordant: except for lack of +8 or 9?, del(12)(p12), der(19)t(19;?)(p13;?)	Findings suggested that karyotypic evolution occurred in the skin at relapse
Kubonishi [34]	1	Therapy-related AML-M5a	SB	*MLL* (11q23) gene rearrangement	SB Prior Concordant: *MLL* gene rearrangement associated with t(10;11)	
Sen [45]	11	AML-M1/M4/M5/NOS	FISH	+8 in 64% (7/11) of cases	KY Prior/concurrent Concordant in 3 of 7 Discordant in 4 of 7: Result (−) FISH Concordant: Additional 2 cases	FISH may detect numerical and structural abnormalities missed by KY; Numerical abnormalities of chr. 8 may predispose to skin involvement by AML
Lillington [64]	1	AML-M4	KY FISH RT-PCR	t(10;11), complex rearrangement of chr. 5, der(1;21), with trisomy 1; *MLL* (11q23) gene rearrangement; *MLL-AF10* fusion product	KY FISH RT-PCR Concurrent Concordant	
Beswick [18]	1	Aleukemic LC of myelo-monocytic lineage	Cytogenetic analysis, NOS	Normal karyotype; Positive for clonal *IgH* receptor gene rearrangement; Negative for clonal *TCR* gene rearrangement	Cytogenetic analysis, NOS Subsequent Concordant: normal karyotype	

(continued)

Table 13.2 (continued)

Study	*n*	Diagnosis	Molecular technique in skin	Molecular findings in skin	Molecular findings in BM (methods; timing and results, in relation to skin findings)	Comments
Chang [21]	1	Aleukemic LC of monocytic lineage	KY RT-PCR	Failed to obtain metaphases; Negative for t(8;21), inv(16), t(6;11), t(9;11), and t(11;19)	NR	Negative for fusion transcripts of nonrandom chromosomal translocations that are commonly associated with AML
Zebisch [32]	1	Therapy-related aleukemic LC	TCR analysis, NOS PCR	Negative for clonal *TCR* gene rearrangement; No *TP53* gene mutation or polymorphism detected	Cytogenetic analysis, NOS Subsequent 46XY, add(15)(p12) karyotype	
Deeb [10]	2	(1) AML-M5a (2) Aleukemic myeloid sarcoma	Conventional cytogenetics, NOS Array-CGH FISH	Gain 8q21.2–q24.3, 19, 21; Gain 5p15.33-p13.1, 8; Loss 10q24.2–q26.3 +8cen	Case (2): array-CGH Concurrent Concordant, except for +19	Demonstration of – Clonal nature of these tumors – Identical genomic abnormalities to those in BM – Patient stratification based on genetics – Genetic markers of follow-up, esp. in absence of BM disease
Zhang [14]	1	Congenital AML-M5	KY	*MLL* (11q23) gene rearrangement	KY Concurrent/subsequent Concordant, with karyotypic evolution in BM	
Alexiev [65]	2	Monoblastic sarcoma	KY	Normal karyotypes	NR	
Pileri [66]	7	Myeloid sarcoma	FISH	+8 in AML-M4 (*n = 1*); -20q associated with *CBF-beta* deletion in AML-M5 (*n = 1*); Negative results: *n = 5*	NR	The clinical behavior and response to therapy of myeloid sarcoma were not influenced by cytogenetic findings, previous history (*de novo* disease or underlying pathology), histotype, phenotype, age, sex, sites involved or clinical presentation (personal communication)
Christopeit [67]	1	AML	KY	47XX, add(6), del(11)(q23), add(12)(p13?), del(13)(q12q14), +19	KY Concurrent Concordant	

Dijkman [61]	6	AML-M4	Oligonucleotide microarray Array-CGH	No highly recurrent alterations; +8 in 33%	NR	Integrated genomic analysis may be used in the differential diagnosis of cutaneous AML and HDN
Szczempański (2008)	1	AML-M4	SB PCR	Germline *MLL*; Positive for clonal *IgH* receptor gene rearrangement; Positive for clonal *TCR* gene rearrangement; Negative for EBV	SB PCR Prior Concordant	Detection of clonally-related AML-M4 in patients with prior precursor B-ALL argued against second malignancy
Stern [9]	1	AML with maturation	FISH	Hyperdiploidy (chr. 6/chr. 17)	NR	
Ferran [69]	1	Acute myeloid dendritic cell leukemia	PCR ISH	Negative for clonal *TCR* gene rearrangement; Negative for EBV	FISH Concurrent Tetrasomy 8	
Kaune [88]	1	AML-M5	FISH	Gain of 8q22 (*ETO/AML1*), 8q23 (*MYC*), Tel8q; Loss of 17p13 (*TP53*)	FISH Prior Negative for *BCR-ABL1*; 8q NR	Partial gain of 8q rather than trisomy 8 may be associated with skin involvement in AML
Balmer [19]	1	Congenital aleukemic AML with monocytic differentiation	FISH	*MLL* (11q23) gene rearrangement	NR	Presence of *MLL* in skin, in the absence of BM disease, raises therapeutic questions
Cappel [39]	1	AML with maturation (histiocytoid Sweet's syndrome-like lesions)	FISH	7q (*AML1*) abn. 11q23 (*MLL*) abn.	FISH Concurrent Concordant	Concordant skin and BM FISH analyses, despite differences in blast morphology and immunophenotype

BM bone marrow, *KY* karyotyping, *SB* Southern blot, *FISH* fluorescence in situ hybridization, *ISH* in situ hybridization, *PCR* polymerase chain reaction, *RT-PCR* reverse transcription-PCR, *CGH* comparative genomic hybridization, *chr.* chromosome, *NOS* not otherwise specified, *IgH* immunoglobulin heavy chain, *TCR* T-cell receptor, *EBV* Epstein-Barr virus, *NR* not reported, *HDN* CD4+/CD56+ hematodermic neoplasm (now termed blastic plasmacytoid dendritic cell neoplasm), *ALL* acute lymphoblastic leukemia

Table 13.3 Molecular studies undertaken in cases of leukemia cutis (LC) in patients with MPN, MDS/MPN, and MDS

Study	*n*	Diagnosis	Molecular technique in skin	Molecular findings in skin	Underlying BM disorder	Molecular findings in BM (methods; results, in relation to skin findings)	Comments
Ohyashiki [74]	1	Granulocytic sarcoma	KY	t(9;22)(+Ph) Trisomy 8 Tetrasomy 8	CML	KY Concordant t(9;22)(+Ph) Tetrasomy 8	Skin lesions associated with blast crisis; Chromosomal analysis confirmed that skin tumor cells originated from the CML clone
Madhumathi [75]	1	Myeloid sarcoma	Cytogenetic analysis, NOS	t(9;22)(+Ph)	CML	t(9;22)(+Ph) Concordant	Skin lesions associated with blast crisis
McCollum [76]	1	CMML	NR	Negative for clonal *TCR* gene rearrangement	–	KY -13q	Skin involvement was initial manifestation of CMML
Yin [47]	1	Myeloid sarcoma	KY FISH	t(9;22)(*BCR-ABL1* fusion signals, +Ph), t(8;21), +8, additional chromosomal abnormalities	CML	KY FISH Concordant t(9;22) (*BCR-ABL1* fusion signals, +Ph), t(8;21), +8, additional chromosomal abnormalities	Skin lesions associated with blast crisis; Evaluation of skin lesions confirms secondary chromosomal aberrations that occur as part of clonal evolution; Discordant morphologic features between skin lesions and BM, despite concordant cytogenetic findings
Pileri [66]	8	Myeloid sarcoma	FISH (*n = 1*)	Monosomy 7 (*n = 1*)	RA	KY Discordant: normal karyotype	The clinical behavior and response to therapy of myeloid sarcoma were not influenced by cytogenetic findings, previous history (*de novo* disease or underlying pathology), histotype, phenotype, age, sex, sites involved, or clinical presentation (personal communication)

MPN myeloproliferative neoplasms, *MDS/MPN* myelodysplastic/myeloproliferative neoplasms, *MDS* myelodysplastic syndromes, *CML* chronic myelogenous leukemia, *CMML* chronic myelomonocytic leukemia, *RA* refractory anemia, *BM* bone marrow, *KY* karyotyping, *FISH* fluorescence in situ hybridization, *NOS* not otherwise specified, *NR* not reported, *+Ph* Philadelphia chromosome, *TCR* T-cell receptor

In up to 10% of patients, cutaneous lesions can precede BM or PB involvement, and are designated as "aleukemic" LC [5, 7, 10, 18–22]. In such instances, a low threshold for biopsy of any unusual skin lesion and a high index of suspicion of LC is required. Even in the presence of "normal" BM findings, the development of LC is associated with a high risk for subsequent BM pathology. Systemic disease generally emerges within 1–20 months, but may not manifest for up to 3–4 years [18, 20]. However, a proportion of patients who present with "aleukemic" extramedullary disease may not develop acute leukemia, if treated appropriately at the time of presentation [23].

Congenital leukemia is defined as leukemia presenting at birth or within the first 4 weeks of life [7, 14, 19, 24–27]. Only ~200 cases have been reported in the literature, of which ~25–30% exhibited cutaneous involvement [7, 14, 19, 24–27]. The prognosis is generally poor, although a few patients have experienced temporary or permanent spontaneous remission of their disease, including skin lesions [7, 14, 19, 24–27]. LC can be the initial manifestation of congenital leukemia, and may even precede PB or BM disease in up to 10% of cases (termed "aleukemic congenital leukemia cutis") [14, 19, 27]. Cutaneous involvement by congenital leukemia must be distinguished from other skin conditions that may mimic it, including: (a) transient myeloproliferative disorder (TMD), a condition commonly seen in neonates with Down syndrome (up to 20% of cases) or trisomy 21 mosaicism, and rarely in neonates with normal karyotypes [24, 25, 28, 29]; (b) other transient leukemoid reactions; (c) extramedullary hematopoiesis; and (d) non-hematopoietic malignancies, such as metastatic neuroblastoma and Langerhans cell histiocytosis [14, 27, 30, 31].

LC is infrequently associated with so-called therapy-related leukemia, which develops as a consequence of radiation therapy and/or cytotoxic chemotherapy (i.e., alkylating agents and topoisomerase inhibitors) for other malignancies [8, 13, 32–34]. Most examples of LC in this setting are of myeloid lineage and demonstrate recurrent rearrangements of the *MLL* gene at chromosome 11q23 [8, 13, 32–34]. Of note, therapy-induced "aleukemic" LC has also been described [32].

Clinical Features

There exists great variability in the clinical spectrum and histopathological features of LC [35]. Patients with LC commonly present with single-to-multiple, variably sized, violaceous, erythematous, or hemorrhagic papules, plaques, and/or nodules [3]. Other common clinical presentations can include marked thickening of the gums, leonine facies, and the so-called "blueberry muffin baby" [3]. Unusual and rare manifestations of cutaneous leukemic infiltration described in the literature include eczematous changes, exfoliative erythroderma, bullous disorders, perifollicular acneiform eruptions, vitiligo, lesions localized to the palms, stasis dermatitis-like changes, chilblain-like lesions, leukemic vasculitis, symmetrical edematous facial erythema, and generalized morbilliform drug or viral exanthem-like eruptions [15, 36–38]. Interestingly, leukemic infiltrates are often localized to sites of prior trauma, burns, intravenous lines, intramuscular injections, herpetic lesions, scars, or other inflammatory processes [36, 38, 39]. Importantly, the clinical morphology of the skin lesions depends not only on the nature of the infiltrating leukemic cells, but can also vary as a result of therapy or other co-existing conditions [40]. Therefore, skin lesions from an individual with a distinct leukemia subtype can show varied clinical appearances over the course of the disease [3]. In addition to specific cutaneous changes caused by direct infiltration of the skin by leukemic cells, there are a variety of nonspecific secondary skin lesions, termed "leukemids", that are commonly associated with leukemia, and seen in ~40% of patients [3, 6, 8]. The latter may be attributed to BM dysfunction or failure, and include ecchymoses, purpura, or petechiae secondary to coagulopathy, pallor secondary to anemia, and/or cutaneous infections as a result of immunosuppression [3, 6, 8]. Cutaneous vasculitis, panniculitis, chilblain-like eruptions, erythema multiforme, and neutrophilic dermatoses (pyoderma gangrenosum and Sweet's syndrome;

see Chap. 14) can also occur in patients with leukemia, in the absence of specific cutaneous infiltration by tumor cells [3, 6, 8].

Histopathological Features

Confirming or establishing a diagnosis of LC on a limited skin biopsy may be particularly challenging. Misclassification of the leukemia subtype or misdiagnosis of LC as cutaneous involvement by high-grade lymphoma (including Burkitt lymphoma, blastoid mantle cell lymphoma, blastoid transformation of follicular lymphoma, or diffuse large B-cell lymphoma) or another so-called "small round blue cell tumor" is not unusual, and has been reported in 40–100% of cases [4, 5, 41]. In "aleukemic" examples, the unavailability of neoplastic cells in the PB or BM aspirates for immunophenotyping and molecular studies can make it extremely difficult to classify LC. There appears to be no correlation between the microscopic findings and the diverse clinical presentations in LC [38]. Similar to clinical findings, LC may show a wide spectrum of histopathological features, from prominent dermal/subcutaneous, diffuse or nodular infiltration of leukemic cells to perivascular and periadnexal infiltrates [35]. In some instances, leukemic cells may be sparse or obscured by an exuberant granulomatous/reactive inflammatory process [42]. Epidermotropism is an unusual finding, but infiltration and destruction of adnexal structures, nerves, muscle bundles, and rarely blood vessels can be seen [3, 35]. Mitoses, apoptotic figures, and stromal fibrosis or edema may also be present [3]. The cytologic features of the infiltrating leukemic cells can vary depending on the lineage and degree of cellular maturation [3, 35], which in some cases may be related to prior therapeutic intervention; for example, all-*trans* retinoic acid (ATRA) for patients with APL [7, 11, 12, 43, 44]. The morphology of blasts in the skin infiltrate is typically similar to that at other body sites in a patient with acute leukemia [42]. However, some investigators have reported significant morphological differences between leukemic cells in the PB or BM and those infiltrating the skin in individual patients [39, 45–47]. This finding may stem from the presence of distinct cells/subclones with skin-homing properties – a hypothesis supported by differential responses to treatment by tumor cells in the skin and BM compartments in some leukemia patients [37]. As discussed in the following sections, adjunct cytochemical, immunophenotypic, and molecular diagnostic techniques are commonly applied to skin biopsies of suspected LC. Electron microscopy has been rarely used to diagnose cutaneous involvement by leukemia [22].

Ancillary Laboratory Testing

Cytochemistry utilizes stains, such as myeloperoxidase (MPO), Giemsa Sudan Black B, chloroacetate esterase, nonspecific esterase, toluidine blue, and periodic acid-Schiff (PAS) to target various enzymes, fats, and other substances in blasts and other hematopoietic cells [48, 49]. For instance, MPO is found in the granules of myeloid cells and serves as the most important marker distinguishing myeloid from lymphoid blasts [48, 49]. While these cytochemical stains are a practical adjunct to identifying and confirming leukemia, they now have diminished utility given the availability of other techniques, such as immunohistochemistry, flow cytometry and molecular assays, which yield more specific results [48, 49]. Cytochemistry has been applied to skin biopsies in the evaluation of LC [7, 21–23, 36, 38, 45, 46, 50]. In most instances, the cutaneous cell type determined by cytochemistry has been found to reflect the subtype of leukemia diagnosed by PB or BM testing [36]. However, some studies report discordant findings [38]. The usefulness of cytochemistry

on cutaneous specimens is especially limited, as staining may be focal [23], and there is a requirement for fresh or archived touch imprints, which are more commonly available for PB and BM smears [48, 49].

Flow cytometry can yield rapid, informative data on the immunophenotype of hematopoietic and suspected leukemic infiltrates in skin biopsies, but has not been widely utilized in the work-up of LC [21, 41, 42, 51–56]. This is mainly due to the difficulty of preparing viable single cell suspensions from skin biopsies – a process that is highly dependent on the method of specimen fixation/transportation. Flow cytometric analysis requires a portion of fresh tissue stored in RPMI medium or similar solution. However, without some index of suspicion, skin biopsies are typically submitted in formalin fixative [51].

Immunohistochemistry is undoubtedly a powerful diagnostic tool in the setting of formalin-fixed paraffin-embedded (FFPE) LC specimens. However, the ever-expanding selection of immunologic markers can promote indiscriminate and inappropriate utilization of this technology. Usage of multiple antibodies (that may not add diagnostic information) is unavoidable, because no singular antibody is specific for any one particular entity. However, a systematic, algorithmic approach which employs a focused panel of antibodies is advisable. Such a panel would include markers of: (a) hematopoietic origin (CD45/LCA); (b) immature hematopoietic cells (CD34, TdT, CD117); and (c) lineage (MPO, lysozyme, PGM1, Factor VIII, and CD41 for myeloid; and CD2, CD3, CD4, CD8, CD20, and PAX-5 for lymphoid). The use of immunohistochemistry should be streamlined to exclude other histopathological mimics, confirm/establish the diagnosis of LC, and identify its hematopoietic lineage [2, 3, 48, 49]. Importantly, most studies have demonstrated similar immunophenotypic patterns between leukemic infiltrates in the skin and those in corresponding BM and lymph node specimens [15, 21, 36, 57]. However, similar to the morphological and cytochemical variation between different sites of leukemic involvement, some investigators have reported that LC can manifest a different immunophenotypic profile than seen in the respective PB and/or BM pathology [2, 38, 39, 46, 58]. Several factors could explain such findings. Firstly, heterogeneity of surface antigen expression by leukemic cells over the disease course has been documented [58]. For example, CLL may contain both CD5+ and CD5- B-cell clones, and conversion from >85% B-cell CD5-positivity at diagnosis to <10% B-cell CD5-positivity during follow-up is noted in some patients [58]. Secondly, immunophenotypically distinct subgroups of leukemic cells may occupy different body sites, with gain or loss of particular antigens promoting or associated with a propensity for cutaneous disease [3, 8, 10, 12, 45, 50, 58–61]. In this regard, a number of immunophenotypic findings in the blast cells examined from cutaneous, PB and BM specimens have been found to be associated with a propensity for extramedullary involvement in patients with acute leukemia. These include cell surface CD2, CD4, CD56, CD82, CD138, MCP-1/CCR2, and CLA expression [3, 8, 10, 12, 45, 50, 58–61]. Of note, increased CD4 and CD56 expression by leukemic blasts is found in >90% and 40–50% of cases of AML with cutaneous disease, respectively [45, 50, 59]. Thirdly, disease progression or transformation can be associated with loss of surface antigen expression [58]. Fourthly, phenotypically distinct populations could be preferentially selected by therapy, or the immunophenotype may otherwise be altered by treatment [2, 58]. Finally, technologies such as flow cytometry and immunohistochemistry can demonstrate different sensitivities for the detection of some antigens, particularly in FFPE tissue samples [51, 58]. For example, anti-surface antigen antibodies from different clones and/or vendors could explain variable phenotypes, or the level of antigen expression may be below the threshold for immunohistochemistry-based detection in some cases [58]. It is possible that a “negative” result on tissue immunohistochemistry could be associated with a “dim positive” pattern by the more sensitive technique of flow cytometry. The potential of variable surface antigen presentation and/or detection in leukemic infiltrates in the skin highlights the importance of correlating the findings of immunohistochemical studies with the clinical history, microscopic features, and results of other tests in patients with LC.

Molecular Diagnostic Strategies

The revised 2009 World Health Organisation (WHO) manual of hematologic neoplasms now lists a significant number of leukemias and related disorders, whose classification is based on the detection of specific chromosomal translocations and/or gene mutations [1]. These can be determined by such molecular techniques as karyotyping, Southern blot, polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR), fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), and array-CGH [1, 10, 49, 62]. In general, molecular studies are dictated by the results of clinical, microscopic, and immunophenotypic studies, and are frequently ordered only after a comprehensive immunohistochemical or flow cytometric work-up has provided adequate diagnostic direction [1, 48, 49]. Genetic abnormalities leading to leukemia are not only heterogeneous, but also complex, and multiple aberrations often cooperate in a multistep fashion to determine the leukemia phenotype. Specific genetic abnormalities identify the pathogenesis of these neoplasms and uncover potential therapeutic targets. As these genetic abnormalities also carry important diagnostic and prognostic implications for patients with these disorders, attempts to test for them must be made as part of the initial evaluation [1]. Given some of the limitations of traditional methodologies in the setting of leukemic skin infiltrates, molecular diagnostic techniques have a number of interesting applications in the diagnosis and management of patients with LC.

A number of studies have described the use of molecular testing on skin samples from patients with LC, including: (a) AML [9, 10, 14, 18, 19, 21, 32, 34, 39, 45, 50, 61, 63–69]; (b) APL [7, 11, 12, 43, 44, 70–73]; (c) MPN, MDS/MPN, and MDS [47, 66, 74–76]; (d) ALL/LBL [41, 52–56, 77, 78]; (e) blastic plasmacytoid dendritic cell (BPDC) neoplasm (formerly known as CD4+/CD56+ hematodermic neoplasm) [61, 79–91]; and (f) mature B- and T-/NK-cell neoplasms [92–103]

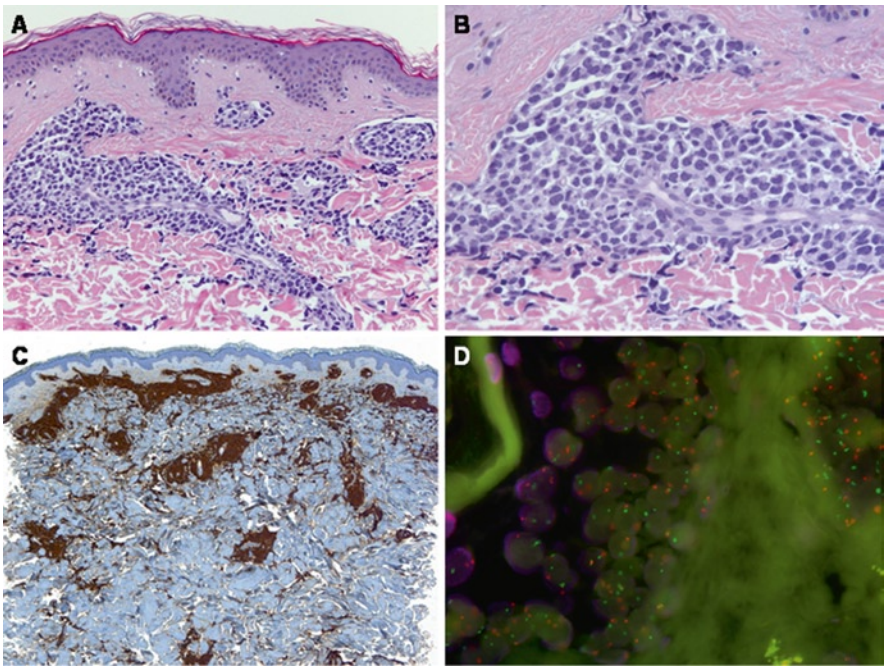

Fig. 13.1 Acute myeloid leukemia (*AML*). (**a**, **b**) Skin biopsy of the abdomen demonstrating histopathological evidence of cutaneous involvement by AML. Diagnosis was confirmed by (**c**) immunohistochemical staining for myeloperoxidase and (**d**) fluorescence in situ hybridization (*FISH*) for centromeres of chromosomes 6 (*red*) and 17 (*green*), revealing a hyperdiploid karyotype in the majority of tumor cells (i.e., >4 signals per nucleus) (Courtesy of Drs. Martin Stern and Peter Häusermann, Departments of Hematology and Dermatology, University Hospital, CH-Basel, Switzerland)

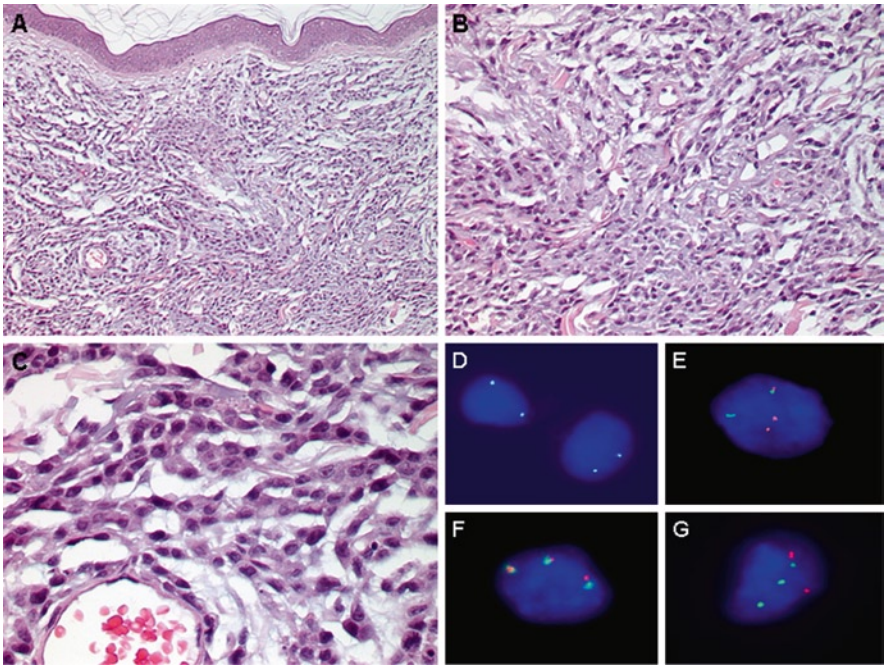

Fig. 13.2 Acute myeloid leukemia (*AML*). (**a–c**) Skin biopsy demonstrating a dermal infiltrate of monomorphous monocytoid cells, consistent with cutaneous involvement by AML. (**d–g**) Evaluation of skin biopsy by fluorescence in situ hybridization (*FISH*). (**d**) Interphase nuclei hybridized with a centromere 8 probe showing a normal pattern of two aqua signals, which excludes aneuploidy of chromosome 8 [i.e., trisomy (three signals) or tetrasomy (four signals)]. (**e**) Interphase nucleus hybridized with dual-fusion dual-color *AML1/ETO* translocation probe. Three *red* signals indicate a gain at chromosome 8q22, whereas the two *green* signals represent normal copies of *AML1*. The absence of *red–green* (*yellow*) fusion signals excludes the occurrence of the t(8;21)(q22;q22) translocation. (**f**) Interphase nucleus hybridized with dual-color break-apart *MYC* rearrangement probe. The three *red* and three *green* signals point to a gain at 8q24. The co-localizations of the *red* and *green* signals exclude the presence of a translocation affecting the *MYC* gene. (**g**) Interphase nucleus hybridized with telomere 8q (*green*) and telomere 8p (*red*) probes. The three *green* signals point to a gain at telomere 8q, while the two *red* signals indicate normal telomere 8p (Courtesy of Dr. Kjell M. Kaune, Department of Dermatology and Venerology, Georg August University Goettingen, Goettingen, Germany)

(Figs. 11.6 and 13.1–13.4). Sézary syndrome and adult T-cell leukemia/lymphoma are discussed in Chaps. 10 and 11, respectively. Technologies used on skin specimens include conventional karyotyping, Southern blot, PCR, RT-PCR, in situ hybridization methods (including FISH), oligonucleotide microarray, and array-CGH. In rare instances, conventional G-banding and Southern blot analyses have been employed to evaluate cutaneous leukemic infiltrates. However, the utility of these older technologies is limited, as both require the submission of fresh tissue, and it may be difficult to culture skin biopsies for karyotype testing [44]. More frequently, the molecular signatures of LC have been evaluated by PCR-based analyses, FISH, and array-CGH methodologies performed on fresh/frozen or FFPE tissue. FISH is a particularly useful technology in this setting, as it allows for correlation of genetic changes with morphological features of the disease. However, as FISH detects only some of the chromosomal abnormalities identified by karyotyping, it has been suggested that the submission of fresh skin samples for conventional cytogenetic analysis should be encouraged in cases of suspected LC, in order to evaluate the full range of potential genetic aberrations [66]. Molecular technologies have been used to evaluate cutaneous leukemic infiltrates for:

1. The presence of genomic aberrations, such as: trisomy 8 and/or *MLL* (11q23) gene rearrangement in AML [14, 19, 45, 61]; *PML-RARA* [t(15;17)(q22;q12)] or *NPM-RARA* [t(5;17)(q35;q12)] fusion genes in APL [7, 11, 12, 43, 44, 70–73]; *BCR-ABL1* [t(9;22)(q34;q11)] fusion gene

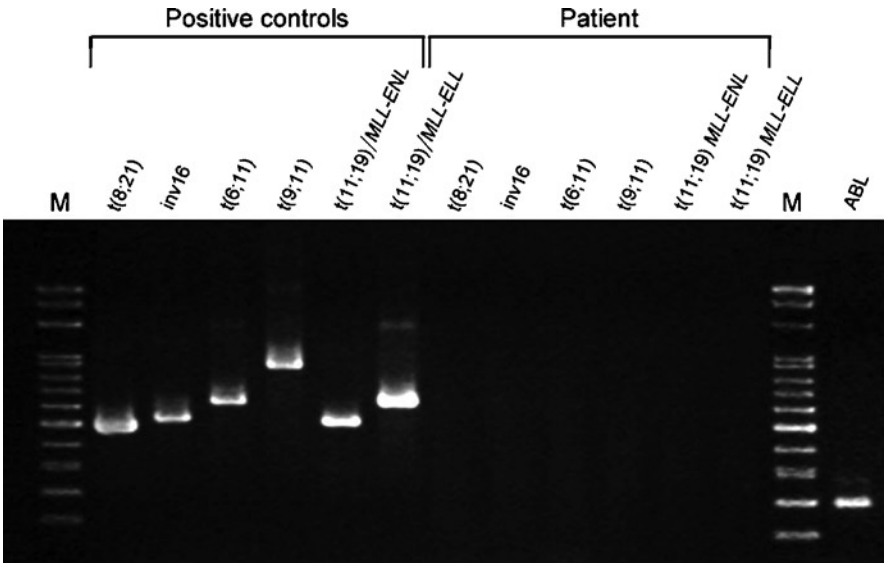

Fig. 13.3 Acute myeloid leukemia (*AML*). RT-PCR analysis for recurrent fusion genes in a single cell suspension prepared from a skin biopsy specimen of AML and positive controls (obtained from bone marrow leukemic cells carrying the fusion transcripts). *M* molecular weight marker, *ABL* amplification of ABL cDNA as an internal control for the RNA quality of the patient sample (Courtesy of Dr. Lee-Yung Shih, Chang Gung University, Taipei, Taiwan)

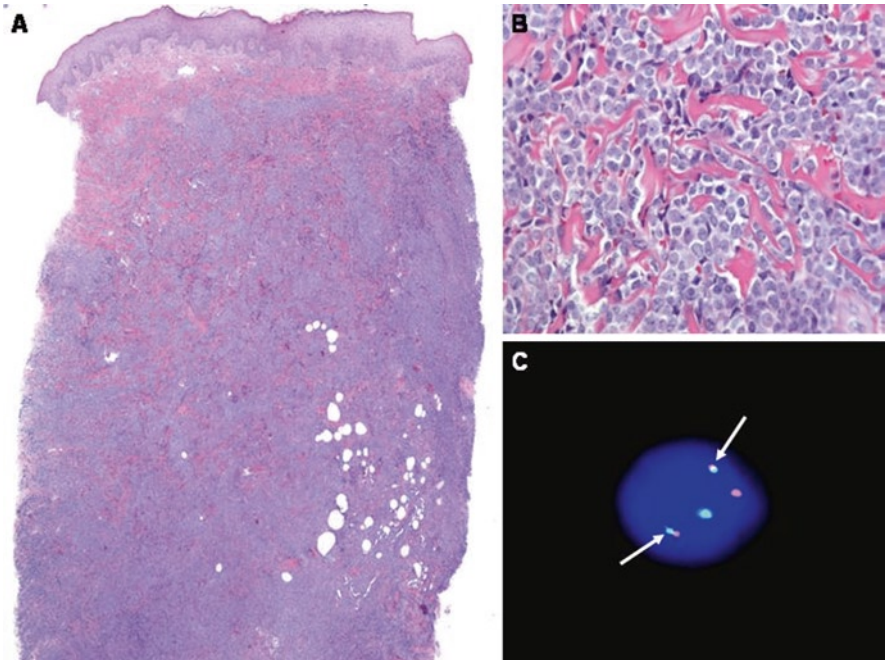

Fig. 13.4 Acute promyelocytic leukemia (*APL*). (**a**, **b**) Skin biopsy of the abdomen demonstrating histopathological evidence of cutaneous involvement by APL. (**c**) Fluorescence in situ hybridization (*FISH*): Representative tumor cell nucleus hybridized with dual-color dual-fusion *PML/RARA* translocation probe. The two fusion signals indicate the fusion products *PML/RARA* and *RARA/PML* (*arrows*), while the separate *red* and *green* signals represent non-rearranged loci (Courtesy of Dr. Tracy George, Clinical Hematology Laboratory, Stanford University, Stanford, CA)

(i.e., Philadelphia chromosome) in chronic myelogenous leukemia (CML) with blast crisis [47, 74, 75]; deletion of the *RB1* retinoblastoma gene in plasma cell myeloma [102]; and *c-kit* gene mutations in mastocytosis [104–106]
2. Gene expression [61]
3. T-cell receptor (*TCR*) and immunoglobulin heavy chain (*IgH*) receptor gene rearrangement status [18, 32, 52, 54, 56, 68, 69, 76, 78–94, 96, 97, 99–103]
4. The presence of infectious agents (i.e., *human T-cell lymphotropic virus type 1* [HTLV-1], *Epstein-Barr virus* [EBV], *Borrelia burgdorferi*, and *human herpesvirus 8* [HHV-8]) [52, 68, 69, 79, 81–87, 89, 90, 92, 94, 100, 102]

Table 13.2 describes molecular studies undertaken in cases of AML with specific cutaneous leukemic infiltrates. Table 13.3 describes molecular studies undertaken in patients showing cutaneous involvement by MPN with blast crisis/transformation, MDS/MPN, and MDS. In addition, 10–20% of cases of neutrophilic dermatoses (i.e., Sweet's syndrome) may be associated with an underlying hematologic disorder, particularly a myeloid dyscrasia. The use of molecular technologies in the evaluation of skin samples of neutrophilic dermatoses is discussed in Chap. 14.

Similar to immunophenotypic features, a number of genetic findings in the blast cells examined from skin, PB, and BM specimens have been shown to be associated with a propensity for extramedullary disease in patients with acute leukemia. Specific cytogenetic abnormalities include: *NPM1* mutations (~16%); inv(16); numerical abnormalities of chromosome 8 (most commonly trisomy 8, seen in 35–63% of cases; or tetrasomy 8); t(8;21) translocation; *MLL* (11q23) gene rearrangement; trisomy 4; trisomy 11; monosomy 7; monosomy 16; and t(9;11) translocation [1, 3, 8, 10, 12, 45, 50, 59–61, 65, 66, 69]. Of note, development of skin disease in the setting of acute leukemia may be associated with the up-regulation of a number of genes on chromosome 8q. In this regard, a number of proteins known to be involved in the homing of leukemic cells are located on chromosome 8 (i.e., fibronectin and tissue plasminogen activator), and it is postulated that numerical aberrations of chromosome 8 may be associated with their significantly up-regulated expression [60]. In addition, it has been postulated that genes such as *C8FW* (8q24), which are known to be consistently up-regulated in association with trisomy 8 in patients with AML, may confer an increased likelihood of cutaneous involvement through the modulation of factors involved in blast adhesion, migration, motility, and tissue invasion [50]. Unlike myeloid disorders, evidence to date suggests that skin infiltration by ALL/LBL is not associated with specific or recurrent karyotypic abnormalities [41, 52–56, 77, 78]. However, *TCR* and *IgH* receptor gene rearrangement analysis by PCR and/or Southern blot are known to be useful adjuncts to the diagnosis of both ALL/LBL [49, 107] and mixed phenotype acute leukemia [108], and have been performed on systemic and cutaneous leukemic infiltrates of these processes [52, 54, 56, 68, 78]. Greater than 90% of T-ALL show clonal *TCR* gene rearrangements and almost 100% of B-ALL show clonal *IgH* receptor gene rearrangements [49, 107]. However, it must be noted that 20–60% of B-ALL can also demonstrate clonal rearrangement of *TCR* genes and up to 20% of T-ALL may also have clonal rearrangement of *IgH* receptor genes ("lineage cross-over") [49, 107], including skin-localized disease [56, 68, 78]. In addition, clonally rearranged *TCR* and *IgH* receptor genes may be identified in up to 5% of AML [49, 107], including LC examples (Table 13.2) [18]. Results of *TCR* and *IgH* receptor gene rearrangement studies must be interpreted in the context of clinical, microscopic, immunophenotypic, and other molecular findings. Receptor gene rearrangement testing has also been used in the evaluation of mature B-cell and T-/NK-cell leukemic infiltrates in the skin [92–94, 96, 97, 99–103]. Furthermore, conventional cytogenetic studies for gross chromosomal aberrations [97], and FISH analysis demonstrating polysomy 8 and *MYC* amplification [96], have been accomplished on cutaneous lesions of T-cell prolymphocytic leukemia (T-PLL). Of note, cutaneous involvement by T-cell large granular lymphocytic leukemia (T-LGLL) may be predictive of a more aggressive clinical course [99, 101]. In contrast, most studies suggest that skin involvement by CLL or T-PLL does not appear to be associated with an adverse prognosis [94, 98]. However, one study has reported that

Table 13.4 Clinical relevance of molecular analyses of skin specimens in patients with leukemia cutis (LC)

Relevance	References
Confirm the diagnosis of LC, in the setting of a known history of leukemia	Most studies
Compare skin and BM specimens for concordant or discordant data	Tables 13.2 and 13.3; Additional refs. [7, 11, 12, 43, 44, 54, 70, 72, 73]
Establish the diagnosis of LC, in the setting of aleukemic skin infiltrates and/or in instances where BM specimens are either unanalyzable or uninformative	[7, 10, 19, 21]
Distinguish 2 or more types of LC	[61, 88, 91]
Differentiate LC from histopathological mimics	[39, 104]
Determine cutaneous relapse of primary tumor or *de novo* secondary malignancy	[68]
Guide effective therapy in patients with LC	[11, 12, 63, 70]
Determine the presence of genomic aberrations that may be relevant to clinical behavior and prognosis	[102, 104–106]

LC leukemia cutis, *BM* bone marrow

the histopathological features of specific skin infiltrates in CLL may be helpful in identifying prognostically different subgroups of patients [95].

The determination of genetic abnormalities in leukemia is important in establishing a diagnosis and identifying prognostic markers of response to induction therapy, remission duration, and patient survival [1]. The identification of such aberrations in LC lesions can take on added relevance in a number of clinical settings, as outlined in Table 13.4. For example, many studies have compared the genetic abnormalities in skin and BM specimens of patients with LC (Tables 13.2 and 13.3). In general, molecular changes in the skin have been found to be concordant with results from prior, concurrent, or subsequent BM testing. However, discordant results have occasionally been reported [11, 12, 45, 54, 66]. Molecular testing of cutaneous samples may be particularly useful in those instances where a genetic aberration or evidence of clonality was found in the skin, but not detected in the corresponding BM disease [11, 12, 45, 54, 66]. In addition, molecular characterization may uncover important information in cases of "aleukemic" LC and/or those instances where corresponding BM specimens are unanalyzable [7, 10, 19, 21]. Such testing could also be used to differentiate two or more types of leukemia. For example, the distinction of cutaneous manifestations of a BPDC neoplasm from (CD56+) AML with skin involvement may be problematic [61, 88, 91]. Of note, foci of plasmacytoid dendritic cell (pDC) differentiation (i.e., CD123+) can be seen in cases of myeloid sarcoma carrying inv(16) [1]. However, BPDC neoplasm and AML have been found to show distinct patterns of chromosomal aberrations and distinct gene expression profiles [61, 88]. Balanced or unbalanced translocations are rarely found in BPDC neoplasm, while cutaneous AML may harbor complex cytogenetic abnormalities, and frequently show specific balanced translocations/inversions (Table 13.2) [88]. Kaune et al. [88] suggest that numerical aberrations of chromosome 8 may qualify as a specific feature for the distinction of AML with skin involvement from BPDC neoplasm, because aberrations of this chromosome are generally found to be absent in the latter. Dijkman et al. [61] report that integrated genomic analysis (oligonucleotide microarray and array-CGH) may also aid in the differentiation of BPDC neoplasm from cutaneous AML, and possibly uncover new therapeutic targets in these tumors. BPDC neoplasm is characterized by recurrent deletions of regions on chromosomes 4 (4q34), 9 (9p11–p13 and 9q12–q34) and 13 (13q12–q31), decreased expression of tumor suppressor genes (RB1, LATS2, CDC14B, DBC1, SYK, KPNA3), and increased expression of both oncogenes (HES6, RUNX2, FLT3) and pDC-related genes (SpiB, TCL1a, CD123, BDCA-2) [61]. In contrast, AML shows specific expression of myelomonocytic genes (CD14, CD33, CLEC5A, MNDA) [61]. Furthermore, molecular technologies could facilitate the differentiation of LC from histopathological mimics. In this respect,

FISH has been used to detect 7q/*AML1* and 1q23/*MLL* abnormalities in both the skin specimen of LC (with microscopic similarity to histiocytoid Sweet's syndrome) and corresponding BM specimen (which demonstrated features of AML with maturation) in the same patient [39]. As a corollary, FISH was employed by another group to rule out a *BCR-ABL1* gene fusion in the skin, in order to support the diagnosis of histiocytoid Sweet's syndrome and exclude cutaneous involvement by CML [109]. Another application is the ability to distinguish cutaneous relapse of a primary leukemia from a *de novo* secondary malignancy. Szczepański et al. [68] determined that (relapsed) myelomonocytic LC was clonally related to a primary ALL, due to the identification of identical clonal TCR and IgH receptor gene rearrangements in both skin and BM specimens by Southern blot and PCR-based testing. Molecular studies of skin samples have also been used to determine effective therapy in patients with LC, including those without definitive microscopic or molecular evidence of BM involvement and/or with skin-isolated relapse of their disease [11, 12, 63, 70]. For example, detection of *PML-RARA* gene fusions in the skin biopsies of patients with APL have been used to select for appropriate treatment (i.e., ATRA) and to follow therapeutic response [11, 12, 70]. In another interesting report, Rubin et al. [63] described the case of an infant with acute monoblastic leukemia and extramedullary involvement, including skin lesions. After achieving remission, the patient later developed a relapse that was isolated to the skin, suggesting that the skin may have harbored the leukemic cells since the time of diagnosis. The authors reported that this hypothesis was supported by cytogenetic data [63]. Finally, the ability to determine the presence of genomic aberrations in skin samples may be relevant to an understanding of the clinical behavior and prognosis of a number of cutaneous hematopoietic disorders. For instance, Requena [102] investigated the cytogenetic characteristics of skin-infiltrating plasma cells in multiple myeloma, and reported that *RB1* retinoblastoma gene deletion (as determined by FISH) may be a poor prognostic marker and identify those patients at risk for aggressive disease. Another example is cutaneous mastocytosis (CM), a mast cell proliferative disease that usually demonstrates a transient benign clinical course with spontaneous remission in children, but may be associated with persistent or progressive disease (i.e., mast cell leukemia) in adults [104–106]. Activating somatic *c-kit* ASP-816-Val mutations have been found in the majority of cases of adult sporadic CM. In contrast, classic pediatric sporadic CM cases (and familial examples) lack these particular aberrations, but may show other dominant inactivating mutations [104–106]. Importantly, the detection of *c-kit* ASP-816-Val mutations in skin biopsies of children with CM identifies a subset of patients with unusual clinical presentations, who are at risk for extensive or persistent disease [104, 105].

References

1. Swerdlow SH, Campo E, Harris NL, Jaffe ES, et al., editors. World Health Organization classification of tumors of hematopoietic and lymphoid tissues. Lyon, France: IARC Press; 2008.
2. Cronin DM, George TI, Sundram UN. An updated approach to the diagnosis of myeloid leukemia cutis. Am J Clin Pathol. 2009;132:101–10.
3. Cho-Vega JH, Medeiros LJ, Prieto VG, et al. Leukemia cutis. Am J Clin Pathol. 2008;129:130–42.
4. de Arruda Câmara VM, Morais JC, Portugal R, et al. Cutaneous granulocytic sarcoma in myelodysplastic syndrome. J Cutan Pathol. 2008;35:876–9.
5. Hejmadi RK, Thompson D, Shah F, et al. Cutaneous presentation of aleukemic monoblastic leukemia cutis – a case report and review of literature with focus on immunohistochemistry. J Cutan Pathol. 2008;35 Suppl 1:46–9.
6. Su WP. Clinical, histopathologic, and immunohistochemical correlations in leukemia cutis. Semin Dermatol. 1994;13:223–30.
7. Kanegane H, Nomura K, Abe A, et al. Spontaneous regression of aleukemic leukemia cutis harboring a NPM/RARA fusion gene in an infant with cutaneous mastocytosis. Int J Hematol. 2009;89:86–90.
8. Weinel S, Malone J, Jain D, et al. Therapy-related leukaemia cutis: a review. Australas J Dermatol. 2008;49:187–90.

9. Stern M, Halter J, Buser A, et al. Leukemia cutis preceding systemic relapse of acute myeloid leukemia. Int J Hematol. 2008;87:108–9.
10. Deeb G, Baer MR, Gaile DP, et al. Genomic profiling of myeloid sarcoma by array comparative genomic hybridization. Genes Chromosomes Cancer. 2005;44:373–83.
11. Selleri C, Pane F, Notaro R, et al. All-trans-retinoic acid (ATRA) responsive skin relapses of acute promyelocytic leukaemia followed by ATRA-induced pseudotumour cerebri. Br J Haematol. 1996;92:937–40.
12. Liso V, Specchia G, Pogliani EM, et al. Extramedullary involvement in patients with acute promyelocytic leukemia: a report of seven cases. Cancer. 1998;83:1522–8.
13. Weinel S, Malone J, Jain D, et al. Leukaemia cutis in a patient treated for breast cancer. Australas J Dermatol. 2009;50:52–5.
14. Zhang IH, Zane LT, Braun BS, et al. Congenital leukemia cutis with subsequent development of leukemia. J Am Acad Dermatol. 2006;54(2 Suppl):S22–7.
15. Papadavid E, Panayiotides I, Katoulis A, et al. Stasis dermatitis-like leukaemic infiltration in a patient with myelodysplastic syndrome. Clin Exp Dermatol. 2008;33:298–300.
16. Longacre TA, Smoller BR. Leukemia cutis. Analysis of 50 biopsy-proven cases with an emphasis on occurrence in myelodysplastic syndromes. Am J Clin Pathol. 1993;100:276–84.
17. Schneider LA, Weber L, Viardot A, et al. Cutaneous leukaemic infiltrations in a patient with previously undiagnosed myelodysplastic syndrome. Clin Exp Dermatol. 2004;29:468–70.
18. Beswick SJ, Jones EL, Mahendra P, et al. Chloroma (aleukaemic leukaemia cutis) initially diagnosed as cutaneous lymphoma. Clin Exp Dermatol. 2002;27:272–4.
19. Balmer N, Castellino S, Sangueza O, et al. Infantile aleukemic myeloid leukemia cutis with monocytic differentiation and MLL translocation. Am J Dermatopathol. 2009;31:411.
20. Van Zuuren EJ, Wintzen M, Jansen PM, et al. Aleukaemic leukaemia cutis in a patient with acute T-cell lymphoblastic leukaemia. Clin Exp Dermatol. 2003;28:330–2.
21. Chang H, Shih LY, Kuo TT. Primary aleukemic myeloid leukemia cutis treated successfully with combination chemotherapy: report of a case and review of the literature. Ann Hematol. 2003;82:435–9.
22. Hansen RM, Barnett J, Hanson G, et al. Aleukemic leukemia cutis. Arch Dermatol. 1986;122:812–4.
23. Menasce LP, Banerjee SS, Beckett E, et al. Extra-medullary myeloid tumour (granulocytic sarcoma) is often misdiagnosed: a study of 26 cases. Histopathology. 1999;34:391–8.
24. Resnik KS, Brod BB. Leukemia cutis in congenital leukemia. Analysis and review of the world literature with report of an additional case. Arch Dermatol. 1993;129:1301–6.
25. Fender AB, Gust A, Wang N, et al. Congenital leukemia cutis. Pediatr Dermatol. 2008;25:34–7.
26. Akcakus M, Patiroglu T, Deniz K, et al. Congenital acute lymphoblastic leukemia: report of a case with leukemia cutis. Clin Pediatr (Phila). 2004;43:487–90.
27. Torrelo A, Madero L, Mediero IG, et al. Aleukemic congenital leukemia cutis. Pediatr Dermatol. 2004;21:458–61.
28. Solky BA, Yang FC, Xu X, et al. Transient myeloproliferative disorder causing a vesiculopustular eruption in a phenotypically normal neonate. Pediatr Dermatol. 2004;21:551–4.
29. Uhara H, Shiohara M, Baba A, et al. Transient myeloproliferative disorder with vesiculopustular eruption: early smear is useful for quick diagnosis. J Am Acad Dermatol. 2009;60:869–71.
30. Haniffa MA, Wilkins BS, Blasdale C, et al. Cutaneous extramedullary hemopoiesis in chronic myeloproliferative and myelodysplastic disorders. J Am Acad Dermatol. 2006;55(2 Suppl):S28–31.
31. Fernández Aceñero MJ, Borbujo J, Villanueva C, et al. Extramedullary hematopoiesis in an adult. J Am Acad Dermatol. 2003;48(5 Suppl):S62–3.
32. Zebisch A, Cerroni L, Beham-Schmid C, et al. Therapy-related leukemia cutis: case study of an aggressive disorder. Ann Hematol. 2003;82:705–7.
33. Deruelle R, Catteau B, Segard M, et al. Cutaneous granulocytic sarcoma arising at the site of radiotherapy for breast carcinoma. Eur J Dermatol. 2001;11:254–6.
34. Kubonishi I, Seto M, Murata N, et al. Translocation (10;11)(p13;q13) and MLL gene rearrangement in a case of AML (M5a) with aggressive leukemia cutis. Cancer Genet Cytogenet. 1998;104:28–31.
35. Cibull TL, Thomas AB, O'Malley DP, et al. Myeloid leukemia cutis: a histologic and immunohistochemical review. J Cutan Pathol. 2008;35:180–5.
36. Ratnam KV, Su WP, Ziesmer SC, et al. Value of immunohistochemistry in the diagnosis of leukemia cutis: study of 54 cases using paraffin-section markers. J Cutan Pathol. 1992;19:193–200.
37. Yazawa H, Saga K, Omori F, et al. The chilblain-like eruption as a diagnostic clue to the blast crisis of chronic myelocytic leukemia. J Am Acad Dermatol. 2004;50(2 Suppl):S42–4.
38. Kaddu S, Zenahlik P, Beham-Schmid C, et al. Specific cutaneous infiltrates in patients with myelogenous leukemia: a clinicopathologic study of 26 patients with assessment of diagnostic criteria. J Am Acad Dermatol. 1999;40:966–78.

39. Cappel MA, Gibson LE. Myeloid leukemia cutis diagnosed with fluorescent in-situ hybridization: presenting with pathergy and mimicking 'histiocytoid' Sweet's syndrome. Am J Dermatopathol. 2009;31:412.
40. Kitamura H, Kaneko T, Nakano H, et al. Juvenile myelomonocytic leukemia presenting multiple painful erythematous lesions diagnosed as Sweet's syndrome. J Dermatol. 2008;35:368–70.
41. Shafer D, Wu H, Al-Saleem T, et al. Cutaneous precursor B-cell lymphoblastic lymphoma in 2 adult patients: clinicopathologic and molecular cytogenetic studies with a review of the literature. Arch Dermatol. 2008;144:1155–62.
42. Tomasini C, Quaglino P, Novelli M, et al. "Aleukemic" granulomatous leukemia cutis. Am J Dermatopathol. 1998;20:417–21.
43. Kishimoto S, Ishii E, Murakami Y, et al. Cutaneous infiltration by leukemic cells in acute promyelocytic leukemia of a child after treatment with all-trans retinoic acid. Pediatr Hematol Oncol. 1997;14:169–75.
44. Wrede JE, Sundram U, Kohler S, et al. Fluorescence in situ hybridization investigation of cutaneous lesions in acute promyelocytic leukemia. Mod Pathol. 2005;18:1569–76.
45. Sen F, Zhang XX, Prieto VG, et al. Increased incidence of trisomy 8 in acute myeloid leukemia with skin infiltration (leukemia cutis). Diagn Mol Pathol. 2000;9:190–4.
46. Kaiserling E, Horny HP, Geerts ML, et al. Skin involvement in myelogenous leukemia: morphologic and immunophenotypic heterogeneity of skin infiltrates. Mod Pathol. 1994;7:771–9.
47. Yin CC, Medeiros LJ, Glassman AB, et al. t(8;21)(q22;q22) in blast phase of chronic myelogenous leukemia. Am J Clin Pathol. 2004;121:836–42.
48. General Haematology Task Force of the BCSH. The role of cytology, cytochemistry, immunophenotyping and cytogenetic analysis in the diagnosis of haematological neoplasms. Clin Lab Haematol. 1996;18:231–6.
49. Olsen RJ, Chang CC, Herrick JL, et al. Acute leukemia immunohistochemistry: a systematic diagnostic approach. Arch Pathol Lab Med. 2008;132:462–75.
50. Kaune KM, Baumgart M, Schmitke E, et al. Papular exanthem discloses acute myeloid leukaemia: interphase fluorescence in situ hybridization revealed deletion of p53 and gain at 8q22/8q24/Tel8q without trisomy 8. Clin Exp Dermatol. 2010;35:160–4.
51. Wu H, Smith M, Millenson MM, et al. Contribution of flow cytometry in the diagnosis of cutaneous lymphoid lesions. J Invest Dermatol. 2003;121:1522–30.
52. Momoi A, Toba K, Kawai K, et al. Cutaneous lymphoblastic lymphoma of putative plasmacytoid dendritic cell-precursor origin: two cases. Leuk Res. 2002;26:693–8.
53. Maitra A, McKenna RW, Weinberg AG, et al. Precursor B-cell lymphoblastic lymphoma. A study of nine cases lacking blood and bone marrow involvement and review of the literature. Am J Clin Pathol. 2001;115:868–75.
54. Biasotti S, Sementa AR, Zupo S, et al. A novel pediatric case of cutaneous pre-B lymphoblastic lymphoma. Haematologica. 2001;86:997–8.
55. Trupiano JK, Bringelsen K, Hsi ED. Primary cutaneous lymphoblastic lymphoma presenting in an 8-week old infant. J Cutan Pathol. 2002;29:107–12.
56. Lin P, Jones D, Dorfman DM, et al. Precursor B-cell lymphoblastic lymphoma: a predominantly extranodal tumor with low propensity for leukemic involvement. Am J Surg Pathol. 2000;24:1480–90.
57. Hudock J, Chatten J, Miettinen M. Immunohistochemical evaluation of myeloid leukemia infiltrates (granulocytic sarcomas) in formaldehyde-fixed, paraffin-embedded tissue. Am J Clin Pathol. 1994;102:55–60.
58. Murphy M, Hoss D. Cutaneous involvement by CD5-negative chronic lymphocytic leukemia (CLL) – aberrant phenotype or false negativity? Implications for immunohistochemical testing on paraffin-embedded skin specimens. J Cutan Pathol. 2011;38:73–5.
59. Seymour JF, Pierce SA, Kantarjian HM, et al. Investigation of karyotypic, morphologic and clinical features in patients with acute myeloid leukemia blast cells expressing the neural cell adhesion molecule (CD56). Leukemia. 1994;8:823–6.
60. Agis H, Weltermann A, Fonatsch C, et al. A comparative study on demographic, hematological, and cytogenetic findings and prognosis in acute myeloid leukemia with and without leukemia cutis. Ann Hematol. 2002;81:90–5.
61. Dijkman R, van Doorn R, Szuhai K, et al. Gene-expression profiling and array-based CGH classify CD4+CD56+ hematodermic neoplasm and cutaneous myelomonocytic leukemia as distinct disease entities. Blood. 2007;109:1720–7.
62. McGrattan P, Campbell S, Cuthbert R, et al. Integration of conventional cytogenetics, comparative genomic hybridisation and interphase fluorescence in situ hybridisation for the detection of genomic rearrangements in acute leukaemia. J Clin Pathol. 2008;61:903–8.
63. Rubin CM, Arthur DC, Meyers G, et al. Leukemia cutis treated with total skin irradiation. Cancer. 1985;55:2649–52.
64. Lillington DM, Jaju RJ, Shankar AG, et al. Cytogenetic and molecular evidence of marrow involvement in extramedullary acute myeloid leukaemia. Br J Haematol. 2000;110:547–51.

65. Alexiev BA, Wang W, Ning Y, et al. Myeloid sarcomas: a histologic, immunohistochemical, and cytogenetic study. Diagn Pathol. 2007;2:42.
66. Pileri SA, Ascani S, Cox MC, et al. Myeloid sarcoma: clinico-pathologic, phenotypic and cytogenetic analysis of 92 adult patients. Leukemia. 2007;21:340–50.
67. Christopeit M, Mueller LP, Hainz M, et al. Cytogenetics detects infiltrations of a primary cutaneous acute myeloid leukemia to the kidney. Ann Hematol. 2007;86:291–3.
68. Szczepański T, de Vaan GA, Beishuizen A, et al. Acute lymphoblastic leukemia followed by a clonally-unrelated EBV-positive non-Hodgkin lymphoma and a clonally-related myelomonocytic leukemia cutis. Pediatr Blood Cancer. 2004;42:343–9.
69. Ferran M, Gallardo F, Ferrer AM, et al. Acute myeloid dendritic cell leukaemia with specific cutaneous involvement: a diagnostic challenge. Br J Dermatol. 2008;158:1129–33.
70. Lederman CA, Weisberger J, Seiter K, et al. Differentiation of extramedullary acute promyelocytic leukemia by all-trans-retinoic acid. Leuk Lymphoma. 1995;18:189–93.
71. Ueda K, Kume A, Furukawa Y, et al. Cutaneous infiltration in acute promyelocytic leukemia. J Am Acad Dermatol. 1997;36:104–6.
72. Itoh K, Gotoh W, Yagasaki F, et al. Acute promyelocytic leukemia relapse as leukemia cutis shortly after complete remission with all-trans retinoic acid. Rinsho Ketsueki. 1998;39:221–6.
73. Lee KW, Yi J, Yun T, et al. Extramedullary relapse confirmed by fluorescence in situ hybridization study of an ear mass in acute promyelocytic leukemia. Int J Hematol. 2004;79:462–4.
74. Ohyashiki K, Oshimura M, Uchida H, et al. Characterization of extramedullary tumors in a case of Ph-positive chronic myelogenous leukemia: possible involvement of immature T lymphocytes. Cancer Genet Cytogenet. 1985;15:119–27.
75. Madhumathi DS, Raman RN, Lakshmi DV, et al. Skin lesions in malignancy. Case 1. Chronic myeloid leukemia in lymphoid blast crisis presenting as multiple cutaneous masses. J Clin Oncol. 2001;19:2098–9.
76. McCollum A, Bigelow CL, Elkins SL, et al. Unusual skin lesions in chronic myelomonocytic leukemia. South Med J. 2003;96:681–4.
77. Millot F, Robert A, Bertrand Y, et al. Cutaneous involvement in children with acute lymphoblastic leukemia or lymphoblastic lymphoma. The Children's Leukemia Cooperative Group of the European Organization of Research and Treatment of Cancer (EORTC). Pediatrics. 1997;100:60–4.
78. Chimenti S, Fink-Puches R, Peris K, et al. Cutaneous involvement in lymphoblastic lymphoma. J Cutan Pathol. 1999;26:379–85.
79. Bekkenk MW, Jansen PM, Meijer CJ, et al. CD56+ hematological neoplasms presenting in the skin: a retrospective analysis of 23 new cases and 130 cases from the literature. Ann Oncol. 2004;15:1097–108.
80. Hallermann C, Middel P, Griesinger F, et al. CD4+ CD56+ blastic tumor of the skin: cytogenetic observations and further evidence of an origin from plasmocytoid dendritic cells. Eur J Dermatol. 2004;14:317–22.
81. Reichard KK, Burks EJ, Foucar MK, et al. CD4(+) CD56(+) lineage-negative malignancies are rare tumors of plasmacytoid dendritic cells. Am J Surg Pathol. 2005;29:1274–83.
82. Ng AP, Lade S, Rutherford T, et al. Primary cutaneous CD4+/CD56+ hematodermic neoplasm (blastic NK-cell lymphoma): a report of five cases. Haematologica. 2006;91:143–4.
83. Martín JM, Nicolau MJ, Galán A, et al. CD4+/CD56+ haematodermic neoplasm: a precursor haematological neoplasm that frequently first presents in the skin. J Eur Acad Dermatol Venereol. 2006;20:1129–32.
84. Hu SC, Tsai KB, Chen GS, et al. Infantile CD4+/CD56+ hematodermic neoplasm. Haematologica. 2007;92:e91–3.
85. Pilichowska ME, Fleming MD, Pinkus JL, et al. CD4+/CD56+ hematodermic neoplasm ("blastic natural killer cell lymphoma"): neoplastic cells express the immature dendritic cell marker BDCA-2 and produce interferon. Am J Clin Pathol. 2007;128:445–53.
86. Ascani S, Massone C, Ferrara G, et al. CD4-negative variant of CD4+/CD56+ hematodermic neoplasm: description of three cases. J Cutan Pathol. 2008;35:911–5.
87. Alencar AJ, Bustinza E, Barker J, et al. Hematodermic tumor presenting with generalized skin involvement. J Clin Oncol. 2009;27:3059–61.
88. Kaune KM, Baumgart M, Bertsch HP, et al. Solitary cutaneous nodule of blastic plasmacytoid dendritic cell neoplasm progressing to overt leukemia cutis after chemotherapy: immunohistology and FISH analysis confirmed the diagnosis. Am J Dermatopathol. 2009;31:695–701.
89. Ji H, Wang L, Li D, et al. CD4/CD56 hematodermic neoplasm: a report of three cases. Int J Dermatol. 2009;48:743–8.
90. Wang P, Wang Z, Kong Y, et al. Primary cutaneous CD4+/CD56+ hematodermic neoplasm (blastic NK-cell lymphoma): a case report. Int J Dermatol. 2009;48:62–5.
91. Magro CM, Porcu P, Schaefer J, et al. Cutaneous CD4+ CD56+ hematologic malignancies. J Am Acad Dermatol. 2010;63:292–308.

92. Cerroni L, Höfler G, Bäck B, et al. Specific cutaneous infiltrates of B-cell chronic lymphocytic leukemia (B-CLL) at sites typical for Borrelia burgdorferi infection. J Cutan Pathol. 2002;29:142–7.
93. Ratnavel RC, Dunn-Walters DK, Boursier L, et al. B-cell lymphoma associated with chronic lymphatic leukaemia: two cases with contrasting aggressive and indolent behaviour. Br J Dermatol. 1999;140:708–14.
94. Plaza JA, Comfere NI, Gibson LE, et al. Unusual cutaneous manifestations of B-cell chronic lymphocytic leukemia. J Am Acad Dermatol. 2009;60:772–80.
95. Kaddu S, Smolle J, Cerroni L, et al. Prognostic evaluation of specific cutaneous infiltrates in B-chronic lymphocytic leukemia. J Cutan Pathol. 1996;23:487–94.
96. Magro CM, Morrison CD, Heerema N, et al. T-cell prolymphocytic leukemia: an aggressive T cell malignancy with frequent cutaneous tropism. J Am Acad Dermatol. 2006;55:467–77.
97. Valbuena JR, Herling M, Admirand JH, et al. T-cell prolymphocytic leukemia involving extramedullary sites. Am J Clin Pathol. 2005;123:456–64.
98. Herling M, Valbuena JR, Jones D, et al. Skin involvement in T-cell prolymphocytic leukemia. J Am Acad Dermatol. 2007;57:533–4.
99. Kato N, Tamura A, Yamanaka Y, et al. CD3+ TCRgammadelta+CD4+ CD8− T-cell large granular lymphocyte leukaemia showing skin infiltrations. Br J Dermatol. 2004;150:382–4.
100. van Steensel MA, van Gelder M, van Marion AM, et al. T-cell large granular lymphocytic leukaemia with an uncommon clinical and immunological phenotype. Acta Derm Venereol. 2009;89:172–4.
101. Nagasawa T, Nakatsuka S, Miwa H, et al. Analysis of T-cell antigen receptor gamma chain gene rearrangement by polymerase chain reaction in combination with denaturing gradient gel electrophoresis in the differential diagnosis of cutaneous T-lymphoproliferative diseases. J Dermatol. 2000;27:238–43.
102. Requena L. Specific cutaneous involvement in patients with multiple myeloma. A clinicopathological, immunohistochemical and cytogenetic study of 40 cases. Actas Dermosifiliogr. 2005;96:424–40.
103. Murphy M, Kristjansson A, Elaba Z, et al. Monoclonal gammopathy of undetermined significance and incidentally identified cutaneous monotypic plasma cell infiltrates in the absence of specific skin lesions. Arch Dermatol. 2010;146:204–6.
104. Verzijl A, Heide R, Oranje AP, et al. C-kit Asp-816-Val mutation analysis in patients with mastocytosis. Dermatology. 2007;214:15–20.
105. Longley Jr BJ, Metcalfe DD, Tharp M, et al. Activating and dominant inactivating c-KIT catalytic domain mutations in distinct clinical forms of human mastocytosis. Proc Natl Acad Sci USA. 1999;96:1609–14.
106. Büttner C, Henz BM, Welker P, et al. Identification of activating c-kit mutations in adult-, but not in childhood-onset indolent mastocytosis: a possible explanation for divergent clinical behavior. J Invest Dermatol. 1998;111:1227–31.
107. Adriaansen HJ, Soeting PW, Wolvers-Tettero IL, et al. Immunoglobulin and T-cell receptor gene rearrangements in acute non-lymphocytic leukemias. Analysis of 54 cases and a review of the literature. Leukemia. 1991;5:744–51.
108. Onozawa M, Hashino S, Kanamori H, et al. Aleukemic leukemia cutis in a patient with Philadelphia chromosome-positive biphenotypic leukemia. Int J Hematol. 2004;80:278–80.
109. Requena L, Kutzner H, Palmedo G, et al. Histiocytoid Sweet syndrome: a dermal infiltration of immature neutrophilic granulocytes. Arch Dermatol. 2005;141:834–42.

Chapter 14
Inflammatory Disorders of the Skin

Michael J. Murphy, Amanda Phelps, and Markus Braun-Falco

Inflammatory disorders of the skin, including eczematous, psoriasiform, lichenoid-interface, autoimmune, and neutrophilic dermatoses, probably represent the group of cutaneous diseases in which molecular pathology currently has the least impact in daily clinical practice. Many of these diseases are readily diagnosed through the correlation of clinical features with histopathological findings on hematoxylin and eosin (H + E)-stained tissue sections. In general, microscopic pattern analysis offers a very useful and reliable method to diagnose inflammatory skin diseases. The application of additional histochemical stains, immunohistochemistry, and/or immunofluorescence analysis is occasionally required. However, in some instances, diagnostic difficulties do arise. For example, the clinical and/or microscopic distinction of allergic contact dermatitis (ACD) from irritant contact dermatitis (ICD), pompholyx (dyshidrotic eczema) from pustular psoriasis, and even classic chronic psoriasis from chronic atopic dermatitis (AD) may be challenging. Although chronic psoriasis and AD show distinct differences with respect to cytokine milieu (i.e., Th1 in AD *vs.* Th2 in psoriasis), bacterial superinfection, surface pH, transepidermal water loss and itch, it is well known that these disorders share many morphological and molecular features [1, 2]. For example, from a dermatopathologist's perspective, the lesional skin of both conditions can demonstrate the presence of T-cell and CD1a+/CD11c+ dendritic cell infiltrates associated with hyperplasia/altered differentiation of keratinocytes [1, 2]. In addition, cutaneous T-cell dyscrasias (i.e., lymphomas) can occasionally masquerade, both clinically and histopathologically, as inflammatory dermatoses (i.e., cutaneous lupus erythematosus) [3–5].

These issues highlight the need to develop novel objective strategies to increase diagnostic accuracy through the use of molecular technologies. Today, the focus of inflammatory skin disease research is largely directed toward the investigation of pathogenic pathways and therapeutic targets [6, 7]. While progress has been made, the genetic/epigenetic mechanisms underlying all cellular and molecular changes in inflammatory dermatoses remain to be fully characterized. Except for lymphocyte clonality assays, the routine clinical use of molecular technologies in the diagnosis and management of these conditions is largely unexplored. The concepts and pitfalls of molecular diagnostic testing for lymphocyte clonality in cutaneous T-cell infiltrates, including both neoplastic and inflammatory skin disorders, are discussed in Chap. 10. In the appropriate setting, the detection of monoclonal T-cell expansion in the skin is used as supportive evidence of cutaneous T-cell lymphoma. However, molecular analysis of T-cell receptor gene rearrangements (TCR-GRs) have also demonstrated monoclonal and/or restricted oligoclonal T-cell profiles in a wide range of

A. Phelps (✉)
Central Connecticut State University,
New Britain, CT 06001, USA
e-mail: mnder410@comcast.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_14,

inflammatory skin conditions, including eczematous dermatoses, psoriasis, drug reactions, lichenoid eruptions (lichen planus), and autoimmune disorders (cutaneous lupus erythematosus, scleroderma/systemic sclerosis). Therefore, the integration of clinical, histopathological, and immunophenotypic features is necessary for an accurate diagnosis of cutaneous lesions with evidence of T-cell monoclonality.

Recent studies on inflammatory dermatoses have employed gene expression profiling strategies, which allow the simultaneous analysis of the transcriptional activity of hundreds-to-thousands of genes at the mRNA level [1]. Both similarities and differences in the expression of numerous genes, including those involved in innate/adaptive immunity and skin barrier function, have been found in lesional/nonlesional skin of a variety of cutaneous inflammatory disorders, including AD and psoriasis [1]. Interestingly, a number of genetic and epigenetic mechanisms may be commonly altered in diverse inflammatory conditions, suggesting a general role in cutaneous inflammation (i.e., S100 family and miR-21/miR-125b in both AD and psoriasis) [8–10]. For example, Wenzel et al. [10] identified 16 common genes, including S100 proteins (S100A2, S100A7, S100A9), keratins (KRT5, KRT6A, KRT15, KRT17), FADS2, JUP, and CFL1, that are differentially expressed in lesional skin of AD, lichen planus, and psoriasis, compared with skin biopsies of healthy controls. Similarly, Kamsteeg et al. [1] noted that the expression of KRT6 and IL-18 does not significantly discriminate between AD, ACD, ICD, and psoriasis. However, other dysregulated mechanisms may be specific to a particular inflammatory dermatosis (i.e., NELL2 in AD *vs.* miR-146a/miR-203 in psoriasis *vs.* CXCL9 in lichen planus) [9–12]. As a consequence, genetic/epigenetic alterations and protein expression among inflammatory dermatoses could determine both similarities and differences in the clinical phenotypes of these conditions. The question that arises is whether results of molecular studies have any direct clinical significance in these inflammatory conditions vis-à-vis diagnosis, activity monitoring, identification of novel therapeutic targets, and/or prevention. Interestingly, the differential expression of a number of genes has been shown to correlate significantly with disease activity scores and/or response to therapy in patients with psoriasis [13–21]. As will be outlined in this chapter, molecular technologies, such as cDNA/oligonucleotide microarrays and polymerase chain reaction (PCR)-based analyses, can facilitate the differentiation of a number of inflammatory dermatoses. Thus, in principal, the diagnostic use of gene expression profiling in this setting is clearly possible. In fact, one group of investigators has already designed a skin-oriented microarray on the basis of their results [10]. However, as stated by Wenzel et al. [10], "its use is clearly limited by the quality of gene expression data available for all skin disorders, by the cost of this technique, and by the more complicated handling of skin biopsies to obtain mRNA of sufficient quality." The use of more widely available technologies, such as in situ hybridization (ISH) and immunohistochemistry (IHC), to apply data from investigative studies may be a more feasible approach. This would allow for rapid and inexpensive investigation of mRNA and/or encoded protein expression in skin samples, and facilitate the correlation of genomic/proteomic changes with the tissue morphological features of a particular disease. Other advantages of these techniques include technical ease, low cost, and rapid turn around.

Eczematous/Spongiotic Dermatoses

Atopic Dermatitis

Atopic dermatitis (AD) is a very common skin disorder, affecting 10–20% of children and 1–3% of adults in industrialized countries [22, 23]. Together with allergic rhinoconjunctivitis and allergic asthma, it is regarded as a manifestation of atopy (defined as a genetic predisposition toward the development of immediate hypersensitivity reactions against common environmental antigens).

Patients with AD suffer from dry, itchy, and inflamed skin with predilection for flexures and folds, but commonly with extension to the trunk or even whole body involvement. The skin of affected individuals is predisposed to bacterial superinfection. The diagnosis is usually relatively straightforward, based primarily upon clinical features, but supplemented in some cases by histopathological review of skin tissue samples in order to confirm the microscopic hallmark of epidermal spongiosis (intercellular edema).

The pathophysiology of AD is highly complex and still not completely understood, but involves both genetic and environmental factors which contribute to disease susceptibility, pathogenesis, and severity. The importance of genetic influences is evident from twin and family studies. Over the last decade, genome-wide screens and gene association studies have identified numerous chromosomal regions and genes that are linked to AD susceptibility [22]. Candidate AD susceptibility genes belong to several biologically and functionally distinct groups, including cytokines, chemokines, antigen-presenting molecules, pattern-recognition receptors, drug-metabolizing enzymes, and epidermal differentiation complex molecules, underscoring the complexity of AD pathophysiology [22]. Of note, the current focus of AD research includes not only immunological abnormalities, but also epidermal barrier dysfunction [such as filaggrin (FLG) and Kazal type 5 gene mutations]. The latter changes also underpin the development of a number of genodermatoses, including ichthyosis vulgaris and Netherton syndrome (see Chap. 19) [23].

A number of gene expression profiling studies have been performed on patients with AD, typically comparing AD lesional skin with AD nonlesional skin or normal skin of healthy volunteers [1, 8, 11, 24]. Hierarchical clustering across samples is purported to provide a clearer picture of which genes are of greater relevance to AD pathophysiology. Noteworthy is the fact that many of the genes found to be differentially expressed in AD skin in these studies are localized to previously described AD susceptibility chromosomal regions [1, 8, 11, 24]. Genes with differential expression in AD are summarized in Table 14.1. Using oligonucleotide microarrays, Sugiura et al. [8] reported abnormal epidermal differentiation and defective defense mechanisms in AD skin, with overexpression of S100A7/S100A8 and alternative pathway epidermal keratin genes (KRT6A, KRT6B, KRT16), and downregulation of cornified envelope genes [FLG and loricrin (LOR)]. In the study by Sääf et al. [24], cDNA microarray analysis identified ~4,000 genes out of 24,500 tested that were differentially expressed in AD skin. A reciprocal expression pattern of upregulated inflammatory genes and downregulated lipid metabolism genes was found in the skin of AD patients [24]. Up regulated inflammatory genes included those encoding a suppressor of cytokine signaling (SOCS3), chemokines (CCL18, CXCL1), interleukin receptors (IL-2Rγ, IL-4R), cell surface antigens (CD5, CD6, CD28), complement pathway components and regulators (C1R, C1S, SERPING1), IgE receptor gamma subunit (FCER1G), T-cell surface glycoproteins (THY1), and eicosanoid lipid messengers and processing enzymes (PTGES, PTGIS, PTGER3) [24]. These factors are involved in leukocyte recruitment, cellular activation signaling, inflammatory mediator release, promotion of Th2 inflammatory responses, regulation of IgE synthesis, and other still incompletely understood pathways. Genes with reduced expression in AD are associated with cholesterol, fatty acid, and arachidonic acid metabolism [24]. Sääf et al. [24] also noted that genes involved in cornified envelope formation are differentially expressed in AD skin, supporting the hypothesis that skin barrier dysfunction is critically involved in the pathogenesis of AD. For instance, AD skin shows upregulation of two members of the transglutaminase family (TGM1 and TGM3), in addition to kallikrein 7 (KLK7), also called stratum corneum chymotryptic enzyme (SCCE) [24]. Transglutaminases are required for epidermal protein cross-linking during normal stratum corneum formation. KLK7 has catalytic activity, capable of degrading desmosomes in the stratum corneum, and thereby promoting normal desquamation [22]. One of its known inhibitors, serine protease inhibitor Kazal type 5 (SPINK5), is the product of a putative susceptibility gene for AD [23].

Epigenetics describes inherited variations in gene expression that occur without a change in the DNA sequence, and include DNA methylation, histone modification, and microRNAs (miRNAs)

Table 14.1 Gene expression profiles in atopic dermatitis

Biological clusters	Differentially expressed genes
Genes with increased expression	
Interleukins	IL-4, IL-5, IL-6, IL-10, IL-13
Interleukin receptor family	IL-2R (γ), IL-4R, IL-10RA, ST2
Chemokine family	CCL5 (RANTES), CCL18, CCL21, CXCL1, SOCS3
Surface antigens	CD5, CD6, CD28, CD37, CD53, CD86
Complement factors	C1R, C1S, C1QB, SERPING1, CFH
IgE receptor family	Receptor gamma subunit FCER1G, TRAα, THY1
Epithelial host defense proteins	β-Defensin 2, elafin
Epidermal barrier function	Involucrin, S100A2, S100A7, S100A8, S100A9, transglutaminase 1 (TGM1), TGM3, calmodulin-like skin protein CALML5 (CLSP), kallikrein 7 (KLK7/SCCE), serine protease inhibitor B-13, chondroitin sulphate proteoglycan 2, small proline-rich protein 1A/B, 2B/C, trichohyalin
Epidermal differentiation-associated keratins	KRT6A/B, KRT16, KRT17
Eicosanoid lipid messengers and processing enzymes	Prostaglandin E synthase (PTGES), prostaglandin I2 Synthase (PTGIS), prostaglandin E receptor 3 (PTGER3)
Genes with decreased expression	
Synthesis and uptake of cholesterol and fatty acids	ATP-citrate lyase (ACL1), acyl-CoA synthetase (ACSL1, ACSL3), HMG-CoA synthase (HMGCS1, HMGCS2), HMG-CoA reductase (HMGCR)
Polyunsaturated fatty acid pathway	FADS1, FADS2, ELOVL5, acyl-transferase (AGPAT3)
Arachidonic acid metabolism	ALOX5AP, ALOX12, ALOX15B
Epidermal barrier function	Filaggrin, loricrin, desmoglein 2 (DSG2), fibronectin 1, small proline-rich protein 3
Epidermal differentiation-associated keratins	KRT1, KRT2, KRT5, KRT10, KRT14
Transcription factors	Lipin-1, SREBPF1

[9, 12, 25–27]. It is widely believed that epigenetic mechanisms, in addition to the genetic mutations previously outlined, may contribute to the development and progression of a number of skin diseases, including AD [27]. For instance, patients with AD show reduced DNA methyltransferase 1 (DNMT1) expression compared with normal controls [27]. It is hypothesized that hypomethylation, as a result of reduced DNMT1, may induce IgE production via Th2-related cytokine mechanisms in these patients. MiRNAs are small endogenous noncoding RNA molecules (~22–25 nucleotides in length) that bind to target mRNAs, and regulate translation through mRNA repression or degradation [9, 12, 25, 26]. It has recently been demonstrated that miRNAs play a role in normal skin and hair follicle development, cutaneous wound healing, and a wide variety of skin cancers (i.e., melanoma and Kaposi's sarcoma), in addition to inflammatory conditions (i.e., psoriasis and AD) [9, 12, 25, 26]. It is postulated that miRNAs contribute to the pathogenesis of inflammatory skin disorders through regulation of protein expression and cellular functions in both infiltrating immune cells and epidermal keratinocytes [9, 12, 25, 26]. AD is associated with a number of deregulated miRNAs, including upregulation of miR-21 and downregulation of miR-122 and miR-125b (the latter putatively contributing to elevated TNF-α production) [9, 12]. Interestingly, some regions of interest are found to overlap with chromosomal loci previously found to be associated with AD [9, 12]. The ability to profile miRNA levels in different cutaneous inflammatory disorders, such as AD, could potentially lead to the development of tools to evaluate specific miRNAs that are associated with disease progression and prognosis. Importantly, unlike genetic mutations, epigenetic alterations are potentially reversible [27]. Therefore, future research will likely investigate the possibility of employing epigenetic changes not only as novel biomarkers for early diagnosis, disease

progression, and treatment monitoring, but also for the development of targeted epigenetic-modifying drugs (i.e., miRNA-directed therapies), for skin diseases, such as AD [9, 12, 25–27].

Despite increasing knowledge of the roles of genetic and epigenetic changes in the pathophysiology of AD, the question remains: Can this information help in the clinical setting, by providing an objective laboratory-based differentiation of AD from other spongiotic dermatoses, such as ACD and ICD, and/or from non-eczematous inflammatory conditions, such as psoriasis? It is important to consider that these disorders share important cellular and molecular characteristics. All conditions show defective barrier function, in addition to upregulation of many cytokines, chemokines, and inflammatory molecules; although, the immune-response phenotype (i.e., Th1 *vs.* Th2) varies [2, 28]. In general, microscopic review is not sufficiently specific to separate AD from ACD or ICD, as these conditions can all show spongiotic changes (i.e., epidermal intercellular edema), variable epidermal hyperplasia, and an intradermal mixed inflammatory infiltrate of T-cells, histiocytes, and/or eosinophils. However, in a study by Kamsteeg et al. [1], real-time PCR-based expression profiling of 15 genes in AD, ACD, ICD, and psoriasis samples identified several differentially regulated transcripts, which may be possible candidates for disease-specific pathogenic changes. This study also demonstrated that molecular testing could potentially be used to distinguish between these inflammatory skin diseases in the clinical setting (Fig. 14.1). A similar expression pattern for some

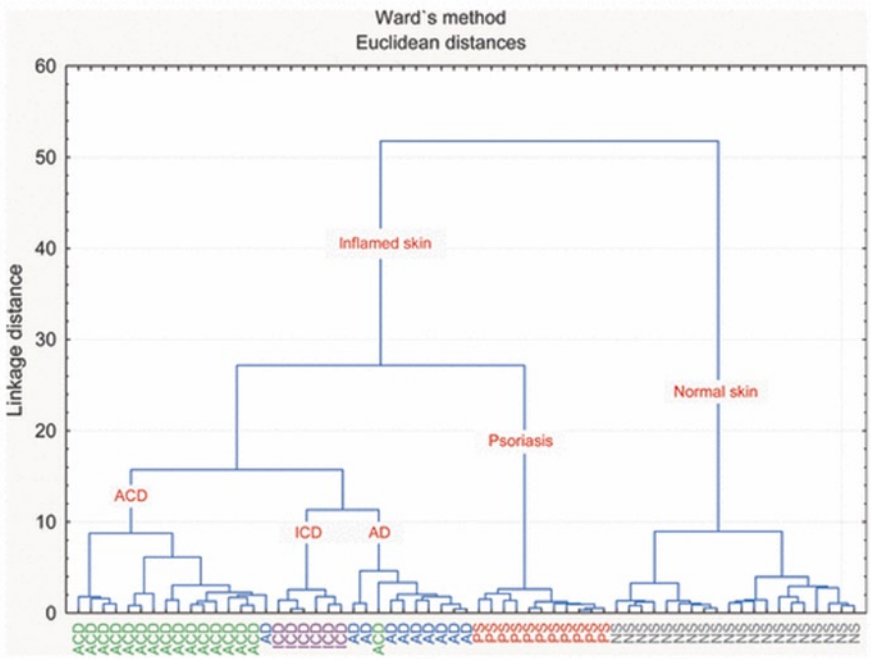

Fig. 14.1 *Molecular discrimination of allergic contact dermatitis, irritant contact dermatitis, atopic dermatitis, and psoriasis*. Real-time quantitative polymerase chain reaction data from skin biopsy samples were analyzed using a one-way clustering approach to assess the similarity between the inflammatory conditions based on the ΔC_t values from CAII, NELL2, hBD-2, IL-1F9, CXCL8, CXCL10, and CCL17. A good separation of diagnosis groups is obtained. Normal skin (*NS*) samples are distinct from all biopsies of inflamed skin. Secondly, psoriasis (*PS*) could be discriminated from the different forms of eczema [atopic dermatitis (*AD*), allergic contact dermatitis (*ACD*), and irritant contact dermatitis (*ICD*)] which are clustered under a separate node. Within the eczematous skin conditions, three distinct clusters emerge for AD, ACD, and ICD. Only two samples (one AD and one ACD) were classified in other clusters. ΔC_t is the difference between the threshold cycle of the target gene and the reference gene (From Kamsteeg et al. [1]. Reprinted with permission from Wiley, Copyright © 2009)

genes, but not all genes, was noted among the eczematous disorders (AD, ACD, and ICD), possibly reflecting their similar, although not identical, cytokine milieu [1]. Of note, psoriasis was found to be molecularly distinct from the other inflammatory conditions [1]. For instance, carbonic anhydrase II (CAII) is found to be highly induced in AD and other eczematous dermatoses, but not in psoriasis [1, 11, 29]. CAII is involved in the regulation of epidermal water transport and intracellular pH maintenance [29]. In contrast, interleukin-1F9 (IL-1F9), elafin, β-defensin-2 (hBD-2), SPRR2C, and vanin-3 are specifically induced in psoriatic lesions (Fig. 14.2) [1, 8, 11]. Neuron-specific Nel-like protein 2 (NELL2) shows strong induction in AD, but not in other eczematous dermatoses. In contrast, the chemokines CCL17 and CXCL10 are predominantly expressed in ACD. CXCL8 and IL-1β are highly expressed in psoriasis, AD, and ACD, but not in ICD [1]. Importantly, ICD does not reveal any specifically regulated marker gene [1]. Of note, the low expression levels of antimicrobial proteins in AD (elafin, hBD-2) may be associated with the relatively high prevalence of cutaneous infections in this condition [1, 8]. Kamsteeg et al. [1] reported that a molecular distinction between normal skin, psoriasis, and eczematous dermatoses (AD, ACD, and ICD) can be made using a set of only seven genes (CAII, NELL2, hBD-2, IL-1F9, CXCL8, CXCL10, and CCL17) (Figs. 14.1 and 14.2). Interestingly, a distinction between ACD and ICD could also be made with this 7-gene set [1]. In another study by Nomura et al. [11], which compared gene expression profiles of AD and psoriasis by oligonucleotide microarray analysis and real-time PCR, a total of 18 genes were found to be significantly upregulated in AD lesions. These included the aforementioned genes NELL2 (with the highest fold increase of 7.6) and CAII, in addition to tenascin C (extracellular matrix molecule) and the chemokines CCL18/PARC, CCL27/CTACK, and CCL13/MCP-4 [11]. While both AD and psoriasis lesional skin demonstrate epidermal hyperplasia and regenerative

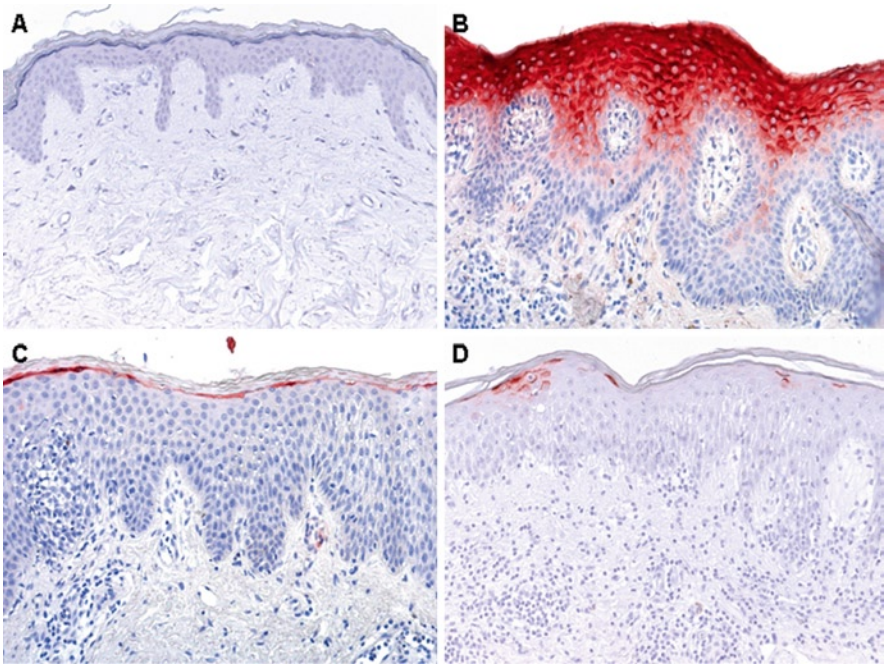

Fig. 14.2 *Immunohistochemical discrimination of normal skin, psoriasis, atopic dermatitis, and allergic contact dermatitis*. Human beta defensin-2 (*hBD-2*) staining by immunohistochemistry in (**a**) normal skin, (**b**) psoriasis, (**c**) atopic dermatitis, and (**d**) allergic contact dermatitis. Together with other markers, hBD-2 distinguishes between psoriasis and eczematous dermatoses (Courtesy of Dr. Joost Schalkwijk, Department of Dermatology and Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands)

epidermal growth, the keratinocyte terminal differentiation program is known to be accelerated in psoriasis and defective in AD [2, 28]. Accordingly, Guttman-Yassky et al. [28] have demonstrated that psoriasis and AD can be distinguished on the basis of terminal differentiation gene expression, with AD showing decreased expression of FLG, LOR, SPRR2C, CDSN, and IVL compared with psoriasis. In another study, this group also confirmed that AD and psoriasis could be differentiated on the basis of Th1 and Th2 gene expression profiling [2]. As expected, AD is characterized by a Th2 pattern (i.e., IL-5, IL-10, IL-13, CCL17, CCL22, TSLPR, CCL5/RANTES, and MMP-12). In contrast, psoriasis demonstrates a Th1 profile [i.e., IL-8, IL-1β, NOS2A, CCL20, and interferon (IFN)-γ] [2]. Finally, Wenzel et al. [10] noted that CCL17 and CCL27 are differentially expressed in AD, when compared with psoriasis and lichen planus.

Gene expression profiling may also have a role in the distinction of inflammatory dermatoses from cutaneous lymphomas. In this regard, Tracey et al. [30] employed a cDNA microarray-based approach to identify 27 transcripts that were differentially expressed between mycosis fungoides (MF) and a range of inflammatory dermatoses, including spongiotic (i.e., eczematous) conditions. These genes included tumor necrosis factor receptor (TNFR)-dependent apoptosis regulators, STAT4, CD40L, other oncogenes and apoptosis inhibitors. A 6-gene (FJX1, STAT4, SYNE1, TRAF1, BIRC3, and Hs.127160) prediction model, capable of differentiating MF from inflammatory conditions, was constructed [30]. This model correctly assigned 97% of cases in a blind test validation, using 24 MF patients with low clinical stages [30].

While miR-21, miR-122a, and miR-125b show similar alterations in both AD and psoriasis, genome-wide analysis has identified a number of miRNAs that are differentially regulated in these conditions (i.e., disease-specific), potentially providing targets for molecular diagnostic tools. For example, miR-203 and miR-146a are found to be upregulated in psoriasis, but do not appear to be altered in AD [9, 12, 26].

Many of these differentially expressed genes have been identified through the use of cDNA/oligonucleotide microarray and/or quantitative PCR technologies. For clinical applications, the goal is to use these findings to develop more user-friendly molecular diagnostic strategies and/or expand the utility of more readily available technologies. Through the use of IHC and flow cytometric analysis, the expression patterns of encoded proteins for these putative disease-specific genes have been evaluated [1]. Importantly, a good correlation between gene transcript and protein levels of CAII is seen in eczematous dermatoses (upregulated) and psoriasis (downregulated) [1, 29]. As predicted by gene expression profiling studies, upregulation of TGase1 (product of TMG1), SOCS3, TSLPR, CCL17, CCL18, and CCL22 proteins is detectable in lesional AD skin by IHC [2, 24]. In contrast, AD skin shows reduced expression and abnormal formation of terminal differentiation proteins (FLG, LOR, CDSN, and IVL) [28]. IHC confirms high levels of hBD-2 protein in lesional skin of psoriasis, with minimal or absent staining in lesional skin of eczematous dermatoses (AD, ACD, and ICD) (Fig. 14.2) [1, 31]. However, elafin protein is strongly expressed by both psoriasis and ICD (within the stratum granulosum), with variable staining in AD and ACD; despite gene expression analysis identifying it as a relatively specific marker of psoriasis [1, 31]. Therefore, as is widely known, the mRNA level of a particular gene may not necessarily correlate with the expression pattern of its encoded protein [32]. Interestingly, both mRNA and protein levels of Ki-67 (a marker of cellular proliferation) and KRT16 (a keratin commonly induced in epidermal hyperproliferative states) are found to be poor discriminators for all these inflammatory disorders (psoriasis, AD, ACD, and ICD) [1, 28, 31].

Allergic Contact Dermatitis

Allergic contact dermatitis (ACD) is a manifestation of specific delayed (type IV) hypersensitivity, T-cell-mediated immune reactions to exogenous chemicals and physical agents. In the sensitization

phase, dendritic Langerhans cells present antigens to naive T-cells, which became specifically sensitized and memorable for these antigens. The elicitation phase takes place after antigen re-exposure, when previously sensitized T-cells become activated and induce an inflammatory response, clinically represented by an acute dermatitis or a more chronic eczematous reaction. Based on gene expression profiling, the induction of two genes, CCL17 and CXCL10, appears to be relatively specific for ACD, contributing to the combined Th1 and Th2 cytokine/chemokine profile seen in some cases [1]. In a study of monocyte-derived dendritic cells (Mo-DCs), treatment with sensitizers (contact allergens, nickel, and Brandrowski's base) resulted in the upregulation of 21 genes, including IL-8, CCL17, CCR7, CCL22, CD86, CXCR4, and PPIA [33].

Irritant Contact Dermatitis

Irritant contact dermatitis (ICD) is induced by contact with irritating chemicals, resulting in a non-allergic inflammatory reaction. In contrast to ACD, the intensity of the reaction is proportional to the dose of the chemical applied. From gene expression studies of lesional skin, it appears that overall transcript induction in ICD is relatively low compared with other inflammatory skin diseases [1]. In addition, Mo-DCs treated with an irritant (SDS) do not show significant gene upregulation (i.e., <1.5-fold induction) [33]. To date, no differentially expressed genes specifically related to ICD have been found.

Psoriasis

Psoriasis is a chronic inflammatory and hyperproliferative skin disease that affects ~2% of Western populations. It is typically characterized by sharply demarcated erythematous scaly plaques that are predominantly localized to the extensor surfaces of the elbows and knees, scalp, and buttocks [34, 35]. However, the clinical picture can be diverse, and patients may demonstrate nail changes, pustular eruptions, and/or erythroderma. Up to 40% of patients have psoriatic arthritis, which can be severe and deforming in ~5% of cases [34, 35]. Psoriasis is a multifactorial disease with a complex pathogenesis, involving polygenic predisposition and environmental triggering factors, such as infection, trauma, and drugs [34, 35]. A genetic basis for this disease is supported by the fact that a concordance rate of 63–73% in seen in monozygotic twins, as compared to 17–20% for dizygotic twins [35, 36]. Several disease susceptibility loci, PSORS1–PSORS9, have been proposed as predisposing factors from the results of genome-wide linkage scans [35, 36]. A recent study by Elder et al. [35] reported an association between psoriasis and seven genetic loci – HLA-C, IL-12B, IL-23R, IL-23A, IL-4/IL-13, TNFAIP3, and TNIP1.

On light microscopy, classic psoriasis plaques demonstrate parakeratosis containing neutrophils, epidermal hyperplasia, increased lesional CD8+ T-cells and myeloid/plasmacytoid dendritic cells, and ectatic blood vessels in the papillary dermis. Lesional keratinocytes are hyperproliferative and show altered expression of early (↑ IVL, ↑ SPRRs) and late (↓ FLG, ↓ LOR, ↓ CASP14) differentiation markers, leading to impairment of skin barrier function. In addition, there is suppression of signaling pathways associated with keratinocyte differentiation (↓ Notch, ↓ PPAR-α); induction of proliferation-associated pathways (↑ c-Myc, ↑ β-integrin, ↑ Wnt5a); activation of signaling pathways involving STAT3, IFN, and MAPK; and dysregulation of both innate and adaptive immunity [34]. These changes lead to a regenerative epidermal phenotype (similar to the normal skin-wound healing response) that is accompanied by increased production of adhesion molecules (ICAM-1, VCAM-1),

antimicrobial peptides (elafin, S100A7, β-defensin 2, and LL-37), and proinflammatory cytokines (TNF-α, IL-1, IL-22, IL-23, iNOS) [34]. Numerous studies have been undertaken in an effort to determine the molecular pathophysiology of psoriasis, but the specific mechanism(s) by which cutaneous inflammation and keratinocytic changes interact to induce the clinical–histopathological features of this disease remain unclear [34, 35]. An in-depth discussion of the current concepts of psoriasis pathogenesis is beyond the scope of this chapter and readers are referred to recent reviews in the literature [34, 35]. Of note, the recent identification of the IL-23/Th17 axis as a major player in the pathobiology of psoriasis has provided a basis for novel therapeutic strategies in this disease. Anti-IL-12p40 monoclonal antibodies, which target the common subunit, p40, of both IL-23 and IL-12, are proving to be beneficial in the treatment of psoriasis patients [37].

A number of molecular studies have uncovered interesting results with immediate clinical utility in the management of patients with psoriasis. In this regard, gene expression profiling technologies have been used to compare lesional psoriatic skin with both nonlesional psoriatic skin and skin samples of other inflammatory dermatoses [1, 2, 10–14, 20, 26, 31, 38–45]. Genes with differential expression in psoriasis are summarized in Table 14.2. Molecular aberrations in established psoriatic plaques are also identifiable in nonlesional psoriatic skin. Of note, the transcript profile of nonlesional psoriatic skin is more similar to that of lesional psoriatic skin than to the gene expression profile of normal skin [17]. In one study, Zibert et al. [26] found 42 upregulated miRNAs and 5 downregulated miRNAs in involved psoriatic skin compared with healthy skin, and 9 deregulated miRNAs in noninvolved psoriatic skin compared with healthy skin. The identified target mRNAs of

Table 14.2 Gene expression profiles in psoriasis

Biological clusters	Genes differentially expressed
Genes with increased expression	
Cytokines, chemokines, and other inflammatory factors	IL-12B, IL-23, IL-1β, IL-8, TNF-α, CCL5, IL-1F5, IL-1F7, IL-1F9, IL-4R, GRO-α, SCYA19/21, SDF, ICAM-1, VCAM-1
IFN pathway	IFN-γ, IFN-inducible protein IFI 16/27/30/35/44, IFN-γ receptor 1, CXCL10, G1P3, myxomavirus resistance MX1, MX2, STAT1/3, OASI1/2, CCL2, CCL20, CD24
Epidermal barrier function	Involucrin, S100A2, S100A7, S100A8, S100A9, S100A12, transglutaminase TGM1/3, small proline-rich family proteins SPRR1A/1B/2A/2B/2C/2D/2E, lipoxygenase ALOXB12, cellular retinoic acid-binding protein CRAB2, corneodesmosin, fatty acid-binding protein FABP5, aquaporin 3 (AQP3), cornifelin, calmodulin-like 5 (CALML5), connexin 26 (GJB2)
Epidermal differentiation-associated keratins	KRT1, KRT6A/B, KRT14, KRT16, KRT17, KRT18
Proteinases and inhibitors	Kallikrein (KLK) 6/7/8/10/13, serine proteinase inhibitor SERPINB 1/2/3/4/5/7, SPINK5, PI3, cystatin A (CSTA), skin-derived protease inhibitor 3 (SKALP), protease inhibitor 3/Elafin, proteasome activator complex PSME2
Antimicrobial activity	β-Defensin 2 (DEFB2), DEFB4, lipocalin LCN2
Transcription factors	JunB, c-ETS-2, NFκB1, AP-1, IRF2-ISRE, eukaryotic translation elongation factor EEF1A1
Signal transduction	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation YWHAB, YWHAG, YWHAQ
Others	Vanin-3, cytochrome CYC1, TNFAIP3 interacting protein TNIP1, translationally controlled tumor protein TPT1, ribosomal protein L14/S4X/S29, integrin ß4 binding protein ITGB4BP, WNT5
Genes with decreased expression	
Combined clusters	KRT15/18, CCL27, JunC, claudin (CLDN) 5/8, zinc finger and BTB domain containing 16 (ZBTB16), monoamine oxidase A, aldehyde dehydrogenase 3 (ALDH3A2), makorin ring finger protein MKRN1, CREBL2, HIBP1, matrilin MATN2

these miRNAs were likely to be involved in cellular growth, proliferation, apoptosis, and extracellular matrix degradation. In addition, miR-221/miR-2/TIMP3 and miR-203/SOCS3 target interactions are postulated to play roles in the pathogenesis of psoriasis, as both TIMP3 and SOCS3 are found to be downregulated in psoriatic skin [12, 26]. These studies have not only provided insight into the pathogenesis of this disorder, but also uncovered potentially specific diagnostic markers and novel therapeutic targets [34, 35]. Many of these investigations are discussed in the preceding section on Eczematous/Spongiotic Dermatoses (Figs. 14.1 and 14.2). Of note, a type I IFN signature, with high expression of chemokines (CCL4, CCL20, CXCL2, CXCL8) and the chemokine receptor CXCR2, appears to be relatively specific to psoriasis [34]. In addition, the gene expression profile of psoriasis lesions has been shown to correlate with disease severity, but not with the clinical type or location of psoriatic plaques, family history of psoriasis, age at onset, or association with psoriatic arthritis [13, 14].

Furthermore, a number of investigators have evaluated the effects of current therapeutic modalities, such as pimecrolimus, cyclosporine, rhIL-11, dithranol, alefacept, IL-10, and phototherapy, on the transcriptomic profile of blood samples and lesional skin of psoriasis patients [15–21]. These studies have identified a panel of differentially expressed candidate genes that may serve as novel therapeutic intervention points, in addition to surrogate and predictive biomarkers of disease activity and treatment response. For instance, four-week treatment with topical pimecrolimus was shown to result in the downregulation of molecules involved in antigen presentation (HLA-E), chemotaxis and leukocyte migration (L-selectin, RANTES, LFA1a), inflammation (prostaglandin endoperoxide synthase 1), and other pathways [19]. In another study, a subset of 41 differentially regulated genes, which returned to normal or uninvolved levels following therapeutic intervention with rhIL-11 or cyclosporine, were identified [20]. These included 12 genes that are localized to psoriasis susceptibility loci, such as ID4, HBP-17, KRT16, S100A2, S100A9, S100A12, GNA15, MTX, PRKMK3, and SCYA2 [20]. Importantly, many of these changes were noted to precede clinical improvement [20]. Genes noted to be upregulated by treatment with rhIL-11, but not cyclosporine, included TNXA, CRIP1, and GATA3 [20]. With respect to dithranol treatment, gene expression profiling identified 18 genes (some well-known, IL-8 and COX-2; some recently identified immune regulatory molecules, G0S2 and CDKN1C) that showed significant up- or downregulation in active versus treated psoriatic lesions [21].

Ongoing investigations into the pathogenesis of psoriasis will likely lead to the development of other effective therapies with highly selective and targeted modes of action and low toxicity profiles [37]. The role of pharmacogenetics and pharmacogenomics in the management of patients with psoriasis is further explored in Chap. 21.

Lichenoid-Interface Dermatoses

The lichenoid-interface dermatoses (LIDs) represent a group of clinically heterogeneous inflammatory skin disorders with variable incidence and different triggering factors/antigens, but all showing a similar tissue reaction pattern on light microscopy. These disorders are characterized by a band-like infiltrate of predominantly mononuclear cells associated with epidermal basal cell injury/degeneration, and include lichen planus (LP), cutaneous lupus erythematosus (CLE), dermatomyositis, acute graft-versus-host disease (aGVHD), erythema multiforme, lichen sclerosus, and lichenoid drug reactions, amongst others [46, 47]. Recent studies suggest that a common inflammatory signaling pathway, involving plasmacytoid dendritic cell (pDC)-mediated type I IFN signaling (via CXCL9 and CXCL10 and their common receptor, CXCR3), underlies the pathogenesis of many LIDs. Similar mechanisms may be at play in lichenoid-interface reactions to verrucae and actinic keratoses [47–49]. An in-depth review of the pathways responsible for these disorders is beyond the scope of

this chapter and available elsewhere [47–49]. A definitive distinction of LIDs from other inflammatory dermatoses is generally possible through the integration of clinical findings and histopathological features [i.e., diagnostic lichenoid-interface reaction pattern, in the absence of spongiosis (i.e., eczematous conditions) and/or psoriasiform epidermal hyperplasia (i.e., psoriasis)] [46]. However, significant microscopic overlap can occur among LIDs, precluding a definitive diagnosis in some instances (i.e., aGVHD *vs.* erythema multiforme). Adjunct direct immunofluorescence (DIF) studies (for immunoglobulin, complement, and fibrinogen deposition) and/or serological testing [such as screening for hepatitis C virus (HCV) infection in LP] may be helpful in some cases [46]. As with other inflammatory dermatoses, research has largely been driven by the desire to elucidate the pathobiology and develop novel therapeutic strategies in these conditions. For example, in contrast to AD, psoriasis and other inflammatory dermatoses, LIDs are characterized by upregulation of CXCR3 ligands, which promote effector cytotoxic T-cells and pDCs [48]. This suggests that the use of chemokine antagonists may be a therapeutic approach in these disorders [48]. Interestingly, many of the uncovered cellular and molecular mechanisms could provide avenues for the development of more specific tools for the diagnosis of LIDs. For example, it has been proposed that future diagnostic tests may include cytokine profiling of lesional tissue [46]. Molecular diagnostic strategies in the setting of LIDs are discussed in the following sections.

Lichen Planus

Lichen planus (LP) is a chronic inflammatory disease that affects skin, mucous membranes, nails, and hair [46]. Cutaneous disease is clinically characterized by itchy, flat-topped polygonal, purple papules and plaques. On light microscopy, cutaneous LP (cLP) shows a lichenoid-interface lymphocytic reaction pattern, necrotic keratinocytes (Civatte bodies), and sawtooth-like epidermal acanthosis with wedge-shaped hypergranulosis [46]. The inflammatory infiltrate is dominated by IFN-promoted cytotoxic CD8+ T-cells, which appear to be autoreactive against epithelial antigens [49]. The pathogenesis of LP is largely unknown; however, a number of viruses have been proposed to play a role in its development [i.e., HCV and reactivated human herpes virus (HHV)-7]. Of note, HCV and HHV-7 DNA/RNA and antigens can be detected in lesional skin of LP by PCR, reverse transcription (RT)-PCR, ISH, and IHC [50–52]. Using expression profiling analysis, Wenzel et al. [10, 49] identified 60 genes that were upregulated in cLP compared with skin from healthy controls. These included: (1) a specific chemokine gene expression pattern; (2) both type I and type II IFN-inducible genes (IRF1, MX1, IFITM1); and (3) several MHC class I and class II molecules (HLA-B, HLA-DRB1). The most significantly increased transcripts belonged to the gene cluster of CXCR3 ligands – in particular CXCL9, which showed >13-fold induction [10, 49]. ISH- and PCR-based analyses have confirmed these results [10, 49]. CXCL9 is known to bind to CXCR3 on activated Th1 T-cells and NK-cells. This result is corroborated by the expression of CXCR3 protein by the majority of lymphocytes in cLP (Fig. 14.3) [10, 49]. In addition, CALML3, plexin D1, TRAM1, COX6A1, ENO1, NF-κB2, and LCP1 are among the most highly expressed genes in LP. By comparing the expression profiles of cLP with those of AD and psoriasis, several genes that are relatively specifically upregulated in LP are noted, including CXCL9, CCL19, and CCL21 [10, 49]. Of note, CCL13, CCL18, and CCL22 are found to be increased in both cLP and AD. Both ISH and IHC have been used to identify the presence and localization of CXCL9, IFN-α, IFN-β, and MxA mRNA and protein – confirming them as relatively specific markers for cLP, and facilitating its distinction from non-LIDs, in particular AD [10, 49]. In addition to their possible applications as diagnostic tools, these observations may provide novel targets for therapeutic intervention in cLP (i.e., anti-CXCL9 antibodies). With regard to mucosal lesions (oral LP), Tao et al. [53] have identified 985 differentially expressed

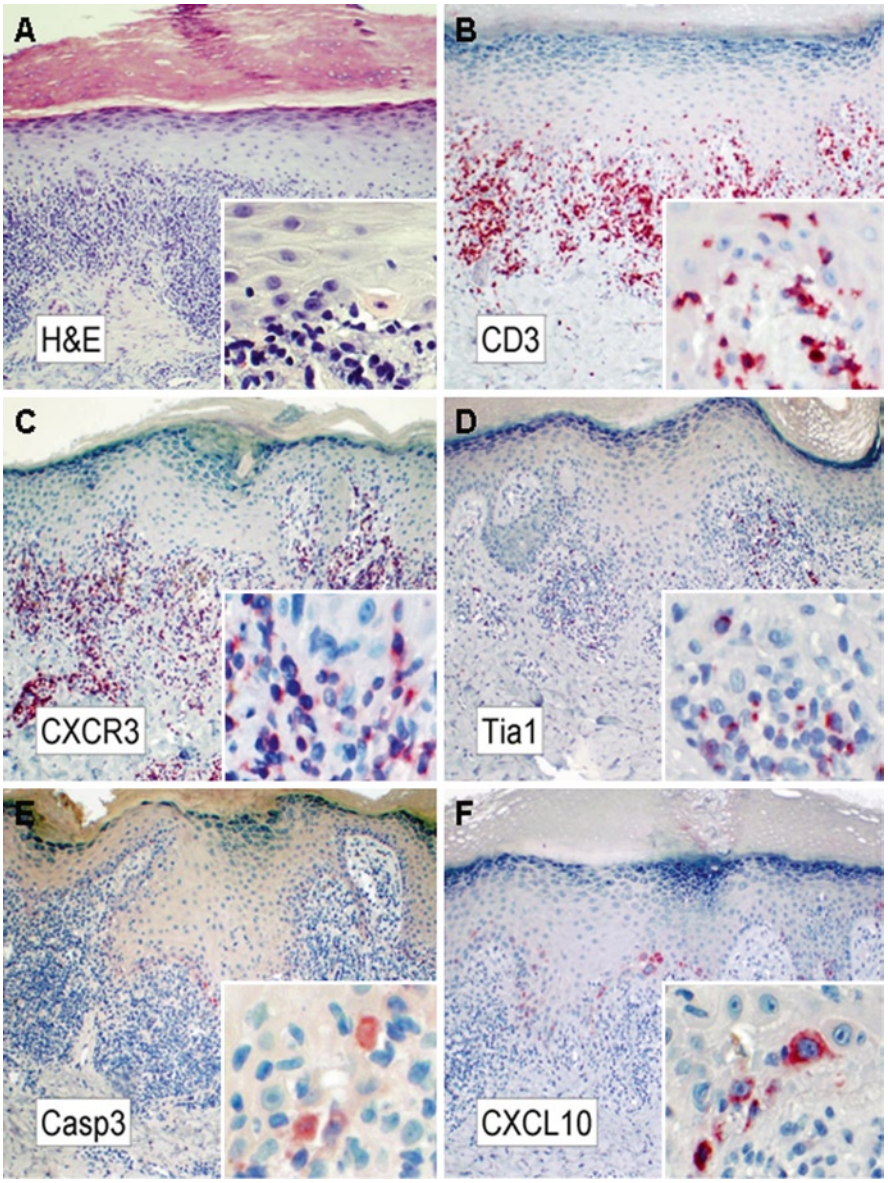

Fig. 14.3 *Lichen planus*. (**a**) Lichen planus is the prototype cell-rich lichenoid-interface dermatosis, demonstrating a typical band-like lichenoid inflammatory infiltrate, vacuolization of the basal layer, hyperkeratosis, hypergranulosis, and sawtooth-like acanthosis. (**b**) CD3-positive T-cells dominate the inflammatory infiltrate. (**c**) Large numbers of CXCR3-positive cytotoxic effector lymphocytes are found at the dermo-epidermal junction and in the upper dermis. The ligand for CXCR3 (IFN-inducible chemokine CXCL9) is the best marker to distinguish lichen planus from other inflammatory skin disorders, such as atopic dermatitis and psoriasis. (**d**) Tia1-positive cytotoxic cells invade the epidermis and induce keratinocyte apoptosis via (**e**) caspase 3, in areas (**f**) where the strongest CXCL10 expression is found. Some infiltrating lymphocytes carry CXCL10-positive granules (Courtesy of Dr. Jörg Wenzel, Department of Dermatology, University of Bonn, Bonn, Germany)

genes (356 downregulated and 629 upregulated, including CXCL9), involved in many biological processes and biochemical pathways, using DNA microarray and quantitative RT-PCR technologies. Another study by Ichimura et al. [54] confirmed upregulation of CXCL9 in oral lesions of LP using RT-PCR analysis.

Graft-Versus-Host Disease

Acute graft-versus-host disease (aGVHD) is a common complication of allogenic hematopoietic stem cell transplantation (HSCT) (up to 80% incidence), but is also rarely seen following blood transfusions or solid-organ transplantation (1–2% incidence) [55]. It arises from the presence of engrafted viable allogenic lymphocytes that are activated against host antigens, typically within an immunocompromised recipient who is unable to recognize or destroy the transplanted cells. Mortality rates for aGVHD vary widely (from <10% to >90%, depending on the disease grade). Rapid and accurate diagnosis is necessary, as early initiation of effective therapy may improve survival in these patients. Skin involvement is common, and typically presents as an initial acrally distributed and later generalized macular erythematous eruption. Diagnosis of aGVHD is commonly based on the correlation of clinical signs/symptoms (fever, diarrhea, pancytopenia) with histopathological features on skin biopsy (lichenoid-interface reaction pattern). Unfortunately, diagnosis is often delayed. The clinical changes may be nonspecific and overlap with a variety of other skin conditions, including drug eruptions and viral exanthems. In addition, the typical light microscopic findings of a band-like lymphocyte infiltrate, vacuolar changes and keratinocyte apoptosis/dyskeratosis are consistent with aGVHD, but not pathognomonic for this disease, as these features may be seen in other LIDs. Other tests, such as HLA analysis of peripheral blood/bone marrow leukocytes, may aid in the diagnosis, but are not 100% sensitive. Accordingly, a number of investigators have studied a possible role for molecular-based testing of skin biopsies for diagnosis and follow-up of patients with aGVHD. In this regard, the detection of mixed chimerism (both recipient and donor alleles present) is used to confirm the diagnosis of aGVHD.

PCR-based analyses of the Y chromosome-specific sex-determining region Y (SRY) gene [56], and DNA polymorphisms associated with variations in the length of dinucleotide or trinucleotide microsatellite repeats [57, 58], were among the first molecular techniques used to detect "foreign" (donor) DNA in skin biopsies of aGVHD. Recently, Schrager et al. [55] described a rapid, reproducible, and efficient multiplex PCR-based DNA fingerprinting method, using nine highly polymorphic short tandem repeats (STRs), in formalin-fixed paraffin-embedded (FFPE) skin biopsies of suspected aGVHD in solid-organ transplant recipients [55].

Unlike PCR-based chimerism testing, ISH-based techniques offer the distinct advantage of allowing the observer to correlate genetic alterations with morphological features. A number of studies have demonstrated that fluorescence ISH (FISH) analysis of FFPE skin biopsies (and samples from other sites), using X and/or Y chromosome probes, is useful in confirming aGVHD following transfusions, solid-organ transplantation, and HSCT [59–64]. The presence of a combined XX and XY pattern in an individual patient, or Y-positive cells in a female, is used to support the diagnosis (Fig. 14.4). Notably, donor cells can comprise from 0–90% of the total skin infiltrate in aGVHD [59, 60]. In addition, studies by Niino et al. [59] and Murata et al. [61] have noted a strong correlation between the frequency of FISH-positive donor-derived cells (lymphocytes, keratinocytes, and endothelial cells) and the histopathological severity of aGVHD (i.e., numbers of dyskeratotic/apoptotic cells), following HSCT. These two studies: (1) confirmed both diagnostic and grading applications of molecular testing; (2) suggested a role for donor cells (other than lymphocytes, and possibly derived from hematopoietic pluripotent stem cells) in the pathogenesis of aGVHD; and (3) identified potential therapeutic targets [59, 61]. However, a major limitation of the FISH-sex chromosome approach could be its lower sensitivity for detecting chimerism compared with PCR-based STR techniques [65], although this is disputed in other studies [64]. Furthermore, it can be applied only to transplant cases with sex-mismatched donors. Recently, a novel FISH approach using copy number polymorphisms to detect chimerism in a gender-independent fashion was developed, and may have some future application to aGVHD diagnosis and follow-up [65].

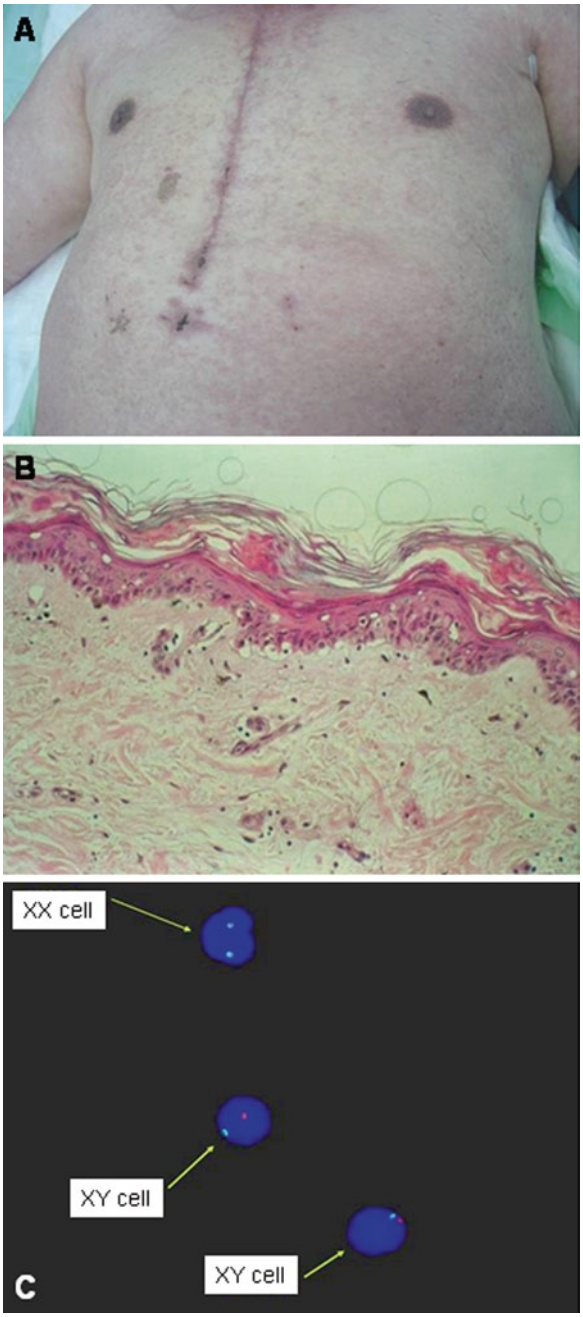

Fig. 14.4 *Transfusion-associated graft-versus-host disease* (GVHD). (**a**) Clinical signs and (**b**) cutaneous histopathological features of acute GVHD. (**c**) Fluorescence in situ hybridization (FISH) analysis of peripheral blood cells demonstrating mixed chimerism (Courtesy of Dr. Meltem Olga Akay, Eskisehir Osmangazi University Medical School, Eskisehir, Turkey)

While not typically considered a monoclonal T-cell disorder (such as cutaneous T-cell lymphoma), it has been suggested that disease-specific antigen-driven T-cell clones may be expanded in lesional skin of aGVHD, and used as "biomarkers" to detect involvement at other sites [66]. In addition, patients with changes of chronic GVHD in the skin and detectable expanded T-cell clones in the peripheral blood appear to be more likely to respond to certain forms of treatment (i.e., photophoresis) [67]. In a recent study, Beck et al. [66] used PCR amplification of rearranged T-cell receptor

genes from skin biopsies of aGVHD, followed by cloning and sequencing, in order to develop clonotype-specific PCR assays. Individual skin-derived disease-associated clones were detectable in the peripheral blood of these patients during active disease [66]. Dominant T-cell clones were identified in the skin biopsies of the majority of cases with histopathological changes of aGVHD, and identical clones were found in serial skin biopsies of an individual patient [66]. The detection of a dominant T-cell clone preceding diagnostic histopathological features of aGVHD (i.e., molecular-positive, microscopic-negative disease) suggests a possible adjunct diagnostic role for T-cell clonality analysis in this disorder [66]. Interestingly, other cutaneous disorders with GVHD-like microscopic features (i.e., Omenn syndrome, an autosomal recessive form of severe combined immunodeficiency) can also demonstrate clonal T-cell populations [68]. Of course, these findings reiterate the need for correlation of molecular clonality results with clinical and histopathological features in the evaluation of cutaneous T-cell infiltrates (see Chap. 10).

A gene expression profiling study of peripheral blood samples of aGVHD patients, using oligonucleotide microarrays, identified 55 differentially expressed genes in the acute and recovery phases of this disease [69]. Of note, five transcripts (TNFSF10/TRAIL, IL-1RN, IFI27, GZMB, and CCR5) were upregulated, and three transcripts (CLK1, TNFAIP3, and BTG1) were downregulated in aGVHD. These genes may be significantly involved in the pathogenesis of this disorder and could represent targets for therapeutic intervention. A role for gene expression profiling as an adjunct to the diagnosis of aGVHD has not been reported to date.

To summarize, diagnostic and prognostic information for patients with GVHD vis-à-vis disease course, prediction and monitoring of therapeutic response, and detection of relapse could potentially be accomplished by (1) quantitative FISH- or PCR-based analysis for chimerism in skin biopsies [55–64] and/or (2) quantitative PCR assays for disease-associated T-cell clones, using individualized markers derived from affected skin tissue (and peripheral blood) [66, 67].

Autoimmune Diseases

Cutaneous Lupus Erythematosus

Cutaneous lupus erythematosus (CLE) is a multifactorial chronic autoimmune disease with well-defined, but heterogeneous skin manifestations, including acute CLE, subacute CLE, chronic CLE, and intermittent CLE [70, 71]. The pathogenesis of CLE has been the subject of numerous investigations, and is found to involve a combination of genetic, environmental (i.e., ultraviolet radiation), and immunological factors (presence of autoantibodies, reduced CD4+/CD25+ regulatory T-cells, and increased proinflammatory cytokines TNF-α and MxA) [70]. CLE shares the common inflammatory signaling pathway involving pDC-derived IFN-α with other LIDs [47–49]. The prognosis for patients with CLE correlates with the extent and severity of systemic involvement [70]. Unfortunately, a clear distinction between CLE and systemic lupus erythematosus (SLE) does not exist. Individuals with primary CLE progress to SLE in 10–40% of cases, and skin lesions may be found in up to 70% of patients with SLE [72]. With currently available parameters (i.e., clinical and serological data), it is difficult to predict outcome in individual patients [70].

Diagnosis of CLE is based on the integration of clinical features, laboratory findings (i.e., ANA/dsDNA/Ro/La autoantibodies), and histopathological changes on skin biopsy [71]. A lichenoid-interface reaction pattern of inflammation is seen; however, the presence of other microscopic features, such as perifollicular/periadnexal inflammation, follicular plugging, basement membrane thickening and/or mucin deposition, would favor a diagnosis of CLE over other LIDs [71]. Supplemental DIF and/or IHC studies may be helpful in some cases. Deposition of immunoglobulins/complement components (i.e., IgG, C3d, and C4d) at the dermoepidermal junction supports the

diagnosis; although, other autoimmune disorders may also show these findings, and results cannot definitively distinguish between the different clinical subtypes of CLE [71, 73]. Of note, a reduction in the number of CD4+/CD25+ T-cells (detected by IHC) has been found in lesional skin of CLE, but not in other inflammatory dermatoses (AD, psoriasis, and LP) [74]. In addition, the lesional expression pattern of IFN-associated proteins (MxA, CXCL9, and CXCL10) reflects the inflammatory distribution (CXCR3+ cytotoxic lymphocytes) in the different subsets of CLE [72].

Similar to other T-cell-mediated autoimmune diseases, it appears that autoreactive T-cells can undergo clonal activation and expansion in SLE and CLE, with monoclonal/restricted oligoclonal TCR-GRs detectable by PCR analysis in peripheral blood and skin samples (see Chap. 10) [4, 75]. Therefore, caution must be exercised in the interpretation of T-cell clonality studies performed on skin biopsies. In addition, some patients can show overlapping features between a subtype of CLE [i.e., lupus erythematosus panniculitis (LEP)] and cutaneous T-cell lymphoma (i.e., subcutaneous panniculitis-like T-cell lymphoma), and the term "atypical lymphocytic lobular panniculitis" has been proposed to describe certain cases [5, 75]. It is suggested that patients with LEP should be carefully monitored for evolution to lymphoma [5]. It remains to be determined if clonally expanded T-cells play a direct role in CLE (and other autoimmune disorders), or are a nonspecific result of chronic antigen stimulation as part of the inflammatory process.

Genomic studies (i.e., gene expression profiling) have uncovered a number of candidate genes and epigenetic changes (i.e., miRNA alterations) that may play a role in the pathophysiology of CLE [6, 76–78]. For example, active and inactive variants of SLE demonstrate common and unique differentially expressed genes; however, the biological significance of these findings remains to be elucidated [77]. Importantly, the molecular basis underlying the phenotypic heterogeneity and propensity for clinical progression in CLE are currently unknown. It is envisioned that future studies will identify biomarkers for accurate diagnosis of CLE subtypes, disease activity, and prognostic assessment, in addition to uncovering specific therapeutic targets in this disease.

Scleroderma/Systemic Sclerosis

Scleroderma/systemic sclerosis (SSc) is a rare, but multigenic complex and heterogeneous, autoimmune connective tissue disorder, that is characterized by microvascular alterations and sclerosis/fibrosis affecting multiple organs [79, 80]. Progressive thickening and sclerosis of the skin is the hallmark of this disease. The pathogenesis of SSc involves activation of profibrotic pathways, with over expression of the cytokines transforming growth factor (TGF)-β and platelet-derived growth factor (PDGF). In addition, PDGF receptors (PDGFR) are found to be upregulated in the skin and at non-cutaneous sites. Scleroderma is divided into two distinct clinical subtypes: (1) a localized form, including morphea and eosinophilic fasciitis; and (2) systemic sclerosis (SSc), which can be further subdivided into two subsets, based on the extent of skin involvement: limited cutaneous SSc (lSSc) and diffuse cutaneous SSc (dSSc) [79, 80]. While the exact cause of SSc is unknown, genetic and environmental factors are believed to be involved in its pathogenesis. Genome-wide association studies have revealed multiple putative, but largely unconfirmed susceptibility loci [79]. These regions include genes encoding extracellular matrix proteins, vascular and oxidative factors, immune-regulatory molecules, cytokines, and growth factors. It has been suggested that some of the suspected genetic loci may be relevant only to certain clinical manifestations of SSc (i.e., diffuse *vs.* limited skin disease *vs.* visceral organ involvement) [79].

Currently, the diagnosis of SSc is based on the clinical presentation (type and extent of organ involvement) and presence of specific serum autoantibodies [79]. The risk for progression of skin disease is difficult to determine on the basis of clinical features; however, the subtype of autoantibody detected may have some utility (i.e., anti-topo I *vs.* anti-ACA) [81]. More objective and reproducible measures for diagnosis, prognosis, follow-up, and treatment selection in patients with SSc are needed.

A number of DNA microarray-based investigations have identified candidate genes and pathways in SSc, and provided insight into the pathogenesis and progression of this disease [6, 7, 78, 80–85]. Studies utilizing skin biopsies from patients with dSSc have identified up to 2,776 differentially expressed genes compared with healthy individuals [81]. Of note, a restricted list of 58 genes was noted to be capable of distinguishing SSc skin from normal controls with 100% specificity [81]. Interestingly, clinically unaffected skin in SSc patients may be involved at the molecular level (i.e., before skin fibrosis is discernable) [81]. Milano et al. [80] have extended these findings to show that differential gene expression can reflect the clinical heterogeneity of SSc skin disease. Patients with dSSc and lSSc showed distinct gene expression profiles. Functional clusters identified included: (1) inflammatory genes associated with B-cells (immunoglobulins), T-cells (PTPRC, CD2, CDW52), cytotoxic T-cells (CD8A, granzyme K/H/B), monocyte/macrophage lineage (LILRB2/3, CD163), antigen presentation (HLA-DRB1, HLA-DPA1, HLA-DMB), and an IFN-related signature (IFIT2, GBP1); (2) fibrosis-related genes, such as collagens (COL5A2/8A1/10A1/12A1), collagen triple helix repeat containing 1 (CTHRC1), and fibrillin-1 (FBN1); and (3) genes associated with cell division and proliferation, including the cell cycle-regulated genes CKS1B, CDKS2, CDC2, MCM8, and E2F7. Milano et al. [80] also reported the identification of three subsets of patients with dSSc and two subsets with lSSc. Four major groups were determined: (1) a diffuse-proliferation group, composed solely of dSSc; (2) an inflammatory group containing patients with dSSc, lSSc, and morphea; (3) a limited group containing most of the patients with lSSc, characterized by only low expression of proliferation and T-cell genes; and (4) a normal-like group, comprised of only a few dSSc and lSSc patients, and showing a gene expression profile similar to normal skin [80]. The broad inflammatory group was explained as reflecting the very earliest stages of all types of SSc, which commonly demonstrate a microscopically evident inflammatory component. The proliferation group reflects the later fibrotic stages of SSc. In order to further define these groups, the expression of three genes was measured by quantitative PCR: TNFRSF12A showed highest expression in dSSc and lowest in lSSc, CD8A had highest expression in the inflammatory group, and WIF1 was highest in normal skin and lowest in dSSc [80]. The evaluation of these three genes allowed a relatively straightforward distinction between the different groups of SSc. In addition, Milano et al. [80] reported that the severity of skin disease in dSSc was associated with a 177-gene signature. Sargent et al. [83] demonstrated that a TGF-β-responsive signature is found in the diffuse-proliferation subset of dSSc patients, identifying it as a potential pathogenic pathway and therapeutic target in these patients. In another study, Chung et al. [84] showed that an imatinib-responsive gene signature is present in most cases of dSSc. Clinical improvement of skin lesions in two patients with early dSSc, who received imatinib mesylate (Gleevec®), was associated with reductions in the cutaneous expression of phosphorylated PDGFR-β and ABL [84]. The goal of future studies will be to identify those genes that correlate with specific morphological changes in the skin. These will serve as biomarkers of disease activity and clinical outcome, predictors of systemic disease, and represent novel targets for therapeutic intervention and possibly early preventive strategies. The development of molecular diagnostic tests for SSc, based on either PCR or microarray technologies, has been envisioned [81].

Expanded clonal T-cell populations can be identified in lesional skin and peripheral blood of 45–100% and 34–61% of patients with SSc, respectively [86–89]. Interestingly, multiple skin biopsies in the same patient, taken at different times and from different sites, can show identical dominant T-cell clones [89]. However, identical T-cell clones have been found in the skin and peripheral blood in only a minority (14%) of patients [88]. Of note, the presence of peripheral blood T-cell clonality is significantly associated with (1) lSSc (34–74%) over dSSc (10–48%), and (2) a favorable response to photophoresis [86–88]. It has been suggested that clonal T-cells may be involved in the development of SSc, particularly the lSSc subtype, but this awaits confirmation [88]. Interestingly, other sclerodermoid cutaneous reactions can also demonstrate clonal T-cell expansions [90]. For example, T-cell clones have been identified in the peripheral blood of 100% (6/6) of

patients with nephrogenic systemic fibrosis (NSF), but their possible role in the pathogenesis and/or clinical course of this disease remains unclear [90]. The T-cell clonality status in skin biopsies of NSF has not been reported to date. The application of molecular diagnostic technologies in the setting of other fibrosing cutaneous lesions (i.e., keloids) is discussed in Chap. 17.

Neutrophilic Dermatoses

Neutrophilic dermatoses (ND) are a group of cutaneous inflammatory disorders, which include Sweet's syndrome (SS) and pyoderma gangrenosum (PG) [91, 92]. SS is characterized by asymmetrically distributed, painful, erythematous plaques of acute onset, accompanied by fever and neutrophilic leukocytosis. PG typically presents as a nodule or pustule, which subsequently ulcerates, and extends centrifugally [91, 92]. The typical histopathological features of ND are dense, nodular or diffuse, dermal and/or subcutaneous predominantly mature neutrophilic infiltrates, with leukocytoclasis (fragmented neutrophil nuclei), edema, and dilated small blood vessels with swollen endothelial cells, but no definitive evidence of vasculitis [91, 92]. It is postulated that the cutaneous neutrophilic infiltration in ND represents a hypersensitivity reaction or occurs secondary to changes in cytokine profiles [92–94]. Up to 80–90% of cases are idiopathic or associated with underlying infections, inflammatory disorders, or ingestion of certain medications [91, 92, 95]. In the remaining 10–20% of cases, ND may be a manifestation of an underlying hematologic disorder, particularly a myeloid dyscrasia [i.e., acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), or myelodysplastic syndrome (MDS)] or myeloma/monoclonal gammopathy [91–93]. In the setting of myeloid dyscrasias, ND may represent: (1) a paraneoplastic phenomenon, heralding either an occult disorder or relapse of a previously treated condition; (2) a drug-induced dermatosis, secondary to systemic therapy with agents such as all-*trans* retinoic acid (ATRA), granulocyte-colony stimulating factor (G-CSF), proteasome inhibitors, or tyrosine kinase inhibitors; or (3) the *bona fide* presence of leukemia cutis, with immature myeloid precursors/blasts admixed with mature neutrophils [92]. The evaluation of patients with ND requires correlation of clinical findings with microscopic features on skin biopsy, in addition to a systemic work-up for a possible underlying disorder (i.e., imaging, laboratory studies) [91]. The histopathological distinction of leukemia cutis from ND in a patient with a history of a myeloid disorder may be difficult, as dermal infiltrates of immature myeloid cells can also be found in the latter [93–96]. A number of studies have utilized molecular technologies to evaluate skin samples of ND (i.e., SS and PG), both in patients with and without underlying myeloid dyscrasias (Table 14.3). The readership may wish to review Chap. 13 for a discussion of molecular diagnostic strategies in leukemia cutis.

In some patients with ND and underlying myeloid dyscrasias, microscopic examination of their skin biopsies did not definitively reveal immature or atypical myeloid cells, and molecular studies were employed to evaluate for evidence of cutaneous infiltration by leukemic cells. For example, SS-like skin lesions of three patients with either underlying MDS [i.e., refractory anemia with excess blasts in transformation (RAEB-t)] [97] or CML [98, 99] did not demonstrate immature granulocytes by light microscopy. However, FISH [97, 98] or Southern blot analysis [99] of skin samples detected specific genomic aberrations (i.e., the presence of leukemic cells) in all three cases. In another study, the presence of t(9;22)/BCR-ABL1+ skin-infiltrating leukemic cells, suggested by the identification of atypical myeloid forms on light microscopy, was confirmed by FISH in a patient with CML and SS-like skin lesions (Fig. 14.5) [96]. Of note, in these four cases, there was patient-specific concordance of cytogenetic features between the skin and peripheral blood/bone marrow specimens [96–99]. However, in all cases this evaluation required the use of alternative or additional cytogenetic techniques, such as karyotyping and RT-PCR, on the non-cutaneous samples [96–99]. Interestingly, none of these four patients was reported to show evidence of

Table 14.3 Molecular studies on neutrophilic dermatoses

Study	*n*	Type	Microscopic features	Molecular technique in skin	Molecular findings in skin	Underlying BM disorder	Findings on BM and/or PB (method; results, in relation to skin findings)	Comments
Van Kamp (1994)	1	SS	"Signs of Sweet's syndrome"	FISH	Monosomy 5	MDS (RAEB-t)	BM: KY Concordant Complex abnormalities, including loss of chr. 5	Circulating and skin infiltrating neutrophils of patients with MDS are clonally derived; Use of G-CSF played a role in pathogenesis of SS; Presence of "leukemic" cells can be detected in the skin lesions of SS in an MDS patient: (a) with "signs of SS" on skin biopsy (b) without evidence of disease progression or blast crisis following further treatment
Urano (1999)	1	SS	Mature neutrophils	SB	+ *BCR* gene rearrangement	CML	BM: RT-PCR Concordant t(9;22) (*BCR-ABL1* fusion gene, +Ph) PB: SB Concordant +*BCR* gene rearrangement	Presence of "leukemic" cells can be detected in the skin lesions of SS in a CML patient: (a) without microscopic evidence of immature granulocytes in the skin biopsy (b) without blast crisis in the BM

(continued)

Table 14.3 (continued)

Study	*n*	Type	Microscopic features	Molecular technique in skin	Molecular findings in skin	Underlying BM disorder	Findings on BM and/or PB (method; results, in relation to skin findings)	Comments
Magro (2001)	1	SS	(a) Predominantly mature neutrophils (b) Some dysplastic hypolobulated forms, suggesting maturation of dysplastic clonal stem cells (pseudo-Pelger-Huet anomaly) (c) Immature metamyelocytes and myelocytes	X-inactivation assay [based on human androgen receptor (HUMARA) gene]	+ Clonal restriction	AML-M2	Post-treatment BM: X-inactivation assay Concordant + Clonal restriction	Same allele was preferentially protected in the skin and BM specimens, suggesting that the dermal infiltrate was derived from the same clone as the BM cells
Liu (2004)	1	SS	Mature neutrophils	FISH	t(9;22) (*BCR-ABL1* fusion signals, +Ph)	CML	BM: RT-PCR Concordant t(9;22) BM: KY/FISH Discordant Negative result	Presence of "leukemic" cells can be detected in the skin lesions of SS in a CML patient: (a) treated with tyrosine kinase inhibitor [Imatinib Mesylate (Gleevec®)] (b) with mature neutrophilic infiltrates on skin biopsy (c) without evidence of disease progression or blast crisis following further treatment
Requena (2005)	41	SS	"Histiocytoid" mononuclear cells (CD68+/MPO+)	FISH	Negative for *BCR-ABL1* gene fusion (14 cases tested)	No underlying myeloid dyscrasias	NR	Early lesions of SS may be characterized by "histiocytoid" immature myeloid cells, without evidence of subsequent myeloid dyscrasia development

Magro (2007)	16	SS/PG	Group (1): SS and PG with underlying AML/MDS (n = 9) Dysplastic neutrophils (hyperlobation, asymmetrical lobation, increase in cell size) or atypical monocytoid cells	X-inactivation assay	+ Clonal restriction (n = 5)	Underlying AML/MDS	BM not analyzed	HUMARA assay to detect neutrophil clonality is limited by: (a) restriction for use in women heterozygous for the locus (b) possible presence of clonal hematopoietic cells in elderly women
			Group (2): SS and PG controls without underlying myeloid dyscrasias (n = 7) Predominantly mature neutrophils; two clonal cases showed neutrophil or monocytoid cell atypia		+ Clonal restriction (n = 5) Subsequent diagnosis of MDS (n = 1) and unexplained neutropenia (n = 1)	No underlying myeloid dyscrasias	BM not analyzed	Clonality in SS/PG, while characteristic of an underlying myeloid dyscrasia, is not observed exclusively in this setting Clonality in SS/PG lesions of patients with known myeloid dyscrasias may be associated with blast crisis, ATRA or G-CSF therapy, or AML-derived G-CSF Clonality in SS/PG lesions of patients without known myeloid dyscrasias, particularly in the setting of neutrophil/monocytic atypia, should prompt close clinical follow-up and/or additional work-up Recurrent SS/PG may represent an "indolent, localized cutaneous neutrophilic dyscrasia"

(continued)

Table 14.3 (continued)

Study	*n*	Type	Microscopic features	Molecular technique in skin	Molecular findings in skin	Underlying BM disorder	Findings on BM and/or PB (method; results, in relation to skin findings)	Comments
Kaune (2008)	1	SS	(a) Predominantly mature neutrophils (b) Some atypical monocytoid and binuclear cells	FISH	t(9;22) (*BCR-ABL1* fusion gene, +Ph)	CML	SITE NR: KY Concordant t(9;22)(+Ph)	Presence of "leukemic" cells can be detected in the skin lesions of SS in a CML patient: (a) treated with newer tyrosine kinase inhibitor (nilotinib) (b) without evidence of disease progression or blast crisis following further treatment
Cappel (2009)	1	SS	Atypical "histiocytoid" cells	FISH	7q (*AML1*) abn. 11q23 (*MLL*) abn.	AML	BM: FISH Concordant 7q (*AML1*) abn. 11q23 (*MLL*) abn.	Presence of "leukemic" cells can be detected in the skin lesions of SS in an AML patient, despite morphologic and immunophenotypic differences from malignant cells in BM

BM bone marrow, *PB* peripheral blood, *SS* Sweet's syndrome, *PG* pyoderma gangrenosum, *FISH* fluorescence in situ hybridization, *RT-PCR* reverse transcription-polymerase chain reaction, *SB* Southern blot, *KY* karyotyping, *MDS* myelodysplastic syndrome, *RAEB-t* refractory anemia with excess blasts in transformation, *CML* chronic myelogenous leukemia, *AML* acute myeloid leukemia, *Ph* Philadelphia chromosome, *G-CSF* granulocyte-colony stimulating factor, *ATRA* all-*trans* retinoic acid, *MPO* myeloperoxidase, *NR* not reported, *CHR* chromosome, *ABN* abnormality

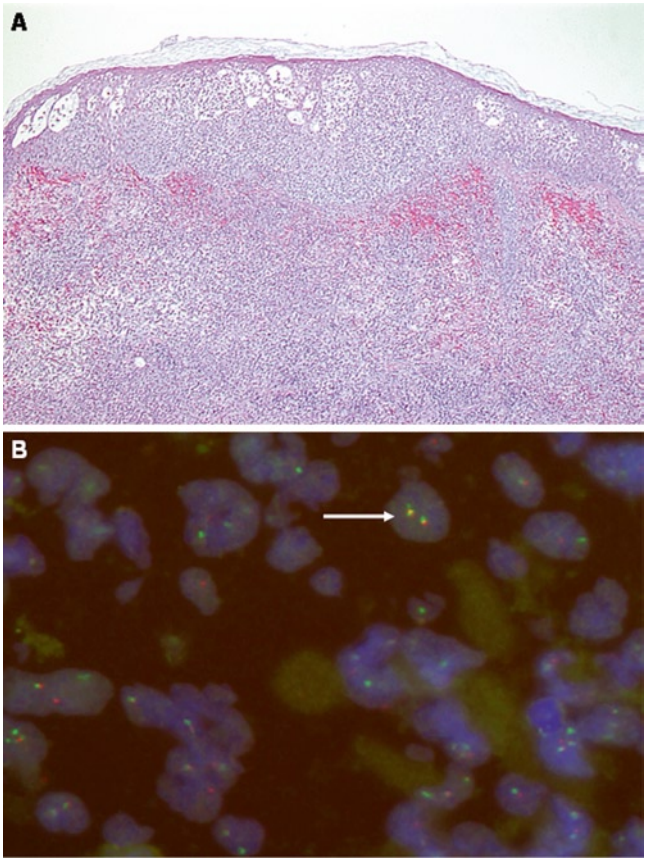

Fig. 14.5 *Sweet's syndrome in a patient with t(9;22)(q34;q11)-positive chronic myelogenous leukemia* (CML) *treated with the tyrosine kinase inhibitor Nilotinib*. (**a**) Spongiotic epidermis, dermal hemorrhage and a dense mixed infiltrate composed of predominantly mature neutrophils with some atypical monocytoid and binuclear cells. (**b**) Fluorescence in situ hybridization (*FISH*) on formalin-fixed paraffin-embedded lesional tissue section. Interphase nuclei were hybridized with a BCR/ABL dual-color, dual-fusion translocation probe – red signal: 9q34 (ABL-gene); green signal: 22q11 (BCR-gene). The presence of fused red-and-green signals (*arrow*) indicates the presence of a t(9;22)(q34;q11) translocation, juxtaposing the BCR- and ABL-loci (Courtesy of Dr. Kjell M. Kaune, Department of Dermatology and Venerology, Georg August University Goettingen, Goettingen, Germany)

disease progression or blast crisis following further therapy [96–99]. Therefore, the significance of molecular findings in skin biopsies of ND from patients with underlying MDS or CML is unclear.

It is important to note that skin lesions of ND may develop prior to the diagnosis of a myeloid dyscrasia, and therefore, surveillance is appropriate for any patient who presents with SS or PG, in the absence of any other predisposing systemic condition. Magro et al. [93, 94] have investigated whether the presence of neutrophil clonality (detected by X-inactivation assay) in SS and PG skin specimens is a marker of an underlying myeloid dyscrasia. Clonality was identified in 81% of ND samples in patients with underlying AML or MDS ($n = 9$) [93]. Histopathological review of the skin biopsies of all these patients demonstrated dysplastic neutrophils (i.e., hyperlobation, asymmetrical lobation, increased cell size) or atypical monocytoid cells [93]. Interestingly, clonality was also identified in 81% of ND samples in a control group who did not have known myeloid dyscrasias ($n = 7$) [93]. One of the patients in this group later developed MDS. Another patient developed an unexplained neutropenia. Skin biopsies from the control group demonstrated dermal/subcutaneous

infiltrates of predominantly mature neutrophils with leukocytoclasia; however, neutrophil or monocytoid cell atypia was seen in two clonal cases, including the patient with subsequent MDS. Clonality is therefore detectable in ND from: (1) patients with and without known myeloid dyscrasias; and (2) from skin samples with and without discernable neutrophilic or monocytic atypia. Accordingly, the presence of clonality in SS/PG, while characteristic of an underlying myeloid dyscrasia, is not observed exclusively in this setting. Magro et al. [93] have suggested that recurrent sterile clonal SS/PG in a patient without a known underlying myeloid disorder may represent an "indolent, localized cutaneous neutrophilic dyscrasia." This term describes a lesion on a morphological continuum with leukemia cutis, and analogous to some cutaneous lymphoid proliferations with molecular evidence of T-cell clonality, but little or no propensity to progress to overt cutaneous T-cell lymphoma (see Chap. 10) [93]. The significance of clonal neutrophilic infiltrates in skin biopsies of ND from patients who do not have underlying myeloid disorders remains to be determined. Nonetheless, the identification of clonality in this setting, particularly in the presence of neutrophil/monocytic atypia, may warrant close clinical follow-up and/or additional studies.

As stated previously, abnormal or immature myeloid cells can be observed by light microscopy in ND lesions of patients with underlying myeloid disorders [93–96]. However, it has also been reported that 0.5–3.0% of dermal neutrophils in ND lesions not associated with a myeloid dyscrasia may also demonstrate cytologic atypia [100]. A histiocytoid variant of SS has recently been described, which demonstrates the presence of immature histiocyte-like myeloid cells (CD68+/MAC-387+/HAM-56+/MPO+ by IHC) on skin biopsy [95]. The authors excluded the possibility of cutaneous involvement by AML or CML by clinical follow-up, peripheral blood examinations, and failure of FISH analysis to detect the BCR-ABL1 fusion gene in skin samples [95]. To date, none of these patients, in whom follow-up is available, has developed a myeloid dyscrasia (L. Requena, personal communication, 2010). Of note, a recent report describes a patient with AML who developed a skin eruption resembling histiocytoid SS on light microscopy, but which was confirmed to be a myeloid leukemia cutis by FISH analysis of the skin biopsy [101].

In conclusion, the demonstration of (1) immature/atypical myeloid cells and (2) clonality in skin biopsies of ND may be suggestive of, but not pathognomonic for, an underlying occult myeloid disorder. Findings must be interpreted in the context of clinical features and results of additional studies (i.e., IHC). Molecular tests (i.e., FISH) on skin specimens for specific genomic abnormalities may have a role in the diagnostic work-up of patients with ND.

References

1. Kamsteeg M, Jansen PA, van Vlijmen-Willems IM, et al. Molecular diagnostics of psoriasis, atopic dermatitis, allergic contact dermatitis and irritant contact dermatitis. Br J Dermatol. 2010;162:568–78.
2. Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, et al. Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis. J Allergy Clin Immunol. 2007;119:1210–7.
3. Veysey EC, Wilkinson JD. Mycosis fungoides masquerading as cutaneous lupus erythematosus and associated with antiphospholipid syndrome. Clin Exp Dermatol. 2008;33:26–9.
4. Park HS, Choi JW, Kim BK, et al. Lupus erythematosus panniculitis: clinicopathological, immunophenotypic, and molecular studies. Am J Dermatopathol. 2010;32:24–30.
5. Pincus LB, LeBoit PE, McCalmont TH, et al. Subcutaneous panniculitis-like T-cell lymphoma with overlapping clinicopathologic features of lupus erythematosus: coexistence of 2 entities? Am J Dermatopathol. 2009;31:520–6.
6. Kunz M. DNA microarray technology in Dermatology. Semin Cutan Med Surg. 2008;27:16–24.
7. Sellheyer K, Belbin TJ. DNA microarrays: from structural genomics to functional genomics. The applications of gene chips in dermatology and dermatopathology. J Am Acad Dermatol. 2004;51:681–92.
8. Sugiura H, Ebise H, Tazawa T, et al. Large-scale DNA microarray analysis of atopic skin lesions shows overexpression of an epidermal differentiation gene cluster in the alternative pathway and lack of protective gene expression in the cornified envelope. Br J Dermatol. 2005;152:146–9.

9. Sonkoly E, Ståhle M, Pivarcsi A. MicroRNAs: novel regulators in skin inflammation. Clin Exp Dermatol. 2008;33:312–5.
10. Wenzel J, Peters P, Zahn S, et al. Gene expression profiling of lichen planus reflects CXCL9+ -mediated inflammation and distinguishes this disease from atopic dermatitis and psoriasis. J Invest Dermatol. 2008;128:67–78.
11. Nomura I, Gao B, Boguniewicz M, et al. Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: A gene microarray analysis. J Allergy Clin Immunol. 2003;112:1195–202.
12. Sonkoly E, Wei T, Janson PC, et al. MicroRNAs: novel regulators involved in the pathogenesis of Psoriasis? PLoS One. 2007;2:e610.
13. Kulski JK, Kenworthy W, Bellgard M, et al. Gene expression profiling of Japanese psoriatic skin reveals an increased activity in molecular stress and immune response signals. J Mol Med. 2005;83:964–75.
14. Quekenborn-Trinquet V, Fogel P, Aldana-Jammayrac O, et al. Gene expression profiles in psoriasis: analysis of impact of body site location and clinical severity. Br J Dermatol. 2005;152:489–504.
15. Haider AS, Lowes MA, Suárez-Fariñas M, et al. Identification of cellular pathways of "type 1", Th17 T cells, and TNF- and inducible nitric oxide synthase-producing dendritic cells in autoimmune inflammation through pharmacogenomic study of cyclosporine A in psoriasis. J Immunol. 2008;180:1913–20.
16. Haider AS, Lowes MA, Gardner H, et al. Novel insight into the agonistic mechanism of alefacept in vivo: differentially expressed genes may serve as biomarkers of response in psoriasis patients. J Immunol. 2007; 178:7442–9.
17. Hochberg M, Zeligson S, Amariglio N, et al. Genomic-scale analysis of psoriatic skin reveals differentially expressed insulin-like growth factor-binding protein-7 after phototherapy. Br J Dermatol. 2007;156:289–300.
18. Jung M, Sabat R, Krätzschmar J, et al. Expression profiling of IL-10-regulated genes in human monocytes and peripheral blood mononuclear cells from psoriatic patients during IL-10 therapy. Eur J Immunol. 2004; 34:481–93.
19. Rappersberger K, Komar M, Ebelin ME, et al. Pimecrolimus identifies a common genomic anti-inflammatory profile, is clinically highly effective in psoriasis and is well tolerated. J Invest Dermatol. 2002;119:876–87.
20. Oestreicher JL, Walters IB, Kikuchi T, et al. Molecular classification of psoriasis disease-associated genes through pharmacogenomic expression profiling. Pharmacogenomics J. 2001;1:272–87.
21. Koczan D, Guthke R, Thiesen HJ, et al. Gene expression profiling of peripheral blood mononuclear leukocytes from psoriasis patients identifies new immune regulatory molecules. Eur J Dermatol. 2005;15:251–7.
22. Kiyohara C, Tanaka K, Miyake Y. Genetic susceptibility to atopic dermatitis. Allergol Int. 2008;57:39–56.
23. Hanifin JM. Evolving concepts of pathogenesis in atopic dermatitis and other eczemas. J Invest Dermatol. 2009;129:320–2.
24. Sääf AM, Tangvall-Linder M, Chang HY, et al. Global expression profiling in atopic eczema reveals reciprocal expression of inflammatory and lipid genes. PLoS One. 2008;3:e4017.
25. Sand M, Gambichler T, Sand D, et al. MicroRNAs and the skin: tiny players in the body's largest organ. J Dermatol Sci. 2009;53:169–75.
26. Zibert JR, Løvendorf MB, Litman T, et al. MicroRNAs and potential target interactions in psoriasis. J Dermatol Sci. 2010;58:177–85.
27. Li Y, Sawalha AH, Lu Q. Aberrant DNA methylation in skin diseases. J Dermatol Sci. 2009;54:143–9.
28. Guttman-Yassky E, Suárez-Fariñas M, Chiricozzi A, et al. Broad defects in epidermal cornification in atopic dermatitis identified through genomic analysis. J Allergy Clin Immunol. 2009;124:1235–44.e58.
29. Kamsteeg M, Zeeuwen PL, de Jongh GJ, et al. Increased expression of carbonic anhydrase II (CA II) in lesional skin of atopic dermatitis: regulation by Th2 cytokines. J Invest Dermatol. 2007;127:1786–9.
30. Tracey L, Villuendas R, Dotor AM, et al. Mycosis fungoides shows concurrent deregulation of multiple genes involved in the TNF signaling pathway: an expression profile study. Blood. 2003;102:1042–50.
31. de Jongh GJ, Zeeuwen PL, Kucharekova M, et al. High expression levels of keratinocyte antimicrobial proteins in psoriasis compared with atopic dermatitis. J Invest Dermatol. 2005;125:1163–73.
32. de Sousa Abreu R, Penalva LO, Marcotte EM, et al. Global signatures of protein and mRNA expression levels. Mol Biosyst. 2009;5:1512–26.
33. Szameit S, Vierlinger K, Farmer L, et al. Microarray-based in vitro test system for the discrimination of contact allergens and irritants: Identification of potential marker genes. Clin Chem. 2008;54:525–33.
34. Rácz E, Prens EP. Molecular pathophysiology of psoriasis and molecular targets of antipsoriatic therapy. Expert Rev Mol Med. 2009;11:e38.
35. Elder JT, Bruce AT, Gudjonsson JE, et al. Molecular dissection of psoriasis: integrating genetics and biology. J Invest Dermatol. 2010;130:1213–26.
36. Das RP, Jain AK, Ramesh V. Current concepts in the pathogenesis of psoriasis. Indian J Dermatol. 2009;54:7–12.
37. Di Cesare A, Di Meglio P, Nestle FO. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. J Invest Dermatol. 2009;129:1339–50.

38. Bowcock AM, Shannon W, Du F, et al. Insights into psoriasis and other inflammatory diseases from large-scale gene expression studies. Hum Mol Genet. 2001;10:1793–805.
39. Zhou X, Krueger JG, Kao MC, et al. Novel mechanisms of T-cell and dendritic cell activation revealed by profiling of psoriasis on the 63, 100-element oligonucleotide array. Physiol Genomics. 2003;13:69–78.
40. Reischl J, Schwenke S, Beekman JM, et al. Increased expression of Wnt5a in psoriatic plaques. J Invest Dermatol. 2007;127:163–9.
41. Gudjonsson JE, Johnston A, Stoll SW, et al. Evidence for altered Wnt signaling in psoriatic skin. J Invest Dermatol. 2010;130:1849–59.
42. Gudjonsson JE, Ding J, Johnston A, et al. Assessment of the psoriatic transcriptome in a large sample: additional regulated genes and comparisons with in vitro models. J Invest Dermatol. 2010;130:1829–40.
43. Yao Y, Richman L, Morehouse C, et al. Type I interferon: potential therapeutic target for psoriasis? PLoS One. 2008;3:e2737. Erratum in: PLoS One. 2009;4.
44. Gudjonsson JE, Ding J, Li X, et al. Global gene expression analysis reveals evidence for decreased lipid biosynthesis and increased innate immunity in uninvolved psoriatic skin. J Invest Dermatol. 2009;129:2795–804.
45. Ito M, Ogawa K, Takeuchi K, et al. Gene expression of enzymes for tryptophan degradation pathway is upregulated in the skin lesions of patients with atopic dermatitis or psoriasis. J Dermatol Sci. 2004;36:157–64.
46. Lehman JS, Tollefson MM, Gibson LE. Lichen planus. Int J Dermatol. 2009;48:682–94.
47. Sontheimer RD. Lichenoid tissue reaction/interface dermatitis: clinical and histological perspectives. J Invest Dermatol. 2009;129:1088–99.
48. Meller S, Gilliet M, Homey B. Chemokines in the pathogenesis of lichenoid tissue reactions. J Invest Dermatol. 2009;129:315–9.
49. Wenzel J, Tüting T. An IFN-associated cytotoxic cellular immune response against viral, self-, or tumor antigens is a common pathogenetic feature in “interface dermatitis”. J Invest Dermatol. 2008;128:2392–402.
50. de Vries HJ, Teunissen MB, Zorgdrager F, et al. Lichen planus remission is associated with a decrease of human herpes virus type 7 protein expression in plasmacytoid dendritic cells. Arch Dermatol Res. 2007;299:213–9.
51. Kurokawa M, Hidaka T, Sasaki H, et al. Analysis of hepatitis C virus (HCV) RNA in the lesions of lichen planus in patients with chronic hepatitis C: detection of anti-genomic- as well as genomic-strand HCV RNAs in lichen planus lesions. J Dermatol Sci. 2003;32:65–70.
52. Lazaro P, Olalquiaga J, Bartolomé J, et al. Detection of hepatitis C virus RNA and core protein in keratinocytes from patients with cutaneous lichen planus and chronic hepatitis C. J Invest Dermatol. 2002; 119:798–803.
53. Tao XA, Li CY, Xia J, et al. Differential gene expression profiles of whole lesions from patients with oral lichen planus. J Oral Pathol Med. 2009;38:427–33.
54. Ichimura M, Hiratsuka K, Ogura N, et al. Expression profile of chemokines and chemokine receptors in epithelial cell layers of oral lichen planus. J Oral Pathol Med. 2006;35:167–74.
55. Schrager JJ, Vnencak-Jones CL, Graber SE, et al. Use of short tandem repeats for DNA fingerprinting to rapidly diagnose graft-versus-host disease in solid organ transplant patients. Transplantation. 2006;81:21–5.
56. Hayakawa S, Chishima F, Sakata H, et al. A rapid molecular diagnosis of posttransfusion graft-versus-host disease by polymerase chain reaction. Transfusion. 1993;33:413–7.
57. Wang L, Juji T, Tokunaga K, et al. Brief report: polymorphic microsatellite markers for the diagnosis of graft-versus-host disease. N Engl J Med. 1994;330:398–401.
58. Warren LJ, Simmer K, Roxby D, et al. DNA polymorphism analysis in transfusion-associated graft-versus-host disease. J Paediatr Child Health. 1999;35:98–101.
59. Niino D, Nakashima M, Kondo H, et al. Correlation of donor-derived keratinocytes and severity of graft-versus-host disease (GVHD) in epidermis. Pathol Res Pract. 2005;200:775–81.
60. Au WY, Ma SK, Kwong YL, et al. Graft-versus-host disease after liver transplantation: documentation by fluorescent in situ hybridisation and human leucocyte antigen typing. Clin Transplant. 2000;14:174–7.
61. Murata H, Janin A, Leboeuf C, et al. Donor-derived cells and human graft-versus-host disease of the skin. Blood. 2007;109:2663–5.
62. Akay MO, Temiz G, Teke HU, et al. Rapid molecular cytogenetic diagnosis of transfusion associated graft-versus-host disease by fluorescent in situ hybridization (FISH). Transfus Apher Sci. 2008;38:189–92.
63. Kanehira K, Riegert-Johnson DL, Chen D, et al. FISH diagnosis of acute graft-versus-host disease following living-related liver transplant. J Mol Diagn. 2009;11:355–8.
64. Meves A, el-Azhary RA, Talwalkar JA, et al. Acute graft-versus-host disease after liver transplantation diagnosed by fluorescent in situ hybridization testing of skin biopsy specimens. J Am Acad Dermatol. 2006;55:642–6.
65. Wu D, Vu Q, Nguyen A, et al. In situ genetic analysis of cellular chimerism. Nat Med. 2009;15:215–9.
66. Beck RC, Wlodarski M, Gondek L, et al. Efficient identification of T-cell clones associated with graft-versus-host disease in target tissue allows for subsequent detection in peripheral blood. Br J Haematol. 2005; 129:411–9.

67. French LE, Alcindor T, Shapiro M, et al. Identification of amplified clonal T cell populations in the blood of patients with chronic graft-versus-host disease: positive correlation with response to photopheresis. Bone Marrow Transplant. 2002;30:509–15.
68. D'hauw A, Seyger MM, Groenen PJ, et al. Cutaneous graft-versus-host-like histology in childhood. Importance of clonality analysis in differential diagnosis. A case report. Br J Dermatol. 2008;158:1153–6.
69. Takahashi N, Sato N, Takahashi S, et al. Gene-expression profiles of peripheral blood mononuclear cell subpopulations in acute graft-vs-host disease following cord blood transplantation. Exp Hematol. 2008;36:1760–70.
70. Kuhn A, Sontheimer RD. Cutaneous lupus erythematosus: molecular and cellular basis of clinical findings. Curr Dir Autoimmun. 2008;10:119–40.
71. Walling HW, Sontheimer RD. Cutaneous lupus erythematosus: issues in diagnosis and treatment. Am J Clin Dermatol. 2009;10:365–81.
72. Wenzel J, Zahn S, Bieber T, et al. Type I interferon-associated cytotoxic inflammation in cutaneous lupus erythematosus. Arch Dermatol Res. 2009;301:83–6.
73. Magro CM, Dyrsen ME. The use of C3d and C4d immunohistochemistry on formalin-fixed tissue as a diagnostic adjunct in the assessment of inflammatory skin disease. J Am Acad Dermatol. 2008;59:822–33.
74. Franz B, Fritzsching B, Riehl A, et al. Low number of regulatory T cells in skin lesions of patients with cutaneous lupus erythematosus. Arthritis Rheum. 2007;56:1910–20.
75. Guitart J, Magro C. Cutaneous T-cell lymphoid dyscrasia: a unifying term for idiopathic chronic dermatoses with persistent T-cell clones. Arch Dermatol. 2007;143:921–32.
76. Nakou M, Knowlton N, Frank MB, et al. Gene expression in systemic lupus erythematosus. Arthritis Rheum. 2008;58:3541–9.
77. Sandrin-Garcia P, Moraes Junta C, Fachin AL, et al. Shared and unique gene expression in systemic lupus erythematosus depending on disease activity. Ann NY Acad Sci. 2009;1173:493–500.
78. Assassi S, Mayes MD, Arnett FC, et al. Systemic sclerosis and lupus: points in an interferon-mediated continuum. Arthritis Rheum. 2010;62:589–98.
79. Granel B, Bernard F, Chevillard C. Genetic susceptibility to systemic sclerosis. From clinical aspect to genetic factor analyses. Eur J Int Med. 2009;20:242–52.
80. Milano A, Pendergrass SA, Sargent JL, et al. Molecular subsets in the gene expression signature of scleroderma skin. PLoS One. 2008;3:e2696.
81. Sargent JL, Milano A, Connolly MK, et al. Scleroderma gene expression and pathway signatures. Curr Rheumatol Rep. 2008;10:205–11.
82. Whitfield ML, Finlay DR, Murray JI, et al. Systemic and cell type-specific gene expression patterns in scleroderma skin. Proc Natl Acad Sci USA. 2003;100:12319–24.
83. Sargent JL, Milano A, Bhattacharyya S, et al. A TGFbeta-responsive gene signature is associated with a subset of diffuse scleroderma with increased disease severity. J Invest Dermatol. 2010;130:694–705.
84. Chung L, Fiorentino DF, Benbarak MJ, et al. Molecular framework for response to imatinib mesylate in systemic sclerosis. Arthritis Rheum. 2009;60:584–91.
85. Tan FK, Hildebrand BA, Lester MS, et al. Classification analysis of the transcriptosome of nonlesional cultured dermal fibroblasts from systemic sclerosis patients with early disease. Arthritis Rheum. 2005;52:865–76.
86. French LE, Lessin SR, Addya K, et al. Identification of clonal T cells in the blood of patients with systemic sclerosis: positive correlation with response to photopheresis. Arch Dermatol. 2001;137:1309–13.
87. Marie I, Cordel N, Lenormand B, et al. Clonal T cells in the blood of patients with systemic sclerosis. Arch Dermatol. 2005;141:88–9.
88. Kreuter A, Höxtermann S, Tigges C, et al. Clonal T-cell populations are frequent in the skin and blood of patients with systemic sclerosis. Br J Dermatol. 2009;161:785–90.
89. Sakkas LI, Xu B, Artlett CM, et al. Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. J Immunol. 2002;168:3649–59.
90. Kreuter A, Höxtermann S, Gambichler T, et al. Detection of clonal T cells in the circulation of patients with nephrogenic systemic fibrosis. Arch Dermatol. 2009;145:1164–9.
91. Wallach D, Vignon-Pennamen MD. From acute febrile neutrophilic dermatosis to neutrophilic disease: forty years of clinical research. J Am Acad Dermatol. 2006;55:1066–71.
92. Cohen PR. Sweet's syndrome–a comprehensive review of an acute febrile neutrophilic dermatosis. Orphanet J Rare Dis. 2007;2:34.
93. Magro CM, Kiani B, Li J, et al. Clonality in the setting of Sweet's syndrome and pyoderma gangrenosum is not limited to underlying myeloproliferative disease. J Cutan Pathol. 2007;34:526–34.
94. Magro CM, De Moraes E, Burns F. Sweet's syndrome in the setting of CD34-positive acute myelogenous leukemia treated with granulocyte colony stimulating factor: evidence for a clonal neutrophilic dermatosis. J Cutan Pathol. 2001;28:90–6.
95. Requena L, Kutzner H, Palmedo G, et al. Histiocytoid Sweet syndrome: a dermal infiltration of immature neutrophilic granulocytes. Arch Dermatol. 2005;141:834–42.

96. Kaune KM, Baumgart M, Gesk S, et al. Bullous Sweet syndrome in a patient with t(9;22)(q34;q11)-positive chronic myeloid leukemia treated with the tyrosine kinase inhibitor nilotinib: interphase cytogenetic detection of BCR-ABL- positive lesional cells. Arch Dermatol. 2008;144:361–4.
97. van Kamp H, van den Berg E, Timens W, et al. Sweet's syndrome in myeloid malignancy: a report of two cases. Br J Haematol. 1994;86:415–7.
98. Liu D, Seiter K, Mathews T, et al. Sweet's syndrome with CML cell infiltration of the skin in a patient with chronic-phase CML while taking Imatinib Mesylate. Leuk Res. 2004;28 Suppl 1:S61–63.
99. Urano Y, Miyaoka Y, Kosaka M, et al. Sweet's syndrome associated with chronic myelogenous leukemia: demonstration of leukemic cells within a skin lesion. J Am Acad Dermatol. 1999;40:275–9.
100. Nogita T, Morioka N, Ishibashi Y, et al. Pelgeroid-like anomalous cells in the diagnosis of neutrophilic dermatosis associated with myelodysplastic syndrome. Int J Dermatol. 1992;31:864–5.
101. Cappel MA, Gibson LE. Myeloid leukemia cutis diagnosed by fluorescent in-situ hybridization: presenting with pathergy and mimicking 'histiocytoid Sweet's syndrome. Am J Dermatopathol. 2009;31:412 [abstract].

Chapter 15
Infectious Diseases of the Skin I: Dermatophytosis/Onychomycosis

Pranab K. Mukherjee, Nancy Isham, and Mahmoud A. Ghannoum

Dermatophytes are a unique group of fungi that infect keratinous tissue, including skin, hair, and nails, resulting in cutaneous mycoses called dermatophytoses, tinea, or ringworm infections. This closely-related group of organisms can be categorized into one of three genera: *Trichophyton, Microsporum,* and *Epidermophyton.* Species within these genera that do not invade keratinous tissue are, by definition, not regarded as dermatophytes. As with a number of fungi, dermatophytes may exhibit two phases in their life cycle: the anamorph state (imperfect or asexual phase), which is isolated in the laboratory; and the teleomorph state (perfect or sexual phase). Not all of the teleomorphs for dermatophyte species have been identified, but the generic name for both *Trichophyton* and *Microsporum* is *Arthroderma* [1]. Dermatophyte infections are generally named according to the anatomic locations involved. For example, infection of the feet, nails, scalp/eyebrows/eyelashes, groin, and other body sites are termed tinea pedis, tinea unguium, tinea capitis, tinea cruris (intertrigo), and tinea corporis, respectively. A single dermatophyte species may infect several anatomic sites, and different species can result in clinically identical lesions.

Onychomycosis is a common, chronic, highly resistant fungal infection in which affected nails become discolored, brittle, thickened, and friable. The disease rarely resolves spontaneously and recurrence after treatment is common. Onychomycosis is most often caused by dermatophytes, although *Candida albicans* and certain nondermatophytic fungi can also be involved [2]. Most dermatophytic nail infections are caused by *T. rubrum* (80% of cases), in addition to *T. mentagrophytes* and *E. floccosum*. Onychomycosis and tinea pedis are widespread in developed countries, with nearly 10% of the population being infected at any one time [3–5]. Tinea pedis is present in nearly 50% of patients with onychomycosis [6]. In susceptible individuals, many cases of toenail fungus begin as tinea pedis [2, 7].

The epidemiology of these infections is influenced by several variables, including geographical region, causative organisms, and age of the infected patients [8–10]. *T. rubrum* is the most common cause of dermatophytoses [8–10]. In a recent study, Panackal et al. [11] performed a cross-sectional analysis of cutaneous fungal infections in the USA during the period 1995–2004, using data from the National Ambulatory Medical Care Survey (NAMCS) and National Hospital Ambulatory Medical Care Survey (NHAMCS). These analyses showed that tinea unguium, tinea corporis, tinea pedis, tinea capitis, and tinea cruris represented 23.2%, 20.4%, 18.8%, 15.0%, and 8.4%, respectively, of all cutaneous fungal infections. Tinea capitis was significantly more common among black than white populations [11]. In a separate study, Neji et al. [12] showed that between 1998

M.A. Ghannoum (✉)
Center for Medical Mycology, Department of Dermatology, University Hospitals Case Medical Center, Case Western Reserve University, 11100 Euclid Avenue, Lakeside 3500, Cleveland, OH 44106, USA
e-mail: Mahmoud.Ghannoum@UHhospitals.org

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_15,

and 2007, the most common type of dermatophytosis (in Sfax, Tunisia) was onychomycosis (30.3%), followed by tinea pedis (24.8%), intertrigo (21.7%), tinea corporis (11.4%), and tinea capitis (9.6%). The most isolated dermatophyte was *T. rubrum* (74.5%), followed by *T. violaceum* (7.9%), *T. mentagrophytes* (7.5%), *M. canis* (3.8%), *E. floccosum* (0.7%), and *T. verrucosum* (0.54%). Other species occasionally isolated were: *T. schoenleinii*, *T. tonsurans*, *M. audouinii,* and *M. ferrugineum*. Godoy-Martinez et al. [13] analyzed the incidence of onychomycosis in Sao Paulo, Brazil between 1996 and 1999, and reported that the most common pathogens isolated were yeasts, found in 52% of positive cultures (*C. albicans,* 18.3%; *C. parapsilosis,* 13.8%; other species of *Candida,* 15.4%; and other, 4.6%), followed by dermatophytes in 40.6% of positive cultures (*T. rubrum*, 33.2%; *T. mentagrophytes*, 6.3%; and other, 1.2%). Nondermatophyte molds were isolated in 7.4% of positive cultures (*Fusarium* spp., 4.5%; *Nattrassia mangiferae,* 2.3%; *Aspergillus* spp., 0.6%). These investigators demonstrated that *T. rubrum* was the primary agent causing toenail onychomycosis, and that *Candida* spp. were the main causative agents of fingernail onychomycosis in this region. Zaki et al. [14] examined dermatophyte infections in Cairo, Egypt during 2004–2005, and reported that the most common dermatophyte infection diagnosed was tinea capitis (76.4%), followed by tinea corporis (22.3%), and tinea unguium (1.2%). *T. violaceum* was the most frequently isolated dermatophyte species (71.1%), followed by *M. canis* (21.09%), *T. rubrum* (6.2%), and *M. boulardii* (0.49%); both *E. floccosum* and *T. tonsurans* were each only rarely isolated (0.24%). Taken together, these studies clearly show that the epidemiology of dermatophyte infections is influenced by geographic location.

An extensive review of the classification, geographic distribution, and clinical manifestations of dermatophytes is beyond the scope of this chapter. Instead, this chapter focuses on traditional and more recent molecular methods for the diagnosis of dermatophyte infections in humans. Traditional methods to identify dermatophytes in the laboratory are based on detection of fungal elements by direct microscopy of clinical specimens, combined with culture-based identification. Direct microscopy, while rapid and cost-efficient, does not differentiate between different genera and species, and can product false-negative results. Culture-based identification is hampered by the fact that many dermatophytes are slow-growing, and need specialized media and other growth conditions for sporulation, and may also be associated with false-negative results. The sensitivity of traditional methods used for diagnosis of onychomycosis varies from 85% to 92% for periodic acid-Schiff (PAS) stain, 57–90% for potassium hydroxide (KOH) dissolution and centrifugation combined with PAS, 53% for fluorescent brightener (calcofluor white), 53% for chlorazol black E, and 23–59% for culture-based methods (dependent on the medium used; 32% for Mycosel agar, 23% for Littman-oxgall agar) [15, 16]. Similar sensitivities have been reported for other cutaneous dermatophytoses [17–20].

Recent technological advances have demonstrated that molecular methods (which are much faster and more specific than traditional identification methods) may be an attractive alternative for rapid and accurate identification of dermatophytes. In this chapter, we briefly summarize the traditional methods for identification of dermatophytes, and present an overview of the state-of-the-art in the molecular methods currently being developed for this purpose.

Traditional Methods

Traditional laboratory methods to demonstrate dermatophytes are based on the detection of fungal elements by direct microscopic analysis of clinical specimens, combined with culture-based identification. Dermatophyte isolates can be identified to genus/species levels by colony appearance, Scotch tape preparation, growth patterns on *Trichophyton* and/or urea agar slants, and hair perforation tests.

Direct Microscopy

One of the simplest methods to identify fungi in clinical samples is by exposure to KOH, which allows the fungal elements to be visualized under light microscopy. With this method, 10% KOH is added to a small sample of clinical material on a glass slide, covered with a cover slip, and incubated at room temperature for 3 min to allow for digestion of host cells. Slides are then examined microscopically at 400× magnification under phase-contrast for the presence of septate hyphae (2–4 μm in diameter) or fungal spores, indicating the presence of fungal disease [21]. Nail samples infected with nondermatophyte molds, such as *Scytalidium, Scopulariopsis brevicaulis,* and certain *Aspergillus* spp., often demonstrate irregular filaments and swollen nodules, which must be distinguished from the cylindrical filaments or regular chains of spherical conidia exhibited by dermatophyte species. The use of calcofluor white stain increases the sensitivity of the direct microscopic exam; however, this requires a microscope equipped with a mercury vapor lamp and broadband excitation filters to achieve radiation in the range of 300–412 nm [22]. The colony and microscopic morphologies of the major dermatophytes are summarized in Table 15.1, and representative pictures are shown in Fig. 15.1. Direct microscopy, while rapid and cost-efficient, does not differentiate between different genera and species, and can product false-negative results.

Culture-Based Methods

The remainder of the clinical sample that is not used for microscopic examination is plated onto selective and nonselective fungal media. Media selective for dermatophytes, such as Mycosel and Mycobiotic agar (Becton Dickinson, Franklin Lakes, NJ), contain cycloheximide to inhibit the growth of saprophytic molds (i.e., *Penicillium* and *Aspergillus*). Nonselective media, such as potato dextrose agar and Sabouraud dextrose agar, may have antibiotics added to them in order to inhibit bacterial contamination. Dermatophyte test medium (DTM), which is supplemented with cycloheximide, gentamicin, and chlortetracycline, was designed for rapid testing of dermatophytes; however, its use is not recommended, as isolates often exhibit atypical colonial and microscopic characteristics, and many nondermatophytes will also turn this medium red in color (giving false-positive results) [23]. Cultures for dermatophytes should be incubated at 30°C for a total of 4 weeks before being considered mycologically negative. Nonetheless, the majority of positive cultures grow within 1–2 weeks. Culture-based identification is hampered by the fact that many dermatophytes are slow-growing, and require specialized media and other growth conditions for sporulation. Importantly, false-negative culture results can delay appropriate treatment.

Scotch Tape Preparation

Fungal isolates are often identified by distinguishing microscopic characteristics, such as (a) the appearance of the hyphae and (b) the production of conidia. The Scotch tape preparation is an easy means of examining a fungal colony for microscopic structures [24]. This preparation involves cutting a piece of clear Scotch tape, which is then folded back on itself with the adhesive-side turned outward, and pressed onto the surface of the colony and pulled away. Aerial hyphae of the colony remain stuck to the adhesive surface. The tape is placed adhesive-side down into a drop of lactophenol-cotton blue or KOH, previously placed on the center of a glass slide. The slide is examined under microscopy for the presence of septate hyphae, chlamydoconidia, microconidia, and/or macroconidia.

Table 15.1 Colony and microscopic morphology of common dermatophytes (see Fig. 15.1).

		Microscopic morphology		
Dermatophyte	Colony morphology	*Hyphae*	*Microconidia*	*Macroconidia*
Trichophyton rubrum	The surface is granular or fluffy, white to buff. The reverse is deep red or purplish, or occasionally brown, yellow-orange, or colorless. Pigment production is better seen on potato dextrose agar than Mycosel agar	Septate	Tear-shaped (2–3.5 × 3–5.5 μm), usually form singly along the sides of the hyphae	Narrow and thin-walled (4–8 × 40–60 μm), with parallel sides and 4–10 cells. They may be abundant, rare, or absent
T. mentagrophytes	The surface may be tan and powdery, becoming yellowish, or white and downy. The powdery form exhibits concentric and radial folds. Reverse is usually brownish-tan, but may be colorless, yellow, or red	Septate	Round (4–6 μm in diameter) and clustered on branched conidiophores. Microconidia in fluffy strains are smaller, fewer in number, and tear-shaped	Sometimes present; cigar-shaped and thin-walled (4–8 × 20–50 μm), have narrow attachments to hyphae, and contain 1–6 cells. Coiled spiral hyphae are often seen
T. tonsurans	Colonies may be white, yellow, rose, or brown. The surface is usually suede-like, with radial or concentric folds. The reverse is usually reddish-brown, with pigment occasionally diffusing into the medium	Septate, with many conidia formed along the hyphae or on short perpendicular conidiophores	Teardrop or club-shaped, but may enlarge into "balloon" forms. Intercalary and terminal chlamydoconidia are common	Rare, irregularly shaped, and slightly thick-walled

Epidermophyton floccosum	The surface of the colony is brownish-yellow to olive-grey. It is folded in the center with radial grooves, becoming velvety. The reverse of the colony is orange to brown, sometimes with a thin yellow border	Septate	None	Smooth, thin to slightly thick-walled, and club-shaped with rounded ends. They contain 2–6 cells, and are found singly or in characteristic clusters
Microsporum canis	Surface is white, coarsely fluffy or furry, with yellow pigment at the edges and closely-spaced radial grooves. The reverse is deep yellow to brown	Septate, with numerous spindle-shaped, rough thick-walled macroconidia (10–25 × 35–110 μm)	Club-shaped and smooth-walled, form sparsely along the hyphae	Characteristically taper to spiny, bent knob-like ends that resemble "dog snouts"

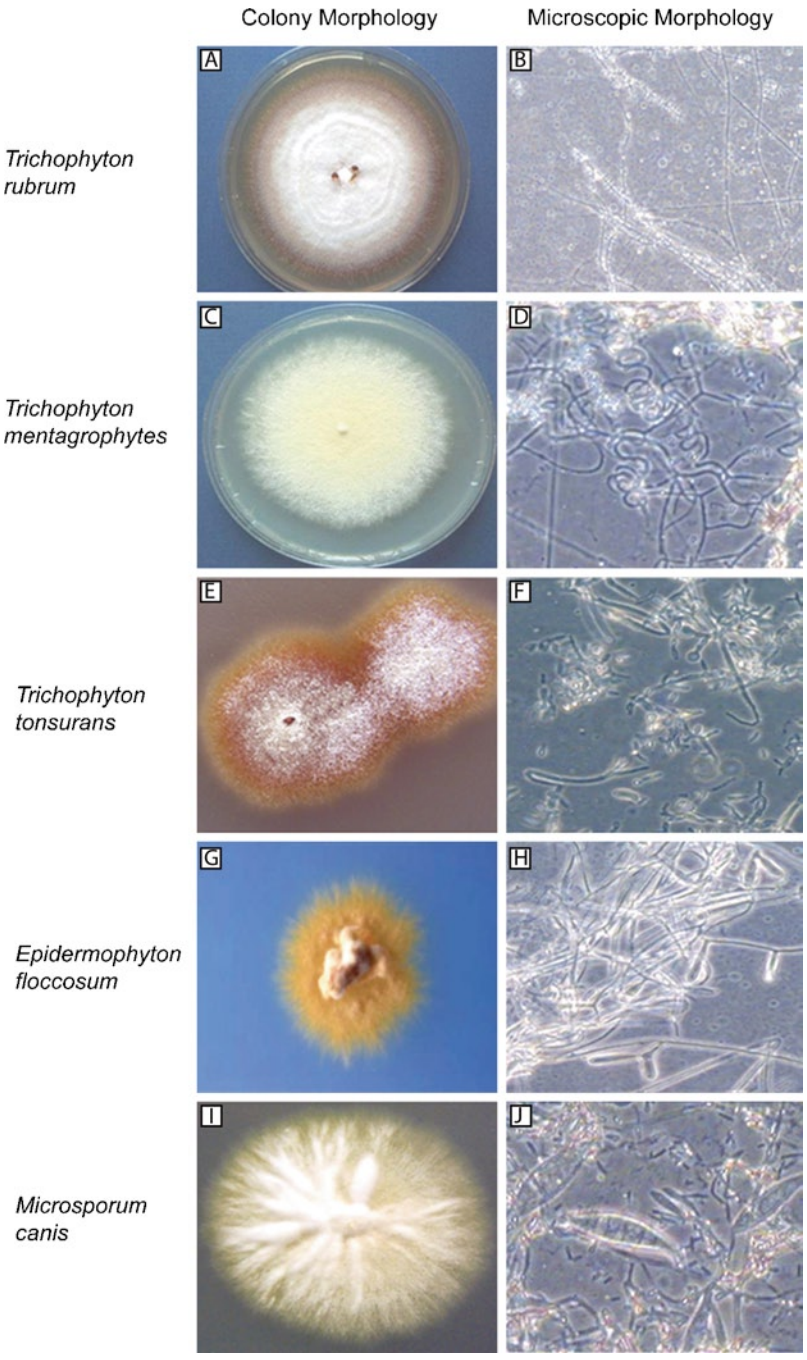

Fig. 15.1 Colony and microscopic morphologies of common dermatophytes (see Table 15.1)

Trichophyton Agar Slants

The *Trichophyton* agar slants are a series of media containing different vitamins or amino acids used to differentiate between *Trichophyton* species. The requirements of different dermatophyte isolates for these compounds are demonstrated by their growth enhancement on the supplemented

media, as compared to poorer growth on a corresponding basal control medium [24]. For example, *T. tonsurans* exhibits more luxuriant growth on *Trichophyton* #4 agar than on *Trichophyton* #1 agar, because of a partial requirement for thiamine. The slants are incubated at 30°C for 7–14 days. If the isolate is suspected to be *T. verrucosum,* which demonstrates long chains of chlamydoconidia and antler-like branches under microscopic examination, the slants should be incubated at 37°C. Growth on the surface of the slants is often graded as follows: (a) 4+, good growth; (b) 2+, intermediate growth; (c) ±, trace; and (d) 0, absence of growth.

Hydrolysis of Urea

Christensen's urea agar slants are also used to differentiate between *Trichophyton* species. Urea hydrolysis in Christensen's medium causes a rise in pH and an indicator color change, following the formation of ammonia [24]. For example, *T. mentagrophytes* produces a bright pink color (positive), while *T. rubrum* will produce no color change (negative). With this method, slants are inoculated as in *Trichophyton* agar slants and incubated at 30°C for 7 days. Change of color from yellow to bright pink indicates a positive reaction.

Hair Perforation Test

Some species of dermatophytes, such as *T. mentagrophytes, M. canis,* and *M. gypseum,* produce specialized hyphae called "perforating organs," which are capable of perforating hair *in vitro.* The hair perforation test is a diagnostic assay to differentiate these species from other dermatophytes that do not perforate hair *in vitro* [25]. This test is performed by preparing a lawn of the test isolate on the surface of a potato dextrose agar (PDA) plate. Several sterile hairs taken from a prepubescent blonde child are then placed onto the fungal lawn. The plates are incubated at 30°C for up to 28 days and examined every 7 days. Some of the hairs are mounted in a drop of lactophenol-cotton blue on a cover-slipped slide. The presence of cone-shaped perforations perpendicular to the long axis of the hair indicate a positive hair-perforation reaction.

Histopathology and Immunohistochemistry

Tissue samples (i.e., skin biopsies and nail clippings) may be taken, and submitted for hematoxylin and eosin (H + E) and histochemical (i.e., PAS and GMS) stains to evaluate for fungal elements. Histopathology and histochemical stains can be used to confirm the presence of fungi, but cannot identify the specific pathogen. Adjunct immunohistochemistry, using specific antibodies directed against dermatophyte components, can be employed for identification of particular fungi [26]. Bound antibodies can be detected by direct immunofluorescence or other indirect methods, such as biotin-avidin reactions.

Flow Cytometry

Flow cytometry has also been used to identify dermatophytes. With this approach, the tissue sample is removed, solubilized with detergent (i.e., Tween-40), and filtered to collect single fungal cells that are then stained with (a) propidium iodide (PI) for DNA and (b) fluorescein isothiocyanate (FITC) for

proteins. The samples are then analyzed with a flow cell sorter coupled with appropriate software for data acquisition. Pierard et al. [26] used a method based on dual flow cytometry with PI and FITC on onychomycosis-associated fungi, and identified fungal "fingerprints" related to granulosity and cell size, in addition to protein and DNA content. A number of disadvantages associated with the immunohistochemical and flow cytometric detection of dermatophytes include the requirement for fungal-specific monoclonal antibodies, availability of standard and reference data, and the requirement for large quantities of the starting material [27].

Molecular Methods

Nonmolecular methods for the identification of dermatophytes are plagued by several pitfalls – the most notable of which is low sensitivity. The sensitivity of culture methods can be influenced by: (a) variable requirements for different growth media, pH, and temperature; (b) time-frame in transferring samples to growth media; (c) availability of optimum amounts of clinical material; (d) possible bacterial contamination; (e) growth of nondermatophyte fungi; and (f) presence of nonviable dermatophytes [28].

The identification of different dermatophyte species and strains can also be performed using nucleic acid-based approaches – molecular changes are considered more stable and precise than phenotypic characteristics [28, 29]. Notably, the use of polymerase chain reaction (PCR)-based molecular methods increases the sensitivity 1,000-fold compared with PAS staining [28]. Molecular methods provide inherent advantages over traditional approaches since they are not dependent on micro organism growth for the purposes of identification. In addition, molecular identification strategies may be adapted for analysis of formalin-fixed paraffin-embedded (FFPE) tissue samples [29]. However, these methods also have some unique disadvantages, primarily due to their extremely high sensitivities. False positivity may result from amplification of any contaminating DNA. Another disadvantage is that a fungus positively identified by PCR amplification may not be the definite cause of infection, since classic PCR can also amplify DNA from dead cells. Recent approaches based on quantitative real-time PCR (qPCR) attempt to overcome this pitfall, by focusing on the gene transcripts rather than the genes themselves. Finally, although PCR is a mainstay of most research laboratories, its widespread use in clinical laboratories is hampered by the infrastructure costs required to set up these assays. Recent technological advances are likely to go a long way in demonstrating the applicability of molecular diagnostic methods for the routine identification of dermatophytes.

Common Technologies

The ability of PCR to amplify minute amounts of target DNA from scant tissue specimens renders approaches based on this technique particularly attractive for the identification of dermatophytes. These methodologies include: (a) nested and seminested PCR; (b) restriction fragment length polymorphism (RFLP) analysis; (c) random amplification of polymorphic DNA (RAPD); (d) Southern blot hybridization; (e) electrophoretic mutation scanning; (f) PCR-enzyme linked immunosorbent assay (PCR-ELISA); (g) PCR-reverse line blot; (h) sequence analyses (rDNA, rRNA regions, and MnSOD); and (i) multiplex qPCR. The most common PCR-based approaches and primer sets used for identification of dermatophytes are outlined in Table 15.2. PCR amplicons are generally visualized by agarose (AE) or polyacrylamide (PAGE) gel electrophoresis. In addition, non-PCR-based approaches, such as oligonucleotide microarray analysis, loop-mediated isothermal amplification (LAMP) and mass spectrometry, have been utilized.

Table 15.2 Representative PCR-based approaches and primer sets used for identification of dermatophytes

Approach	Target	Details	Organisms identified	Study
PCR	18S rRNA (581-bp fragment)	Primer set: TR1: 5′-GTTTCTAGGACCGCCGTA TR2: 5′-CTCAAACTTCCATCGACTTG	*T. rubrum* *T. mentagrophytes* *T. verrucosum* *T. terrestre* *M. canis* *M. gypseum* *E. floccosum,* Yeasts	[35, 36]
	rDNA ITS	LR1: GGTTGGTTTCTTTTCCT SR6R: AAGTAAAAGTCGTAACAAGG	*Trichophyton* spp.	[54]
	rDNA ITS1	18SF1: AGGTTTCCGTAGGTGAACCT 58SR1: TTCGCTGCGTTCTTCATCGA	*Trichophyton* spp. *Microsporum* spp. *Epidermophyton* spp.	[92–94]
	CHS1 gene (620-bp fragment)	Primer 1: CTGAAGCTTACT(ACG)ATGTAT(C)AAT(C)GAG(A)GAT(C) Primer 2: GTTCTCGAG(C)TTT(A)GTA(C)TTC(A)GAA(A)GTT(T)CTG	*Trichophyton* spp *Microsporum* spp.	[95]
	28S rRNA	Primer 1: GGTTGGTTTCTTTTCCT Primer 2: AAGTAAAAGTCGTAACAAGG	*T. mentagrophytes*	[48]
Nested PCR	Topoisomerase II	Common primer set (dPsD1) for species Species-specific primer sets (PsT and PsME)	*T. rubrum* *T. mentagrophytes* *T. violaceum* *M. gypseum* *M. canis* *E. floccosum*	[96]
RT-nested PCR	*ACT*–associated exon/intron	171-bp amplicon External primers: DF: ATCATCTTCGAGACCTTCAACGCCCCAG CA18S86F: ACTGCGAATGGCTCATTAAATCAG Internal primers: NF: TATCCACGTCACCACCTACAA NR: ATGATCTTGACCTTCATCGAC	*Trichophyton* spp. *Microsporum* spp. *Epidermophyton* spp.	[65]

(continued)

Table 15.2 (continued)

Approach	Target	Details	Organisms identified	Study
qPCR	rDNA ITS1	Species-specific primers and probes	*Trichophyton* spp. *Microsporum* spp. *Epidermophyton* spp.	[69]
PCR-RLB	rDNA ITS1	Genus-specific and species-specific probes	*Trichophyton* spp. *Microsporum* spp. *Epidermophyton* spp.	[70]
PCR-AFCE	rDNA ITS2	ITS86: GTGAATCATCGAATCTTTGAAC ITS4: TCCTCCGCTTATTGATAGC	*Candida* *Aspergillus* *Trichophyton* spp. *Microsporum* spp. *Epidermophyton* spp.	[63]
PCR-ELISA	Topoisomerase II gene	Species-specific primers	*T. rubrum* *T. interdigitale* *T. violaceum* *M. canis* *E. floccosum*	[64]
AP-PCR RAPD	Random primer	GAGCCCGACT	*T. mentagrophytes* var. *interdigitale* *T. mentagrophytes* var. *mentagrophytes*	[37]
	Random primer	OPAA11: ACCCGACCTG OPD18: GAGAGCCAAC	*T. rubrum* *T. mentagrophytes* *T. tonsurans* *Microsporum* spp. *E. floccosum*	[38]
	Random primer	OPI-03: CAGAAGCCCA OPI-07: CAGCGACAAG OPI-17: GGTGGTGATG OPI-20: AAAGTGCGGG OPK-01: CATTCGAGCC OPK-08: GAACACTGGG OPK-09: CCCTACCGAC OPK-17: CCCAGCTGTG OPK-19: CACAGGCGGA OPK-20: GTGTCGCGAG	*T. mentagrophytes* *T. rubrum* *T. tonsurans* *M. canis* *Arthroderma benhamiae* *A. vanbreuseghemii* *A. gypsea* *A. otoae*	[41]

	Random primer	$(AC)_{10}$: ACACACACACACACACAC $(GTG)_5$: GTGGTGGTGGTGGTG M13 core sequence: GAGGGTGGCGGTTCT AP3: TCACGATGCA	*T. mentagrophytes* *T. tonsurans*	[42]
	Random primer	T3B: AGGTCGCGGGTTCGAATCC	*Trichophyton* spp. *Microsporum* spp. *Epidermophyton* spp.	[54]
	Random primer	$(GACA)_4$	*Trichophyton* spp. *Microsporum* spp. *Epidermophyton* spp.	[44]
AFLP	EcoRI- and MseI-digested genomic DNA fragments	Pair 1: EcoRI-TGC (GACTGCGTACCAATTC**TGC**) MseI-CTA (GATGAGTCCTGAGTAA**CTA**) Pair 2: EcoRI-ATG (GACTGCGTACCAATTC**ATG**) MseI-TGC (GATGAGTCCTGAGTAA**TGC**)	*T. mentagrophytes* *T. tonsurans*	[54]
RFLP	18S rRNA gene/HaeIII digestion	Primers: TR1: GTTTCTAGGACCGCCGTA TR2: CTCAAACTTCCATCGACTTG NS5: AACTTAAAGGAATTGACGGAAG NS6: GCATCACAGACCTGTTATTGCCTC B2F: ACTTTCGATGGTAGGATAG B4R: TGATCGTCTTCGATCCCCTA CA18S86F: ACTGCGAATGGCTCATTAAATCAG CA18S1176R: AGTCAAATTAAGCCGCAG	*T. rubrum* *T. mentagrophytes* *Candida albicans* *Aspergillus flavus*	[27]
	18S rDNA	NS5: AACTTAAAGGAATTGACGGAAG ITS4: TCCTCCGCTTATTGATATGC	*T. rubrum* strains	[55]

PCR polymerase chain reaction, *RT* reverse transcription, *qPCR* quantitative real-time PCR, *PCR-RLB* PCR-reverse line blot, *AFCE* automated fluorescent capillary electrophoresis, *ELISA* enzyme-linked immunosorbent assay, *AP* arbitrarily primed, *RAPD* random amplified polymorphic DNA, *AFLP* amplified fragment length polymorphism, *RFLP* restriction fragment length polymorphism

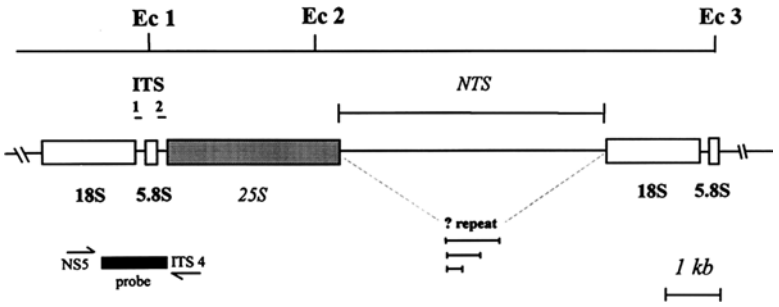

Fig. 15.2 Regions of ribosomal DNA (rDNA). *Eco*RI restriction map of the rDNA repeat unit of *Trichophyton rubrum*. The fragment between restriction sites Ec 1 and Ec 2 may encompass the whole of the 25S gene and is of constant length (~3 kb) in all strains. The fragment between sites Ec 2 and Ec 3 represents the NTS region and the 18S gene, and shows fragment length polymorphisms in several *T. rubrum* strains. One hypothesis to account for these length variations is the presence of a repetitive element located in the NTS region (From Jackson et al. [55]. Reprinted with permission from the American Society for Microbiology. Copyright © 1999)

Ribosomal DNA

The most common target for PCR-based identification of dermatophytes is ribosomal DNA (rDNA), including genes encoding ribosomal RNA, and the intergenic transcribed and nontranscribed regions (Fig. 15.2). These target regions present the attractive advantage of being present in all fungi, yet exhibit substantial hypervariabilities that allow rapid identification of species and strains. PCR-based analyses of these genes are highly sensitive, since these targets are present in high copy numbers. Genes encoding for fungal ribosomal RNA (rRNA) belong to a multicopy gene family, consisting of sequential repetitive arrays of highly conserved sequences of DNA (8–12 kb each). Each repetitive unit codes for one major transcript (the 35S pre-RNA), that consists of two regions transcribed internally into the pre-RNA, but not retained in the mature RNA (Fig. 15.2). These internally transcribed spacers (ITS1 and ITS2) separate the 18S, 5.8S, and 25S regions. Therefore, each repetitive unit of the rDNA is organized as 18S-ITS1-5.8S-ITS2-25S. In addition, a nontranscribed spacer (NTS) region separates each repetitive unit. In some fungi, the NTS region also has a separately transcribed coding region for 5S RNA with variable position and direction of transcription. Of note, this 5S region is not detected in *T. rubrum*. While the repetitive units are used to identify fungi, the sequences of the ITS and NTS regions (i.e., the Tandemly Repetitive Sequences, TRS1 and TRS2) vary between different species and strains, and can be employed for species and/or strain identification. Several restriction sites are conserved within the rDNA genes, which makes it possible to clone these sequences for sequence-based identification.

Apart from rDNA genes, other specific genes that may be targeted for dermatophyte identification include subtilisin, DNA topoisomerase II, chitin synthase 1 (*CHS1*), and the polymorphic microsatellite marker, T1.

Mitochondrial DNA

Some early studies investigated the utility of mitochondrial DNA (mtDNA) analysis for identification of dermatophytes. In this regard, de Bievre et al. [30] showed that differences in mtDNA can be used to classify *T. rubrum* into two groups (I and II). However, subsequent mtDNA analysis was

unable to differentiate between *T. rubrum* Type II, *T. tonsurans*, and *A. vanbreuseghemii* – all of which exhibited identical profiles [31]. In addition, analysis of mtDNA regions did not allow distinction between the genera *Arthroderma* and *Nannizzia* [32]. Testing based on mtDNA can differentiate between pleomorphic strains, and some studies have suggested a potential use for this approach [33, 34]. However, the employment of mtDNA as a target for identification of different dermatophyte species and strains is limited [29].

PCR Amplification of Target Genes

In one of the first studies to report the use of molecular techniques to identify dermatophytes, Bock et al. [35] employed a PCR-based approach using a primer set targeting a fragment of the gene coding for the fungal small ribosomal subunit 18S rRNA. These primers (TR1, 5′-GTTTCTAGGACCGCCGTA; TR2, 5′-CTCAAACTTCCATCGACTTG) bind to sequences which are homologous within the fungi, but differ from corresponding DNA fragments of plants and animals, thereby minimizing cross-reactivity with other eukaryotes or prokaryotes. The amplified fragment is 581 base pairs (bp) in length and contains variable species-specific regions. The DNA of seven dermatophytes (*T. rubrum*, *T. mentagrophytes*, *T. verrucosum*, *T. terrestre*, *M. canis*, *M. gypseum,* and *E. floccosum*) and several yeast species was amplifiable using these primers, but not DNA from 42 normal human skin samples [35]. Furthermore, other DNA preparations from plants and animals did not show amplification reactions. In a follow-up study, investigators from the same group reported the identification of several yeasts, molds, and seven common dermatophytes, using TR1 and TR2 primers [36]. In addition, these investigators collected 69 routine skin and nail specimens, and showed that PCR was more sensitive than culture methods in detecting dermatophytes. Among 38 positive specimens, 35 were detected by PCR, while only 28 were detected by culture, demonstrating the potential clinical relevance of a PCR-based approach [36].

PCR Amplification of DNA Obtained Directly from Infected Tissues

A key element in PCR-based analysis is the isolation of template DNA – which can be accomplished from organisms that are cultured from nail or skin samples. However, culture of these organisms can take up to 4 weeks, thus delaying the diagnostic process. An alternative is direct isolation and amplification of template DNA from tissue specimens (hair, skin, or nails).

Baek et al. [27] evaluated the sensitivity and specificity of culture-independent PCR amplification of fungal DNA in differentiating between different onychomycosis-causing fungi. Affected nails were collected and cleaned with alcohol to remove potential contaminants. These investigators extracted DNA from both the nail plate and subungual hyperkeratotic material. As shown in Fig. 15.3, amplification of template DNA using different primers generated specific fragments of the fungal 18S rRNA gene. For example, primers TR1 and TR2 amplified a 581 bp region of the 18S rRNA for all fungi tested. Digestion of the amplicons with the restriction endonuclease HaeIII resulted in characteristic banding patterns, and allowed differentiation of *T. rubrum* isolates (Fig. 15.3). Combined digestion with two or more restriction enzymes allows differentiation between dermatophytes, yeasts, and molds.

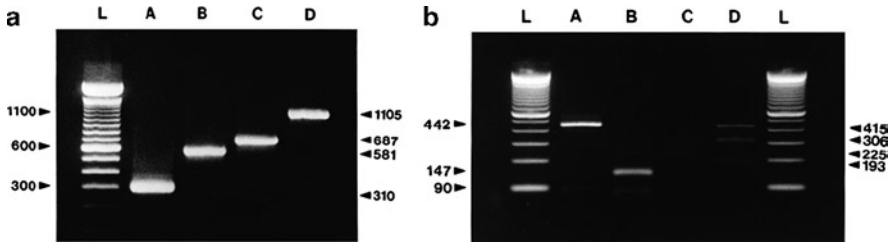

Fig. 15.3 PCR amplification of *T. rubrum* 18S rRNA gene and RFLP pattern. (**a**) Amplification products of *Trichophyton rubrum* using various universal primers for fungus-specific 18S rRNA gene. Lanes: L, 100 bp ladder; A, primers NS5 and NS6; B, primers TR1 and TR2; C, primers B2F and B4R; D, primers CA18S86F and CA18S1176R. (**b**) HaeIII digestion pattern of *T. rubrum* 18S rRNA gene according to amplified products of different primer pairs. Lanes: L, 100 bp ladder; A, primers TR1 and TR2; B, primers NS5 and NS6; C, primers B2F and B4R; D, primers CA18S86F and CA18S1176R (From Baek et al. [27]. Reprinted with permission from John Wiley and Sons. Copyright © 1998)

Arbitrarily Primed PCR/Random Amplified Polymorphic DNA

This approach involves low stringency PCR amplification of polymorphic DNA using a single primer of relatively short and arbitrary nucleotide sequence. The primer randomly, and usually only partially, binds to the template DNA and following the action of DNA polymerase, generates an array of short, nonspecific amplification products (amplicons) or random amplified polymorphic DNAs (RAPDs). These are strain-specific, and can be used as fingerprints to identify different strains within the same species. Several probes, including $(GACA)_4$, OPA-, OPD-, OPI- and OPK-series, and $(GTG)_5$, have been used to identify dermatophytes (see Table 15.2 for a list of representative probes).

Liu et al. [38] used the random primers OPAA11 (5′-ACCCGACCTG-3′) and OPD18 (5′-GAGAGCCAAC-3′) in an arbitrarily primed PCR (AP-PCR)-based approach to distinguish *T. rubrum*, *T. mentagrophytes,* and *T. tonsurans*. By examining 8 *Microsporum* spp., 16 *Trichophyton* species/subspecies, and *E. floccosum*, the authors demonstrated that, except for *T. rubrum*, *T. gourvilii,* and three *T. mentagrophytes* varieties, most of the dermatophyte fungi investigated formed distinct DNA banding patterns on gel electrophoresis [38]. Subsequent studies from this group demonstrated the utility of AP-PCR for identification of a wide array of dermatophytes [39, 40]. Zhong et al. [41] reported the use of OPI- and OPK-series probes to identify the most common dermatophytes (*T. rubrum, T. mentagrophytes*, *M. canis, Arthroderma,* and *E. floccosum*). Graser et al. [42] used the nonspecific $(AC)_{10}$, $(GTG)_5$, M13 core sequence, and AP3 primers to identify 17 species belonging to *Trichophyton*, *Microsporum*, and *Epidermophyton* genera. While all four primers tested were able to differentiate between species, the $(GTG)_5$ primer failed to distinguish between varieties of *T. mentagrophytes*. Mukherjee et al. [43] used the OPK-17 primer and performed RAPD identification of six *T. rubrum* isolates, obtained sequentially from an onychomycosis patient who had failed oral terbinafine therapy. All six sequential isolates demonstrated identical results.

Faggi et al. [44] demonstrated the utility of $(GACA)_4$ as a probe to identify the most common dermatophytes. They reported that this primer was able to amplify and differentiate between *M. canis*, *M. gypseum*, *T. rubrum*, *T. ajelloi*, and *E. floccosum*, with no intraspecies variability [44]. In a subsequent study, the same group suggested that this primer can be used to analyze colonies that (a) do not demonstrate species-specific morphological characteristics and/or (b) are not identifiable with the classical methods [45]. Shehata et al. [46] compared the utility of ITS- and $(GACA)_4$-based PCR approaches in identifying species and strains of 21 dermatophyte isolates. These investigators determined the agreement of culture techniques with PCR-based methods, by amplification of ITS1,

5.8S, and ITS2 regions using the ITS1/ITS4 primer set, followed by MvaI endonuclease digestion (Fig. 15.4). The target region was amplified in all 21 strains tested, resulting in amplified products of approximately 690 bp in the *T. violaceum, T. rubrum*, and *T. mentagrophytes* species, and 740 bp for *M. canis* (Fig. 15.4). MvaI digestion of these amplified products from each of the four isolated species revealed unique restriction patterns, with no intraspecies variation (Fig. 15.4). In the same study, the authors demonstrated that $(GACA)_4$-based PCR could identify different *T. mentagrophytes* varieties [46]. As shown in Fig. 15.5, all of the studied strains were amplified, with 4–11 resulting bands that ranged from 300 to 2,500 bp in size. All *T. violaceum* strains demonstrated almost identical banding patterns, which consisted of three bright bands (of approximately 600, 900, and 1,000 bp) and one to three additional faint bands with sizes ranging from 1,600 to 2,500 bp (Fig. 15.5). *T. rubrum* strains could be distinguished from *T. violaceum* strains by the sizes of the

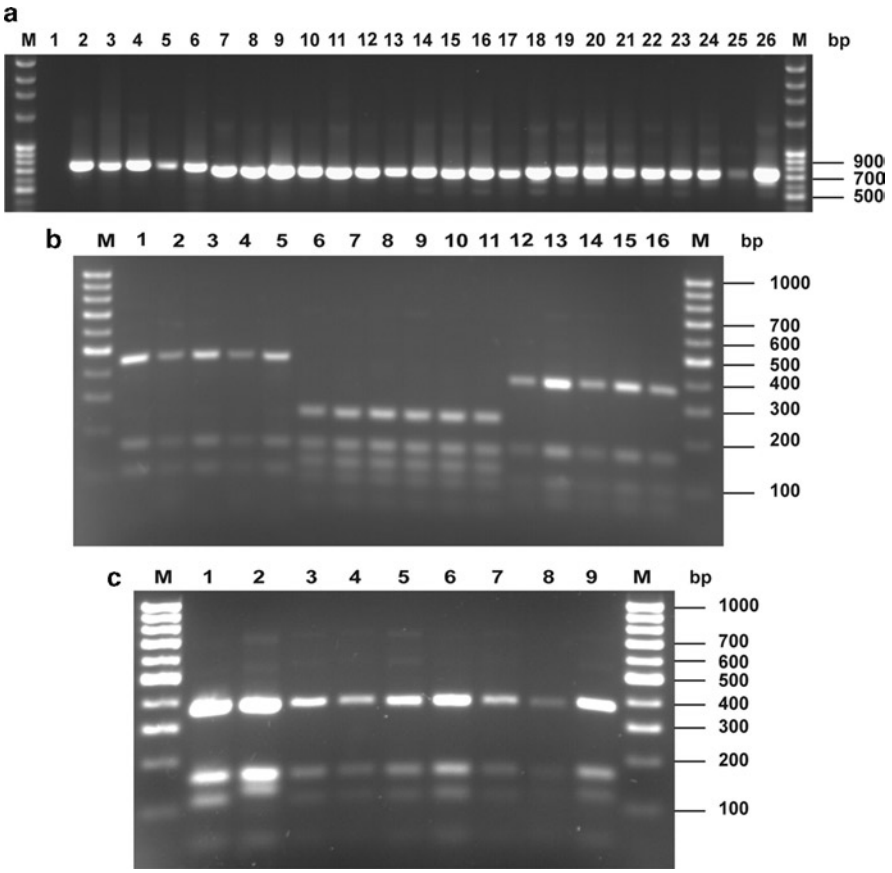

Fig. 15.4 Agarose gel electrophoresis analysis of PCR-amplified ITS regions of dermatophytes. (**a**) PCR products of the ITS1, 5.8S, and ITS2 regions of the four phenotypically identified species. Lanes: M, molecular weight marker (Fisher Scientific International, Inc.); 1, negative control (no template DNA); 2, *M. canis* MRL 2117; 3–6, *M. canis* clinical strains; 7, *T. mentagrophytes* ATCC 9533; 8–12, *T. mentagrophytes* clinical strains; 13, *T. rubrum* ATCC 28188; 14–17, *T. rubrum* clinical strains; 18, *T. violaceum* MRL 2135; 19–26, *T. violaceum* clinical strains. (**b**) MvaI restriction products of *M. canis, T. mentagrophytes*, and *T. rubrum* species. Lanes: M, molecular weight marker (Fisher Scientific International, Inc.); 1, *M. canis* MLR 2117; 2–5 *M. canis* clinical strains; 6, *T. mentagrophytes* ATCC 9533; 7–11, *T. mentagrophytes* clinical strains; 12, *T. rubrum* ATCC 28188; 13–16, *T. rubrum* clinical strains. (**c**) MvaI restriction products of *T. violaceum* isolates. Lanes: M, molecular weight marker (Fisher Scientific International, Inc.); 1, *T. violaceum* MRL 2135; 2–9, *T. violaceum* clinical strains (From Shehata et al. [46]. Reprinted with permission from the American Society for Microbiology. Copyright © 2008)

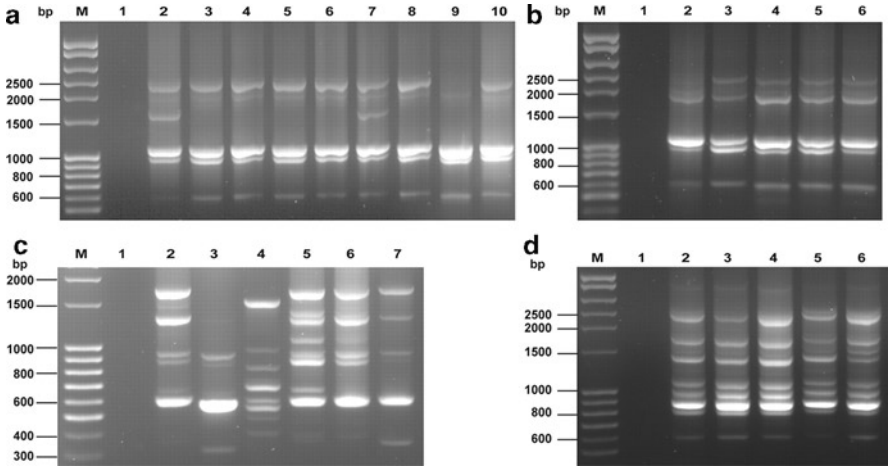

Fig. 15.5 Agarose gel electrophoresis for PCR products using the $(GACA)_4$ primer. (**a**) *T. violaceum* strains. Lanes: M, molecular weight marker (Fisher Scientific International, Inc.); 1, negative control (no template DNA); 2, *T. violaceum* MRL 2135; 3–10, *T. violaceum* clinical strains. (**b**) *T. rubrum* strains. Lanes: M, molecular weight marker (Fisher Scientific International, Inc.); 1, negative control (no template DNA); 2, *T. rubrum* ATCC 28188; 3–6, *T. rubrum* clinical strains. (**c**) *T. mentagrophytes* strains. Lanes: M, molecular weight marker (Fisher Scientific International, Inc.); 1, negative control (no template DNA); 2, *T. mentagrophytes* ATCC 9533; 3–7, *T. mentagrophytes* clinical strains. (**d**) *M. canis* strains. Lanes: M, molecular weight marker (Fisher Scientific International, Inc.); 1, negative control (no template DNA); 2, *M. canis* MRL 2117; 3–6, *M. canis* clinical strains (From Shehata et al. [46]. Reprinted with permission from the American Society for Microbiology. Copyright © 2008)

three largest bands (1,900–2,500 bp) (Fig. 15.5). These studies suggested that $(GACA)_4$-based PCR has utility as a simple, rapid, single-step method for identification of dermatophyte species, as well as for differentiation of *T. mentagrophytes* variants.

PCR Amplification: Sequencing

Sequencing of amplified DNA fragments is another dermatophyte identification method. This approach is especially useful when ascertaining phylogenetic relationships between different species. In this regard, Kano et al. [47] used PCR to amplify a 620 bp genomic DNA fragment of the *CHS1* gene of the dermatophytes, *A. benhamiae*, *A. simii*, *A. vanbreuseghemii*, *T. mentagrophytes* var. *interdigitale* (*T. interdigitale*), and *T. rubrum*, and sequenced the resulting amplicons. Sequencing analysis showed >90% similarity between the species. Phylogenetic analysis of these sequences revealed that *A. benhamiae*, *A. simii*, *A. vanbreuseghemii*, and *T. rubrum* were genetically distinct from one another, but *T. interdigitale* was genetically very close to *A. vanbreuseghemii* [47]. Moreover, the *CHS1* gene fragment of *T. rubrum* contains a restriction site for the endonuclease HinfI, while fragments of *A. benhamiae*, *A. simii, A. vanbreuseghemii*, and *T. interdigitale* lacked this restriction site. In another recent study, Ninet et al. [48] amplified and sequenced a part of the large-subunit rRNA (28S rRNA), and showed that two taxa (type I and II strains *vs.* type III strains) causing distinct dermatophytoses (tinea pedis and unguium *vs.* tinea manuum, corporis, cruris, faciei, barbae, and capitis) were clearly distinguishable among isolates of the *T. mentagrophytes* species complex. Sequencing of two or more target genes can also be used to differentiate between clades of dermatophytes. Frealle et al. [49] differentiated 41 *T. mentagrophytes* isolates of either

human (14 isolates) or animal (27 isolates) origin by DNA sequencing of (a) the variable ITS regions (ITS1-ITS2) flanking the 5.8S rDNA and (b) the housekeeping gene encoding the manganese-containing superoxide dismutase (MnSOD), an enzyme involved in defense against oxidative stress. These investigators classified the isolates into two major clades exhibiting a similar topology, with a higher variability when the ITS marker was used. Summerbell et al. [50] also used the ITS regions to differentiate between anthropophilic and zoophilic dermatophytes, and showed that resequencing the ITS regions of several anomalous isolates eliminates the confusion between anthropophilic *T. tonsurans* and the horse-associated *T. equinum*. Zaki et al. [14] characterized dermatophyte infections in 403 patients, and identified the fungal species by sequencing the ITS1-5.8S-ITS2 rDNA region. Other studies have targeted the complete ITS region [51], subtilisin gene [52], and 28S rDNA [53] for identification of dermatophytes.

Amplified Fragment Length Polymorphism

The amplified fragment length polymorphism (AFLP)-based approach for dermatophyte identification involves digestion of genomic DNA with specific restriction endonucleases, followed by selective amplification of the resulting fragments. Graser et al. [54] used this method, in combination with PCR fingerprinting and sequencing of the ITS region of the ribosomal operon, in order to identify different *Trichophyton* species. Genomic DNA was digested with both EcoRI and MseI, with the resulting fragments ligated with enzyme-specific adapters, and amplified using AFLP-specific primers. PCR amplification was based on the primers $(AC)_{10}$, the M13 core sequence, and T3B (derived from the intergenic spacer region of the tRNA). The fungal ITS region was amplified using the universal primers LR1 and SR6R, corresponding to positions 73–57 of the 25S and positions 1744–1763 of the 18S nuclear rDNA gene of *Saccharomyces cerevisiae*, respectively (Table 15.2). This combined approach was successful in validating the taxa around *T. mentagrophytes* and *T. tonsurans*, and reducing 24 species or varieties to 5 taxa, which were reclassified or synonymized as *T. tonsurans*, *T. interdigitale*, *T. mentagrophytes*, *T. simii,* and *T. erinacei* [42].

Restriction Fragment Length Polymorphism

Evaluation of interstrain restriction fragment length polymorphism (RFLP) in the spacer regions of fungal rDNA repeat units has been used for the typing of a range of clinically important species. Jackson et al. [55] differentiated between different dermatophyte strains by analyzing molecular variation in the rDNA repeats of *T. rubrum* and other dermatophytes, and length variations in the NTS region. *T. rubrum* genomic DNA was digested with EcoRI and transferred to nylon membrane (Southern blotting). The transferred blot was hybridized with a probe amplified from the small-subunit (18S) rDNA and adjacent ITS regions, using the universal primers NS5 and ITS4. The resulting 1,219 bp probe consisted of a 550 bp fragment from the 3′ end of the 18S rDNA plus the adjacent ITS1, 5.8S rDNA, and ITS2 regions. The rDNA RFLPs mapped to the NTS region of the rDNA repeat and appeared similar to those caused by short repetitive sequences in the intergenic spacers of other fungi. Additionally, these investigators amplified the contiguous ITS and 5.8S rDNA regions from 17 common dermatophyte species, using the universal primers ITS1 and ITS4. The resulting amplicons were digested with the restriction endonuclease MvaI, producing unique and easily identifiable fragment patterns for a majority of species. However, some closely related taxon pairs, such as *T. rubrum-T. soudanense* and *T. quinkeanum-T. schoenleinii* could not be distinguished.

Shin et al. [56] recently amplified the ITS region of dermatophytes, digested the amplified fragments with four restriction enzymes (BsYiI, DdeI, HinfI, and MvaI), and identified different species- and strain-specific RFLPs. These investigators were able to differentiate all tested species using any combination of two different restriction enzymes, except for *T. rubrum* and *T. raubitschekii*, both of which produced identical banding patterns after all four restriction enzyme digestions. In the case of *T. mentagrophytes,* MvaI and DdeI each produced two distinct RFLP patterns [56]. Brillowska-Dabrowska et al. [57] combined a two-step DNA extraction method and multiplex PCR to detect dermatophytes, specifically *T. rubrum*, in pure cultures and 118 clinically diseased nails (Fig. 3.3). Probes based on the fungal ITS1 region were used to detect *E. floccosum*, *M. audouinii*, *M. canis*, *M. gypseum*, *M. nanum*, *T. mentagrophytes*, *T. rubrum*, *T. schoenleinii*, *T. soudanense*, *T. terrestre*, *T. tonsurans*, *T. verrucosum*, and *T. violaceum.*

Other investigators have used the PCR-RFLP method to identify different dermatophyte species and strains, targeting topoisomerase II, ITS, and 28S rDNA [53, 58–62]. Monod et al. [53] used PCR/sequencing and RFLP analysis to demonstrate the presence of *Fusarium* spp. and other nondermatophyte fungi in nails. Nail fragments were dissolved in sodium sulfide solution, and the fungal DNA was extracted using a commercial kit. Universal primers were used to amplify fragments of the 28S rDNA, and fungi were identified by sequencing. Results showed dermatophytes, *Fusarium* spp., and other less frequently isolated nondermatophyte fungi as single fungal agents in the majority of cases of onychomycosis [53]. RFLP analysis of PCR products demonstrated mixed infections in ~10% of cases. Identification of dermatophytes within 2 days of procedure was possible with this technology. The rapid and reliable molecular identification of the infectious fungus expedites the choice of appropriate antifungal therapy, thereby potentially improving the cure rate of onychomycosis.

Taken together, these studies clearly demonstrate the advantage of RFLP analysis for the rapid identification of dermatophytes, while highlighting the fact that caution should be exercised before applying it to all dermatophyte species and strain determinations.

Automated Fluorescent Capillary Electrophoresis (AFCE)

Turenne et al. [63] used an automated capillary electrophoresis sequencer to detect fluorescently tagged PCR amplicons from the ITS2 region. The potential advantage of this method lies in its greater sensitivity of differentiating amplicons compared with AE and PAGE.

PCR-ELISA

A recent modification of the PCR approach is its combination with enzyme-linked immunosorbent assay (ELISA), which facilitates rapid identification of species-specific DNA segments directly from clinical samples. Isolated genomic DNA from skin scrapings and/or nails is amplified with species-specific primers, and PCR products are subsequently detected using biotin-labeled probes. Beifuss et al. [64] recently reported using the PCR-ELISA method for detection (within 24 h) of the five common dermatophytes – *T. rubrum*, *T. interdigitale*, *T. violaceum*, *M. canis,* and *E. floccosum* – from clinical specimens. Genomic DNA was isolated from skin and nail samples of patients with suspected dermatophyte infections, and amplified with species-specific digoxigenin-labeled primers targeting the topoisomerase II gene. Two hundred and four microscopy-positive samples from two university mycological laboratories, and 316 consecutive

specimens – regardless of mycological findings – in a dermatological practice laboratory, were utilized. The technique was confirmatory for one of the five dermatophytes in 79.9% (163/204) of clinical samples found to be positive using microscopy [64]. Culture was positive for dermatophytes in 59.8% of the same cases. A statistically significant difference between these two methods of dermatophyte detection was demonstrated [64]. These findings suggest that direct DNA isolation from clinical specimens coupled with PCR-ELISA provide a rapid, reproducible, and sensitive tool for detection and discrimination of five major dermatophytes at species level, independent of morphological and biochemical characteristics.

Nested PCR

A modification of the PCR approach is "nested PCR" – in which conventional amplification is followed by a second amplification reaction, that targets an internal sequence within the initial amplicon, using a different set of primers. Nested PCR reduces the effect of interfering agents, resulting in an increased amount of the final amplicon. Okeke et al. [65] amplified a 725–762 bp sequence of the *ACT* gene (which encodes actin) from genomic DNA of 12 dermatophyte species, and then sequenced the amplicons. Reverse transcription (RT)-nested PCR of dermatophyte ACT mRNA was performed using a primer pair that amplified an *ACT*-associated intron region to produce a dermatophyte-specific 171 bp amplicon. This fragment was subsequently targeted by RT-nested PCR to determine the viability of dermatophytes in skin scales. Results were correlated with culture-based evaluation. The advantage of the RT-nested PCR approach is that it allows the determination of dermatophyte viability in clinical samples. Nagao et al. [66] also used the nested PCR approach, targeting the ITS region to identify *T. rubrum* in glabrous skin. Yang et al. [67] identified dermatophyte species in clinical specimens, using a seminested PCR approach (targeting the ITS region, with NS5, ITS1, and ITS4 primers) combined with RFLP (digestion with BciT130I and DdeI). RFLP-based identification of the strains involved matched results obtained by culture-based methods [67].

Multiplex Real-Time PCR Detection/Identification

Arabatzis et al. [68] used gene sequences to design a quantitative real-time PCR (qPCR) assay for rapid detection and identification of dermatophytes in clinical specimens (Fig. 15.6). Two assays, based on amplification of ribosomal ITS regions and employing probes specific to relevant species and species-complexes, were designed, optimized, and clinically evaluated. One assay was used to detect the *T. mentagrophytes* species complex, *T. tonsurans* and *T. violaceum*. The second assayed for the *T. rubrum* species complex, *M. canis* and *M. audouinii*. Results showed that the analytical sensitivity of both assays was 0.1 pg of DNA per reaction, corresponding to 2.5–3.3 genomes per sample [68]. The protocol was clinically evaluated over 6 months by testing 92 skin, nail, and hair specimens from 67 patients with suspected dermatophytosis. qPCR detected and correctly identified the causative agent in specimens from which *T. rubrum, T. interdigitale, M. audouinii,* or *T. violaceum* were cultured, and also detected a dermatophyte species in an additional seven specimens that were negative by microscopy and culture [68]. This highly sensitive assay also proved to have high positive and negative predictive values (95.7% and 100%, respectively), facilitating rapid and accurate diagnosis conducive to targeted rather than empirical therapy for dermatophytosis. However, this protocol cannot discriminate between all clinically relevant dermatophyte species and two PCR

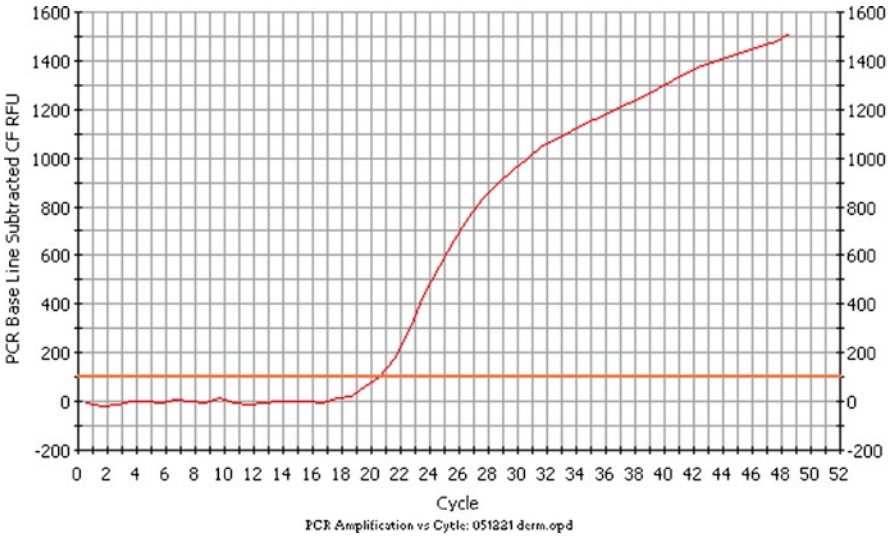

Fig. 15.6 Amplification curve derived from real-time *Trichophyton violaceum*-specific PCR assay of a hair sample. The x-axis shows the cycle number and the y-axis indicates the fluorescence units. An increase in fluorescence above the threshold (*horizontal orange line*) indicates detection of PCR products after 20–21 cycles (Courtesy of Dr. Michael Arabatzis, Medical School, University of Athens, Greece)

reactions are required [68]. More recently, Bergmans et al. [69] developed a single-tube dermatophyte-specific qPCR assay, based on ITS1 sequences. The detection and identification of 11 clinically important species (within *Trichophyton*, *Microsporum*, and *Epidermophyton* genera) in nail, hair, and skin samples was possible, using species-specific probes (Fig. 15.7) [69]. Of note, qPCR yielded significantly more positive results than conventional dermatophyte culture and direct microscopic methods (61.7% *vs.* 47.5%). Most importantly, single-tube qPCR-based dermatophyte identification and quantification may be completed in as little as 4 h (following overnight lysis), with minimal hands-on time, making it a very suitable diagnostic assay for dermatophytosis [69].

PCR-Reverse Line Blot

In a recent study, Bergmans et al. [70] developed and successfully employed a PCR-reverse line blot (PCR-RLB) method for rapid detection and identification of nine dermatophyte species in nail, skin, and hair samples. The method was based on ITS1 sequences, using genus-specific and species-specific probes for nine species within three genera, in isolates obtained from 819 clinical samples (596 nail, 203 skin, and 20 hair). Membranes containing immobilized oligonucleotide probes were exposed to denatured PCR products, allowed to hybridize for 30 min, then subjected to stringency washes and detection using streptavidin-peroxidase and chemiluminescence. The investigators reported a positive PCR-RLB reaction in 93.6% of 172 culture-positive and microscopy-positive samples [70]. More recently, the same authors have shown good concordance (92%) between PCR-RLB and qPCR assays on clinical specimens [69]. PCR-RLB holds great promise, as it facilitates easy detection and identification of dermatophytes directly from nail, skin, and hair samples. However, compared with qPCR, it requires slightly greater hands-on time (~5 h) and post-PCR analysis, with a risk of amplicon contamination and false-positive results [69].

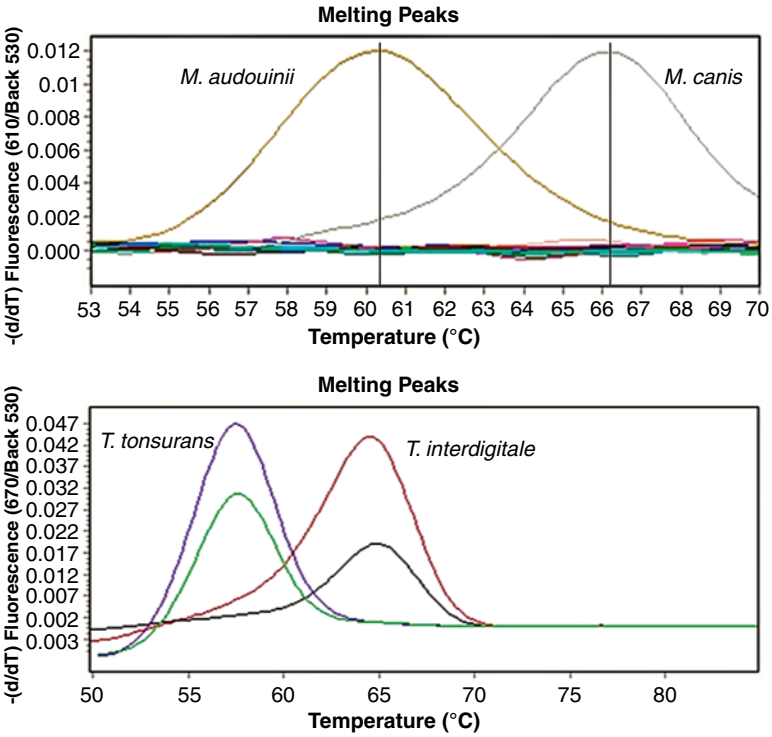

Fig. 15.7 Typical melting curve analyses following real-time PCR amplification of dermatophyte DNA. *Upper panel*: *Microsporum audouinii* (Tm 60.5°C) and *Microsporum canis* (Tm 66°C), using DNA from cultured isolates spiked into DNA from negative clinical samples, with the species-specific hybridization probe set Mcaau1S + Mcaau1A. *Lower panel*: *Trichophyton tonsurans* (Tm 57.5°C) and *Trichophyton interdigitale* (Tm 65°C) from clinical samples, using the species-specific hybridization probe set Tinmeto1S + Tinmeto3A. Values on the y-axis are the first negative derivative of the change in fluorescence (dFluorescence) divided by the change in temperature (dT). *Vertical lines* indicate the melting temperatures (Tm) of the dermatophyte species with the probe sets (Courtesy of Dr. Anneke Bergmans, Laboratory of Medical Microbiology, Franciscus Hospital, Roosendaal, The Netherlands)

Single Strand Conformational Polymorphism (SSCP)

Variations in target sequences can be identified by electrophoretic separation of single-stranded nucleic acids, and have been used to identify dermatophytes. These variations lead to alterations in the secondary structure, influencing the conformation of the biomolecules, and hence affecting mobility during electrophoresis. Cafarchia et al. [71] reported the development of a PCR-SSCP approach, targeting *CHS1*, as a tool to identify dermatophytes.

Loop-Mediated Isothermal Amplification

Routine PCR applications involve multiple cycles of amplification reactions at different temperatures, thus necessitating the use of a thermal cycler. The loop-mediated isothermal amplification (LAMP)

method amplifies a few copies of DNA to 10^9 in less than an hour, under isothermal conditions and with greater specificity [72]. Therefore, it has the potential for routine use in clinical laboratories. Ohori et al. [73] performed LAMP, using species-specific primers based on the D1/D2 domain of the 28S rDNA sequence, in order to identify the dematiaceous fungus *Ochroconis gallopava*, which is recognized as a causative agent of zoonotic and emerging fungal infections. These investigators reported successful detection of the target gene from both fungal DNA and experimentally infected tissues [73].

Microarray Analysis

Microarray-based analysis of ITS1 and ITS2 was recently used to identify 17 species (198 strains) of dermatophytes [74]. The amplified ITS fragments were labeled with digoxigenin and then exposed to (i.e., hybridized with) an immobilized array of 17- to 30-mer oligonucleotides on a nylon membrane. The sensitivity and specificity of this method was reported to be 99.5% and 97.8%, respectively. However, one *M. audouinii* strain was not identified. In addition, two nontarget strains, *M. equinum* and *T. gourvilii* var. *intermedium*, were misidentified as *M. canis* and *T. soudanense*, respectively. Nonetheless, a microarray-based method has potential to become an alternative to conventional identification technologies.

Mass Spectrometry

Mass spectrometry has been used to identify fungal isolates, including dermatophytes [52, 75–81]. This approach is based on the presence of highly abundant low-molecular weight proteins (2–20 kDa), which are believed to be involved in the degradation of keratin. Giddey et al. [80] used a combined 2D-PAGE and shotgun mass spectrometry approach to analyze proteins secreted by *T. rubrum* and *T. violaceum*, and identified 80 proteins that included endo- and exo-proteases, other hydrolases, and oxidoreductases. In another study, Jousson et al. [52] isolated seven genes encoding putative serine proteases of the subtilisin family (*SUB*) in *T. rubrum*, and showed that Sub3 and Sub4 proteins (33 and 31 kDa, respectively) exhibited activity against keratin, suggesting a potential role in fungal invasion of keratinized tissues. These investigators used proteolysis and mass spectrometry to identify orthologous Sub proteins secreted by other dermatophyte species [52]. Recently, Erhard et al. [81] used matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry to identify isolates causing onychomycosis and tinea pedis, and showed similar or better specificity than an ITS/PCR-based approach. Mass spectrometry analysis is rapid and relatively simple to implement. However, (a) contamination and interference are not uncommon; (b) differentiation between different species may be problematic; and (c) results can be influenced by the fungal morphology (i.e., blastospore, conidia, and filaments) [76, 79]. Therefore, although promising, the utility of this technique for rapid and accurate identification of dermatophytes requires further validation.

Molecular Testing: Advantages and Need for Caution

Molecular methods for identification of dermatophytes have gained significant popularity and acceptance in recent years, driven by the fact that these methods allow fast and accurate detection and quantification of fungi. However, widespread adoption of these techniques in clinical laboratories is hampered by several disadvantages. Most recent advances in molecular methods require specialized

training of personnel, in addition to expensive equipment and reagents, and are therefore often available only as research tools. Other major deterrents to the application of molecular diagnostic methods include: (a) risk of false-positive test results; (b) risk of false-negative test results; (c) hypersensitivity with no clinical significance; and (d) potential for narrow range of specificity. False positives are commonly traced to background contamination (i.e., from other samples or from previous amplification reactions) and/or related to an inability to provide any information about cellular viability (i.e., even DNA from dead fungal elements will result in a positive signal by classic PCR). The exclusion of a requirement for post-PCR analysis (i.e., through the use of single-tube qPCR) can reduce the risk of contamination [69]. In addition, the incorporation of a reverse transcription (RT) step can overcome some of the risks of false positivity, since the target by this approach is mRNA, which is expressed only by live fungal cells. However, such modifications can be cost-prohibitive for routine assays. False-negative results may be related to the presence of interfering agents in the tested samples, insufficient starting material, or inefficient DNA extraction from infected tissues. The improper selection of targets, nonspecific primers, and suboptimal assay conditions can also lead to reduced sensitivity and/or specificity, which are crucial determinants that need to be considered before the widespread acceptance of these techniques in clinical practice. In addition, a lack of protocol standardization and reproducibility for molecular diagnostic tests across different laboratories has also precluded their routine use to date.

Cost Effectiveness of Molecular Diagnostic Methods

Molecular diagnostic tests not only demonstrate the advantages of fast and accurate identification of dermatophytes, but also have the potential of providing significant cost benefits in the management and treatment of patients. For example, Kardjeva et al. [82] report that the cost of a single PCR test for *T. rubrum* would be only slightly higher than that of conventional direct microscopy/culture diagnostic testing (2.90 *vs.* 2.30 euros). However, any cost benefit must be balanced with the increased expenditure required to develop and perform these tests; for example, measures instituted to reduce contamination (i.e., building separate laboratory space). Recently, Louie et al. [83] reported that the cost of PCR-based diagnosis of infectious agents can range anywhere from Can$8 to Can$40 per sample, excluding the cost of initial equipment purchase, reagents, and labor. The cumulative cost of PCR analysis can be as high as Can$125 per test, if the latter are included [83]. Of course, reimbursement rates must also be factored into any cost analysis (see Chap. 23). Variable and inadequate reimbursement by third-party payers and managed-care organizations have also hampered widespread acceptance of these methods into the clinical diagnostic laboratory [84]. However, the cost effectiveness of molecular diagnostic testing may be more broadly related to a net reduction in the number of infections, unnecessary treatments, disabilities, hospital stays, and mortality, in addition to societal benefits, such as decreased drug resistance due to targeted therapy, facilitated by such methods [63, 68, 82–91]. For example, Bergmans et al. [70] estimated that the costs of PCR-RLB for dermatophyte identification are US$90 per person, as compared to US$31 per person for traditional diagnostic techniques (i.e., direct microscopy and culture), a premium of US$59 for molecular testing. However, as PCR-RLB results are obtained quickly (3–4 days *vs.* 3–6 weeks for culture), this procedure results in ~50% fewer patients receiving unnecessary antimycotic therapy (i.e., those with PCR-negative results, who would have been treated empirically for 1 month pending culture results), producing an average cost saving of ~US$35 per patient [70].

In summary, molecular methods for dermatophyte identification show increasing potential for routine use in the diagnosis and follow-up (i.e., evaluation of efficacy of antifungal therapy) of skin, hair, and nail infections. For example, molecular tests could be used to determine if relapse after treatment is due to reinfection with either the same or a different dermatophyte strain. Continued development and optimization of some of the more recently described technologies is warranted.

References

1. Rippon JW. Medical mycology: the pathogenic fungi and the pathogenic actinomycetes. 3rd ed. Philadelphia, PA: WB Saunders; 1988.
2. Hainer BL. Dermatophyte infections. Am Fam Physician. 2003;67:101–8.
3. Hay R. Literature review. Onychomycosis. J Eur Acad Dermatol Venereol. 2005;19 Suppl 1:1–7.
4. Elewski BE. Tinea capitis: a current perspective. J Am Acad Dermatol. 2000;42:1–20.
5. Elewski BE. Onychomycosis. Treatment, quality of life, and economic issues. Am J Clin Dermatol. 2000;1:19–26.
6. Jennings MB, Pollak R, Harkless LB, et al. Treatment of toenail onychomycosis with oral terbinafine plus aggressive debridement: IRON-CLAD, a large, randomized, open-label, multicenter trial. J Am Pediatr Med Assoc. 2006;96:465–73.
7. Rupke SJ. Fungal skin disorders. Prim Care. 2000;27:407–22.
8. Deng S, Bulmer GS, Summerbell RC, et al. Changes in frequency of agents of tinea capitis in school children from Western China suggest slow migration rates in dermatophytes. Med Mycol. 2008;46:421–7.
9. Havlickova B, Czaika VA, Friedrich M. Epidemiological trends in skin mycoses worldwide. Mycoses. 2008;51 Suppl 4:2–15.
10. Seebacher C, Bouchara JP, Mignon B. Updates on the epidemiology of dermatophyte infections. Mycopathologia. 2008;166:335–52.
11. Panackal AA, Halpern EF, Watson AJ. Cutaneous fungal infections in the United States: analysis of the National Ambulatory Medical Care Survey (NAMCS) and National Hospital Ambulatory Medical Care Survey (NHAMCS), 1995–2004. Int J Dermatol. 2009;48:704–12.
12. Neji S, Makni F, Cheikhrouhou F, et al. Epidemiology of dermatophytoses in Sfax, Tunisia. Mycoses. 2009;52:534–8.
13. Godoy-Martinez P, Nunes FG, Tomimori-Yamashita J, et al. Onychomycosis in Sao Paulo, Brazil. Mycopathologia. 2009;168:111–6.
14. Zaki SM, Ibrahim N, Aoyama K, et al. Dermatophyte infections in Cairo, Egypt. Mycopathologia. 2009;167:133–7.
15. Lawry MA, Haneke E, Strobeck K, et al. Methods for diagnosing onychomycosis: a comparative study and review of the literature. Arch Dermatol. 2000;136:1112–6.
16. Weinberg JM, Koestenblatt EK, Tutrone WD, et al. Comparison of diagnostic methods in the evaluation of onychomycosis. J Am Acad Dermatol. 2003;49:193–7.
17. Miranda MFR, Silva AJG. Vinyl adhesive tape also effective for direct microscopy diagnosis of chromomycosis, lobomycosis, and paracoccidioidomycosis. Diagn Microbiol Infect Dis. 2005;52:39–43.
18. Mohanty J, Mohanty S, Sahoo R, et al. Diagnosis of superficial mycoses by direct microscopy – A statistical evaluation. Indian J Dermatol Venereol Leprol. 1999;65:72–4.
19. Brasil KW, Pinheiro RL, Pimentel IC. Laboratory diagnosis of superficial and cutaneous mycosis: a comparison of the potassium hydroxide and calcofluor white methods. An Bras Dermatol. 2003;78:547–51.
20. Elston DM. Fluorescence of fungi in superficial and deep fungal infections. BMC Microbiol. 2001;1:21.
21. Murray PR, Baron EJ. Manual of clinical microbiology. 9th ed. Washington, DC: ASM Press; 2007.
22. Haldane DJ, Robart E. A comparison of calcofluor white, potassium hydroxide, and culture for the laboratory diagnosis of superficial fungal infection. Diagn Microbiol Infect Dis. 1990;13:337–9.
23. McGinnis MR. Laboratory handbook of medical mycology. New York: Academic; 1980.
24. St-Germain G, Summerbell RC. Identifying filamentous fungi: a clinical laboratory handbook. Belmont CA: Star Publishing; 1996.
25. Sutton DA, Fothergill AW, Rinaldi MG. Guide to clinically significant fungi. 1st ed. Baltimore, MA: Williams & Wilkins; 1998.
26. Pierard GE, Arrese JE, De Doncker P, et al. Present and potential diagnostic techniques in onychomycosis. J Am Acad Dermatol. 1996;34:273–7.
27. Baek SC, Chae HJ, Houh D, et al. Detection and differentiation of causative fungi of onychomycosis using PCR amplification and restriction enzyme analysis. Int J Dermatol. 1998;37:682–6.
28. Binstock JM. Molecular biology techniques for identifying dermatophytes and their possible use in diagnosing onychomycosis in human toenail: A review. J Am Pediatr Med Assoc. 2007;97:134–44.
29. Kanbe T. Molecular approaches in the diagnosis of dermatophytosis. Mycopathologia. 2008;166:307–17.
30. de Bievre C, Dauguet C, Nguyen VH, et al. Polymorphism in mitochondrial DNA of several *Trichophyton rubrum* isolates from clinical specimens. Ann Inst Pasteur Microbiol. 1987;138:719–27.
31. Nishio K, Kawasaki M, Ishizaki H. Phylogeny of the genera *Trichophyton* using mitochondrial DNA analysis. Mycopathologia. 1992;117:127–32.
32. Kawasaki M, Aoki M, Ishizaki H, et al. Phylogenetic relationships of the genera *Arthroderma* and *Nannizzia* inferred from mitochondrial DNA analysis. Mycopathologia. 1992;118:95–102.

33. Kawasaki M, Ishizaki H, Aoki M, et al. Phylogeny of *Nannizzia incurvata*, *N. gypsea*, *N. fulva* and *N. otae* by restriction enzyme analysis of mitochondrial DNA. Mycopathologia. 1990;112:173–7.
34. Mochizuki T, Takada K, Watanabe S, et al. Taxonomy of *Trichophyton interdigitale* (*Trichophyton mentagrophytes* var. interdigitale) by restriction enzyme analysis of mitochondrial DNA. J Med Vet Mycol. 1990;28:191–6.
35. Bock M, Maiwald M, Kappe R, et al. Polymerase chain reaction-based detection of dermatophyte DNA with a fungus-specific primer system. Mycoses. 1994;37:79–84.
36. Bock M, Nickel P, Maiwald M, et al. Diagnosis of dermatomycoses with polymerase chain reaction. Hautarzt. 1997;48:175–80.
37. Liu D, Coloe S, Baird R, et al. PCR identification of *Trichophyton mentagrophytes* var. interdigitale and *T. mentagrophytes* var. mentagrophytes dermatophytes with a random primer. J Med Microbiol. 1997;46:1043–6.
38. Liu D, Coloe S, Baird R, et al. Molecular determination of dermatophyte fungi using the arbitrarily primed polymerase chain reaction. Br J Dermatol. 1997;137:351–5.
39. Liu D, Coloe S, Baird R, et al. Application of PCR to the identification of dermatophyte fungi. J Med Microbiol. 2000;49:493–7.
40. Liu D, Pearce L, Lilley G, et al. PCR identification of dermatophyte fungi *Trichophyton rubrum, T. soudanense and T. gourvilii.*. J Med Microbiol. 2002;51:117–22.
41. Zhong ZR, Li R, Li D, et al. Typing of common dermatophytes by random amplification of polymorphic DNA. Jap J Med Mycol. 1997;38:239–46.
42. Graser Y, el Fari M, Presber W, et al. Identification of common dermatophytes (*Trichophyton*, *Microsporum*, *Epidermophyton*) using polymerase chain reactions. Br J Dermatol. 1998;138:576–82.
43. Mukherjee PK, Leidich SD, Isham N, et al. Clinical *Trichophyton rubrum* strain exhibiting primary resistance to terbinafine. Antimicrob Agents Chemother. 2003;47:82–6.
44. Faggi E, Pini G, Campisi E, et al. Application of PCR to distinguish common species of dermatophytes. J Clin Microbiol. 2001;39:3382–5.
45. Faggi E, Pini G, Campisi E. PCR fingerprinting for identification of common species of dermatophytes. J Clin Microbiol. 2002;40:4804–5.
46. Shehata AS, Mukherjee PK, Aboulatta HN, et al. Single-step PCR using (GACA)4 primer: Utility for rapid identification of dermatophyte species and strains. J Clin Microbiol. 2008;46:2641–5.
47. Kano R, Nakamura Y, Watari T, et al. Molecular analysis of chitin synthase 1 (*CHS1*) gene sequences of *Trichophyton mentagrophytes* complex and *T. rubrum*. Curr Microbiol. 1998;37:236–9.
48. Ninet B, Jan I, Bontems O, et al. Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. J Clin Microbiol. 2003;41:826–30.
49. Frealle E, Rodrigue M, Gantois N, et al. Phylogenetic analysis of *Trichophyton mentagrophytes* human and animal isolates based on MnSOD and ITS sequence comparison. Microbiology. 2007;153:3466–77.
50. Summerbell RC, Moore MK, Starink-Willemse M, et al. ITS barcodes for *Trichophyton tonsurans* and *T. equinum*. Med Mycol. 2007;45:193–200.
51. Fumeaux J, Mock M, Ninet B, et al. First report of *Arthroderma benhamiae* in Switzerland. Dermatology. 2004;208:244–50.
52. Jousson O, Lechenne B, Bontems O, et al. Secreted subtilisin gene family in *Trichophyton rubrum*. Gene. 2004;339:79–88.
53. Monod M, Bontems O, Zaugg C, et al. Fast and reliable PCR/sequencing/RFLP assay for identification of fungi in onychomycoses. J Med Microbiol. 2006;55:1211–6.
54. Graser Y, Kuijpers AF, Presber W, et al. Molecular taxonomy of *Trichophyton mentagrophytes* and *T. tonsurans*. Med Mycol. 1999;37:315–30.
55. Jackson CJ, Barton RC, Evans EG. Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J Clin Microbiol. 1999;37:931–6.
56. Shin JH, Sung JH, Park SJ, et al. Species identification and strain differentiation of dermatophyte fungi using polymerase chain reaction amplification and restriction enzyme analysis. J Am Acad Dermatol. 2003;48:857–65.
57. Brillowska-Dabrowska A, Saunte DM, Arendrup MC. Five-hour diagnosis of dermatophyte nail infections with specific detection of *Trichophyton rubrum*. J Clin Microbiol. 2007;45:1200–4.
58. Ding J, Li J, Liu Z, et al. Clinical identification of common species of dermatophytes by PCR and PCR-RFLP. J Huazhong Univ Sci Technol Med Sci. 2004;24:642–4.
59. Gutzmer R, Mommert S, Kuttler U, et al. Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR. J Med Microbiol. 2004;53:1207–14.
60. Kamiya A, Kikuchi A, Tomita Y, et al. PCR and PCR-RFLP techniques targeting the DNA topoisomerase II gene for rapid clinical diagnosis of the etiologic agent of dermatophytosis. J Dermatol Sci. 2004;34:35–48.
61. He G, Li J, Ding J, et al. Identification of common species of dermatophytes by PCR-RFLP. J Huazhong Univ Sci Technol Med Sci. 2005;25:458–60.

62. Pounder JI, Williams S, Hansen D, et al. Repetitive-sequence-PCR-based DNA fingerprinting using the Diversilab system for identification of commonly encountered dermatophytes. J Clin Microbiol. 2005;43:2141–7.
63. Turenne CY, Sanche SE, Hoban DJ, et al. Rapid identification of fungi by using the ITS2 genetic region and an automated fluorescent capillary electrophoresis system. J Clin Microbiol. 1999;37:1846–51.
64. Beifuss B, Bezold G, Gottlober P, et al. Direct detection of five common dermatophyte species in clinical samples using a rapid and sensitive 24-h PCR-ELISA technique open to protocol transfer. Mycoses. 27 Sept 2009. [Epub ahead of print].
65. Okeke CN, Tsuboi R, Kawai M, et al. Isolation of an intron-containing partial sequence of the gene encoding dermatophyte actin (ACT) and detection of a fragment of the transcript by reverse transcription-nested PCR as a means of assessing the viability of dermatophytes in skin scales. J Clin Microbiol. 2001;39:101–6.
66. Nagao K, Sugita T, Ouchi T, et al. Identification of *Trichophyton rubrum* by nested PCR analysis from paraffin embedded specimen in trichophytia profunda acuta of the glabrous skin. Nippon Ishinkin Gakkai Zasshi. 2005;46:129–32.
67. Yang G, Zhang M, Li W, et al. Direct species identification of common pathogenic dermatophyte fungi in clinical specimens by semi-nested PCR and restriction fragment length polymorphism. Mycopathologia. 2008;166:203–8.
68. Arabatzis M, van Coppenraet LE Bruijnesteijn, Kuijper EJ, et al. Diagnosis of common dermatophyte infections by a novel multiplex real-time polymerase chain reaction detection/identification scheme. Br J Dermatol. 2007;157:681–9.
69. Bergmans AM, van der Ent M, Klaassen A, et al. Evaluation of a single-tube real-time PCR for detection and identification of 11 dermatophyte species in clinical material. Clin Microbiol Infect. 2010;16:704–10.
70. Bergmans AM, Schouls LM, van der Ent M, et al. Validation of PCR-reverse line blot, a method for rapid detection and identification of nine dermatophyte species in nail, skin and hair samples. Clin Microbiol Infect. 2008;14:778–88.
71. Cafarchia C, Otranto D, Weigl S, et al. Molecular characterization of selected dermatophytes and their identification by electrophoretic mutation scanning. Electrophoresis. 2009;30:3555–64.
72. Notomi T, Okayama H, Masubuchi H, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000;28:E63.
73. Ohori A, Endo S, Sano A, et al. Rapid identification of *Ochroconis gallopava* by a loop-mediated isothermal amplification (LAMP) method. Vet Microbiol. 2006;114:359–65.
74. Li HC, Bouchara JP, Hsu MM, et al. Identification of dermatophytes by an oligonucleotide array. J Clin Microbiol. 2007;45:3160–6.
75. Sun L, Teramoto K, Sato H, et al. Characterization of ribosomal proteins as biomarkers for matrix-assisted laser desorption/ionization mass spectral identification of Lactobacillus plantarum. Rapid Commun Mass Spectrom. 2006;20:3789–98.
76. Fenselau C, Demirev PA. Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev. 2001;20:157–71.
77. Amiri-Eliasi B, Fenselau C. Characterization of protein biomarkers desorbed by MALDI from whole fungal cells. Anal Chem. 2001;73:5228–31.
78. Vargha M, Takats Z, Konopka A, et al. Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. J Microbiol Methods. 2006;66:399–409.
79. Santos C, Paterson RR, Venancio A, et al. Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Appl Microbiol. 2010;108:375–85.
80. Giddey K, Monod M, Barblan J, et al. Comprehensive analysis of proteins secreted by *Trichophyton rubrum* and *Trichophyton violaceum* under *in vitro* conditions. J Proteome Res. 2007;6:3081–92.
81. Erhard M, Hipler UC, Burmester A, et al. Identification of dermatophyte species causing onychomycosis and tinea pedis by MALDI-TOF mass spectrometry. Exp Dermatol. 2008;17:356–61.
82. Kardjeva V, Summerbell R, Kantardjiev T, et al. Forty-eight-hour diagnosis of onychomycosis with subtyping of Trichophyton rubrum strains. J Clin Microbiol. 2006;44:1419–27.
83. Louie M, Louie L, Simor AE. The role of DNA amplification technology in the diagnosis of infectious diseases. CMAJ. 2000;163:301–9.
84. Pfaller MA. Molecular approaches to diagnosing and managing infectious diseases: Practicality and costs. Emerg Infect Dis. 2001;7:312–8.
85. Scherer LC, Sperhacke RD, Ruffino-Netto A, et al. Cost-effectiveness analysis of PCR for the rapid diagnosis of pulmonary tuberculosis. BMC Infect Dis. 2009;9:216.
86. Ninove L, Tan C, Nougairede A, et al. Impact of diagnostic procedures on patient management and hospitalization cost during the 2000 and 2005 enterovirus epidemics in Marseilles, France. Clin Microbiol Infect. 2010; 16:651–6.

87. Khairnar K, Martin D, Lau R, et al. Multiplex real-time quantitative PCR, microscopy and rapid diagnostic immuno-chromatographic tests for the detection of *Plasmodium* spp: performance, limit of detection analysis and quality assurance. Malar J. 2009;8:284.
88. Mahony JB, Blackhouse G, Babwah J, et al. Cost analysis of multiplex PCR testing for diagnosing respiratory virus infections. J Clin Microbiol. 2009;47:2812–7.
89. Yang S, Rothman RE. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. Lancet Infect Dis. 2004;4:337–48.
90. Chandrasekar P. Diagnostic challenges and recent advances in the early management of invasive fungal infections. Eur J Haematol. 2010;84:281–90.
91. Alexander BD, Pfaller MA. Contemporary tools for the diagnosis and management of invasive mycoses. Clin Infect Dis. 2006;43:S15–27.
92. Makimura K, Tamura Y, Mochizuki T, et al. Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions. J Clin Microbiol. 1999;37:920–4.
93. Mochizuki T, Kawasaki M, Ishizaki H, et al. Identification of several clinical isolates of dermatophytes based on the nucleotide sequence of internal transcribed spacer 1 (ITS 1) in nuclear ribosomal DNA. J Dermatol. 1999;26:276–81.
94. Makimura K, Tamura Y, Murakami A, et al. Cluster analysis of human and animal pathogenic *Microsporum* species and their teleomorphic states, *Arthroderma* species, based on the DNA sequences of nuclear ribosomal internal transcribed spacer 1. Microbiol Immunol. 2001;45:209–16.
95. Kano R, Okabayashi K, Nakamura Y, et al. Differences among chitin synthase I gene sequences in *Trichophyton rubrum* and *T. violaceum*. Med Mycol. 2000;38:47–50.
96. Kanbe T, Suzuki Y, Kamiya A, et al. PCR-based identification of common dermatophyte species using primer sets specific for the DNA topoisomerase II genes. J Dermatol Sci. 2003;32:151–61.

Chapter 16
Infectious Diseases of the Skin II: Non-Dermatophytic Infections

Michael J. Murphy and Avery LaChance

Identification of the pathogenic microbe is essential for selection of the most appropriate treatment in the majority of cutaneous infections. Historically, the diagnosis of cutaneous pathogens has been based on the results of immunological studies, lesional culture, and/or microscopic examination of tissue samples, in combination with histochemical stains (i.e., PAS, Gram) or immunohistochemical studies. Microscopic review of clinical specimens allows for rapid microbe detection. However, this method lacks sensitivity and specificity, and typically results in a preliminary determination only. In addition, not every pathogen is identifiable by microscopic analysis, and special stains are often less sensitive than culture methods. Thus, definitive characterization requires growth of the pathogen in culture, which remains the gold-standard methodology for laboratory diagnosis of microbial infection. While the latter generally demonstrates improved sensitivity and specificity as compared with histopathological examination, microbe growth may require days to weeks of culture, delaying both diagnosis and the institution of appropriate therapy. Further complicating matters is the fact that not all pathogens grow outside of their host.

Molecular diagnostic strategies for microbe detection have a number of distinct advantages over traditional methodologies [1–5]. For example, polymerase chain reaction (PCR)-based and fluorescence in situ hybridization (FISH)-based assays generally have no requirement for pathogen growth in culture media. Therefore, a variety of microorganisms (i.e., bacteria, viruses, and fungi) which may be difficult or impossible to culture (due to the need for specialized media and/or protracted incubation times) can be rapidly characterized using these technologies. The high sensitivity of molecular methods allows for the detection of infectious agents that may be present in only minute numbers, in addition to the identification of microbes from nonculturable resources (i.e., formalin-fixed paraffin-embedded tissue) [1–7]. For many infectious diseases, these newer assays are employed in conjunction with traditional methods. For example, information gained from histochemical staining may be used to select for one or a small number of molecular-based bacterial or fungal family-, genus-, or species-specific probes from a larger probe set. In some instances, however, molecular technologies are now beginning to replace culture-, biochemical-, and immunological-based microbial methods [1–5].

Nucleic acid-based testing is being increasingly employed in the diagnosis and management of dermatologic infections (Tables 16.1–16.3) [6–92]. Molecular technologies have been used to detect pathogenic mucocutaneous bacteria (Figs. 16.1–16.4, 17.2 and 17.3), spirochetes (Fig. 16.5), fungi (Fig. 16.6 and Chap. 15), viruses (Fig. 16.7), parasites (Figs. 16.8 and 16.9), and infestations. In terms of practical applications, there are multiple clinical and/or pathobiological questions regarding

A. LaChance (✉)
University of Connecticut Medical School, 263 Farmington Ave., Farmington, CT 06030, USA
e-mail: avery.lachance@gmail.com

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_16,

Table 16.1 Applications of molecular diagnostic tests in the management of mucocutaneous infectious diseases

Facilitate highly sensitive and specific microbial detection
– Necessary for definitive diagnosis before the initiation of treatment
Identify to species level
– Because of the established link between some microbe species and disease severity and/or treatment response
Detect and specifically identify infectious agents that are difficult to culture and/or distinguish by other methodologies
– Fastidious or slow-growing microorganisms; anaerobes; mycobacteria; viruses; unknown species
Demonstrate the viability of detected microbes in host tissues
Quantify microbial load in host tissues
Define patterns of genotypic and phenotypic expression under variable clinical and morphologic conditions
Characterize virulence factors and potential drug resistance mechanisms in microbes
Determine drug efficacy and/or identify disease persistence or relapse
Detect infectious agents that play a role in the pathogenesis of certain skin tumors
Microbe tracking
Identify emerging cutaneous infectious diseases
Reservoir host investigations and vector surveys

mucocutaneous infections that may be answered by nucleic acid-based methods (Table 16.1). Molecular technologies allow for highly sensitive and specific microbial detection (up to 100% in some instances), irrespective of species or genus, and necessary for definitive diagnosis before the initiation of treatment. Of note, the performance characteristics of a variety of molecular assays have been shown to be consistently better than microscopy or tissue culture, particularly in samples with low microbe loads. In addition, species identification may be accomplished. This is important information for the clinical management of patients with many mucocutaneous infections, because of the established link between some microbe species and disease severity and/or treatment response. Results may also support the development of targeted and effective treatment protocols. Infectious agents that are difficult to culture and/or distinguish by other methodologies, including fastidious or slow-growing microorganisms, anaerobes, mycobacteria, viruses, or unknown species, can be detected and often specifically identified. Molecular assays can also be used to demonstrate the viability of detected microbes (i.e., through RNA analysis using reverse transcription PCR [RT-PCR] or other methods) and to quantify microbial loads in host tissues (i.e., real-time quantitative PCR [qPCR]). Such data may be relevant to the monitoring of disease progression, assessing drug efficacy and predicting treatment outcomes. Molecular diagnostic techniques can be employed to define patterns of genotypic and phenotypic expression under variable clinical and morphologic conditions, identify microbe-specific features, such as virulence factors and specific genes associated with antimicrobial drug resistance (Table 16.3), and determine treatment response, including the identification of disease persistence or relapse in mucocutaneous infections (Fig. 16.4). The characterization of infectious agents that play a role in the pathogenesis of certain skin tumors is also possible. Finally, molecular methods may be used for microbe tracking, the identification of emerging cutaneous infectious diseases, and reservoir host studies and vector surveys (i.e., rodents and sand flies in *Leishmaniasis*), which have broader epidemiological and public health implications, including the tracking of drug-resistant strains and outbreak investigations. Of course, implementation of these molecular tests depends on the clinical relevance of the results, and is limited by the availability of alternative methods of testing, the need for specialized equipment, and the technical expertise of the laboratory personnel.

More widespread use of molecular assays for the detection of mucocutaneous microbes will improve patient care in dermatologic clinics. This is a function of their greater sensitivity and specificity, user-friendliness, and reduced test turn-around times, compared with traditional laboratory methods. Theoretically, a more rapid and accurate characterization of an infectious agent would

Table 16.2 Infectious agents and infestations with mucocutaneous manifestations for which molecular diagnostic techniques have shown clinical utility

Bacteria	Spirochetes	Fungi	Viruses	Parasites	Infestations
Mycobacterium tuberculosis [8–11]	*Borrelia burgdorferi* [42–44]	*Candida* spp. [48–51]	*Human papillomavirus* [60–69]	*Leishmania* spp. [85–87]	*Sarcoptes scabiei* [89]
Atypical Mycobacteria, nontuberculosis [11–15]	*Treponema pallidum* [45–47]	*Aspergillus* spp. [48–50]	*Herpes simplex virus* [69–73]	*Toxoplasma* spp. [88]	
Mycobacterium leprae [16–19]		*Cryptococcus neoformans* [48–50]	*Varicella zoster virus* [70–72]		
Staphylococcus aureus, including MRSA [20–26]		*Histoplasmosis capsulatum* [52]	*Epstein-Barr virus* [69–72, 74, 75][a]		
Pseudomonas aeruginosa and other spp. [20–23, 27, 28]		*Blastomyces dermatitidis* [53, 54]	*Cytomegalovirus* [69–71, 76]		
Streptococci, groups A and B [20, 21, 29]		*Coccidioides* spp. [54, 55]	*Human herpesvirus 6* [70, 71, 77]		
Chlamydia trachomatis [30–32]		*Alternaria* spp. [56, 57]	*Human herpesvirus 8* [78, 79]		
Neisseria gonorrhoeae [30–33]		*Sporothrix schenckii* [58, 59]	*Parvovirus B19* [80]		
Bartonella spp. [34–37]		Dermatophytes[b]	*Merkel Cell Polyomavirus* [81, 82][c]		
Rickettsia rickettsii [38–40]			*Human T-cell lymphotropic virus type 1 (HTLV-1)* [83, 84][d]		
Bacillus anthracis [41]					
Other [20–23]					

MRSA methicillin-resistant *Staphylococcus aureus*
[a] Fig. 11.3
[b] Discussed in Chap. 15
[c] Fig. 3.1
[d] Fig. 11.6

Table 16.3 Drug resistance genes of particular interest in mucocutaneous infections

Microbe	Genes conferring resistance	Drug
Mycobacterium tuberculosis	Mutations in *KatG, inhA,* and *aphC*	Isoniazid
	Mutations in *rpoB*	Rifampin
	Mutations in *embB*	Ethambutol
	Mutations in *pncA*	Pyrazinamide
	Mutations in *rpsL* and *rrs*	Streptomycin
	Mutations in *gyrA*	Fluoroquinolones
	Mutations in *rrs*	Amikacin/capreomycin
Mycobacterium leprae	Mutations in *folP1*	Dapsone
	Mutations in *rpoB*	Rifampin
	Mutations in *gyrA*	Fluoroquinolones (i.e., ofloxacin)
Staphylococcus aureus	Presence of *blaZ*, *blaI,* and *blaR*	Penicillin and β-lactam antibiotics
	Presence of SCC*mec*	B-lactams, clindamycin, gentamicin, fluroquinolones, methicillin
	Presence of *femA*, *femB,* and *femX*	Methicillin
	Presence of *luk*S-PV and *luk*F-PV	Methicillin
	Presence of *Tn1546*	Vancomycin
	Presence of *aadD*	Neomycin, kanamycin, paromomycin, and tobramycin
	Presence of *ant4*	Tobramycin
	Presence of *ars*RBC	Arsenate, antimonite
	Presence of *ble*	Bleomycin
	Presence of *cad*A,B	Cadmium (and potentially zinc)
	Presence of *cad*D,X	Cadmium
	Presence of *cat*	Chloramphenicol
	Presence of *cfr*	Chloramphenicol, florfenicol, and clindamycin
	Presence of *dfrA* and *dfrK*	Trimethoprim
	Presence of *erm*B,C	Macrolides, erythromycin, lincosamides, clindamycin, streptogramin B
	Presence of *far1*	Fusidic acid
	Presence of *fus*B	Fusidic acid
	Presence of *ile*S-2	Mupirocin
	Presence of *mer* operon	Mercury
	Presence of *mphBM*	Macrolide antibiotics
	Presence of *msrA*	Macrolide antibiotics
	Presence of *mupA*	Mupirocin
	Presence of *qac*A,B and *smr*	Quaternary ammonium compounds and biocides
	Presence of *str*	Streptomycin
	Presence of *tetK* and *tetL*	Tetracyclines
	Presence of *vat*	Streptogramins type A
	Presence of *vga*	Streptogramins type A, lincosamides, and pleuromutilins
	Presence of *vgb*	Streptogramins type B
	Presence of transposon *aacA* and *aphD*	Gentamicin, kanamycin, and tobramycin
	Presence of transposon *cadB* and *cadC*	Cadmium
	Presence of transposon *erm*A,B	Macrolides, erythromycin, lincosamides, clindamycin, and streptogramin B
	Presence of transposon *fexA*	Florfenicol, chloramphenicol
	Presence of transposon *mer*A,B	Inorganic and organic mercury
	Presence of transposon *sat4*	Streptothricin
	Presence of transposon *spc(ant9)*	Spectinomycin

(continued)

Table 16.3 (continued)

Microbe	Genes conferring resistance	Drug
	Presence of transposon *tetM*	Tetracycline, minocycline
	Presence of transposon *vanRSHAXYZ*	Vancomycin
Treponema pallidum	Mutations in 23S rRNA gene	Clindamycin and 14-,15-, and 16-lactone ring macrolides (i.e., azithromycin, erythromycin, spiramycin)
	Mutations in *rpoB*	Rifampin
Pseudomonas aeruginosa	Presence of transposon Tn*6061* components:	
	– bla_{VEB-1} and bla_{OXA-10}	β-lactams
	– *ant(2')-Ia, ant(3")-Ia, ant(4')-IIb*	Aminoglycosides
	– *tet(G)*	Tetracycline
	– *arr-2*	Rifampin
	– *cmlA5* and *floR*	Chloramphenicol
	– *sul*1	Sulfonamides
	Mutations in *gyrA/gyrB*	Fluoroquinolones
	Mutations in *parC/parE*	Fluoroquinolones
	Mutations in *ampD*	Carbenicillin, ticarcillin, piperacillin, azlocillin, ceftazidime, aztreonam, cefepime, and cefpirome
	Mutations in *nalB* at *mexR*	Fluoroquinolones, carbenicillin, ticarcillin, piperacillin, azlocillin, ceftazidime, aztreonam, cefepime, cefpirome, and meropenem
	Mutations in *nalC* at *nfxB*	Fluoroquinolones, carbenicillin, ticarcillin, piperacillin, azlocillin, ceftazidime, aztreonam, cefepime, cefpirome, and meropenem
	Mutations in *nfxC* at *mex*T	Fluoroquinolones, carbenicillin, ticarcillin, piperacillin, azlocillin, ceftazidime, aztreonam, cefepime, cefpirome, imipenem, and meropenem
	Mutations in *OprD* and *nfxC* at *mexT*	Imipenem and meropenem
	Mutations in *nalC*	Fluoroquinolones and all β-lactams, except imipenem
	Mutations in *ampR* and *dacB*	Penicillins and cephalosporins
Streptococci Groups A and B	Presence of *ermA, ermB, mefA,* and/or *mefE*	Macrolides (i.e., erythromycin and clindamycin)
Chlamydia trachomatis	Mutations in 23S rRNA gene	Macrolides (i.e., erythromycin, azithromycin, and josamycin)
	Mutations in *rpoB*	Rifampin
Neisseria gonorrhoeae	Mutations in *gyrA* and *parC*	Fluoroquinolones (i.e., ciprofloxacin)
	Mutations in *erm* genes	Macrolides
	Mutations in *mtr*CDE	Triton X, crystal violet, erythromycin, and fusidic acid
	Mutations in *mtr*R	Erythromycin and azithromycin
	Mutations in *mtrR* with mutations in *penA* and *ponA*	Penicillin
	Mutations in *penB* with mutations in *penA* and *ponA*	Penicillin
	Mutations in 23S rRNA gene	Azithromycin
Bartonella spp.	Mutations in *gyrA*	Fluoroquinolones
	Mutations in *rpoB*	Rifampin
	Mutations in 23S rRNA gene	Erythromycin

(continued)

Table 16.3 (continued)

Microbe	Genes conferring resistance	Drug
Bacillus anthracis	Mutations in *gyrA/B* and *parC/E*	Fluoroquinolones (i.e., ciprofloxacin)
Candida spp.	Mutations in *FUR1, FCY1,* and *FCY2*	5-fluorocytosine
	Mutations in *CDC21*	Fluorinated analogs
	Mutations in *CgPDR1*	Azoles
	Mutations in *CDR1* and *CDR2*	Fluconazole
	Mutations in *fks*	Micagungin
	Mutations in *erg5* and *erg11*	Azoles and amphotericin B
Aspergillus spp.	Mutations in *cyp51A*	Azoles
Cryptococcus neoformans	Mutations in *MDR1*	Fluconazole and itraconazole
	Mutations in *AFR1*	Azoles
	Mutations in *ubc6-2*	Fluconazole
	Mutations in *ssk1, ssk2, pbs2, skn7,* and *hog1*	Fluconazole and ketoconazole
Histoplasmosis capsulatum	Mutations in *cyp51p*	Fluconazole
Herpes simplex virus and *varicella zoster virus*	Mutations in *thymidine kinase*	Acyclovir, penciclovir, brivudin, valacyclovir, and famciclovir
	Mutations in *DNA polymerase*	Foscarnet
Cytomegalovirus	Mutations in *UL97*	Ganciclovir, foscarnet, and cidofovir
	Mutations in *UL54*	Ganciclovir
	Mutations in *UL27*	Maribavir
Human herpes virus 6	Mutations in *U69*	Ganciclovir
	Mutations in *U38*	Ganciclovir and cidofovir
	Mutations in *DNA polymerase*	Foscarnet
Toxoplasma gondii	Mutations in *alpha-1 tubulin*	Dinitroanilines
Sarcoptes scabiei	Mutations in *Vssc*	Permethrin

allow for the administration of a narrow-spectrum and/or equally sensitive and less expensive antimicrobial drug. This would not only benefit patients, but potentially impact drug resistance patterns, in addition to reducing health care expenditures. Dollar savings could be realized through better use of ancillary diagnostic services and hospital beds, as well as reduced pharmacy costs [51, 93, 94]. Improvements in automation and reductions in cost-per-test, in addition to physician awareness and education, should result in wider availability and broader usage of these testing strategies in dermatology. There are three nucleic acid-based approaches that are employed for the detection of infectious agents at mucocutaneous sites: (a) target amplification; (b) signal amplification; and (c) nonamplified probes [1–7].

Target amplification is the most commonly used molecular application for nucleic acid-based diagnosis of mucocutaneous infections. This approach includes such techniques as PCR (and its variants, including qPCR, multiplex PCR, broad-range PCR, and RT-PCR) and non-PCR-based strategies, including ligase chain reaction (LCR), transcription-mediated amplification (TMA), strand displacement amplification (SDA), nucleic acid sequence-based amplification (NASBA), and signal-mediated amplification of RNA technology (SMART) [1–7]. All of these methods combine the use of an enzyme (polymerase or ligase) with primers (short synthetic oligonucleotides that

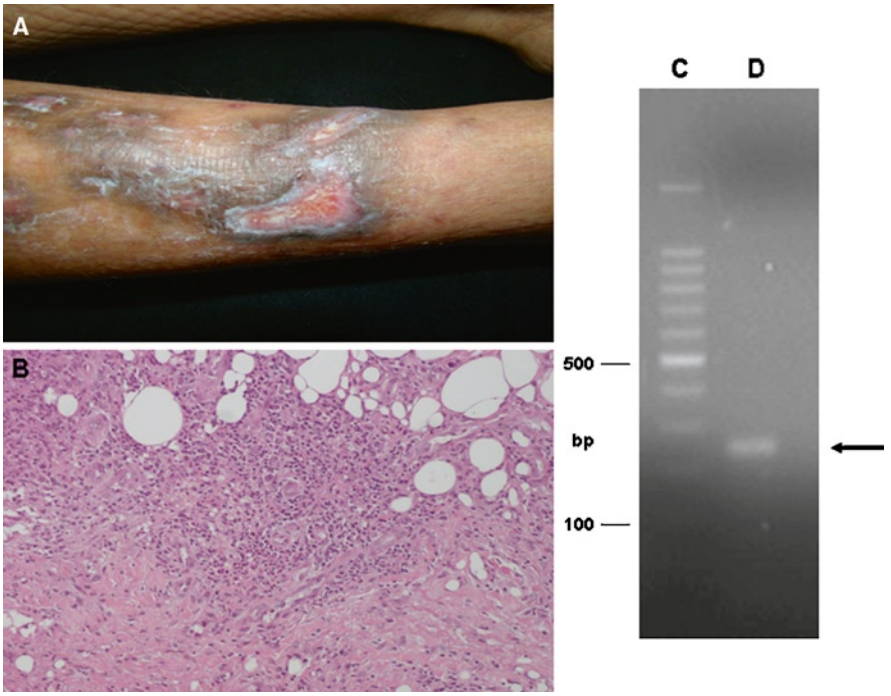

Fig. 16.1 Cutaneous *Mycobacterium tuberculosis*. (**a**) Clinical signs and (**b**) histopathological features of cutaneous tuberculosis. *Right panel*: Analysis of PCR products on 1.5% agarose gel. (**c**) 100-bp ladder. (**d**) Skin sample demonstrating a 245-bp product when analyzed with primers for the right-arm of the insertion element IS*6110* of *M. tuberculosis* (Courtesy of Drs. Oliverio Welsh, Lucio Vera-Cabrera, and Alberto de la Fuente-Garcia, University Hospital, UANL, Monterrey, México)

specifically bind to complementary DNA or RNA sequences in the infectious agent). Early molecular assays were monoparametric (i.e., one analyte, one assay) and qualitative (i.e., positive or negative result). The introduction of multiparametric assays, such as multiplex PCR, now allows for the parallel identification of different targets and acquisition of other data, such as multiple antimicrobial resistance determinants. Furthermore, the development and utilization of closed-tube systems (i.e., qPCR technology) has sped up microbe detection, improved sensitivity, and facilitated quantification (i.e., microbial load), in addition to reducing the risk of test contamination. The latter is particularly important for microbe diagnostics, because of the ubiquitous presence of environmental pathogens. The quantification of microbe nucleic acid could be used to differentiate true disease from colonization, as well as monitoring the efficacy of antimicrobial therapies. A number of different post-amplification technologies can be employed for further amplicon characterization. These include melt curve analysis (qPCR), restriction fragment length polymorphism, DNA sequencing (traditional [Sanger] and pyrosequencing), reverse hybridization, bead-based flow cytometric assays (Luminex technology), and solid- and liquid-phase microarray analysis.

Signal amplification and nonamplified single-probe methods utilize fluorescent, enzymatic (peroxidase) or chemiluminescent-labeled RNA, DNA, or PNA (peptide nucleic acid) probes that bind to the target nucleic acid and generate a signal from the attached reporter molecule [1–7]. These strategies do not require the amplification of the target molecule and, therefore, are not prone to the risks of amplicon contamination. Signal amplification technologies include hybrid capture and branched-chain DNA assays. These methods result in the production of multiple signaling molecules,

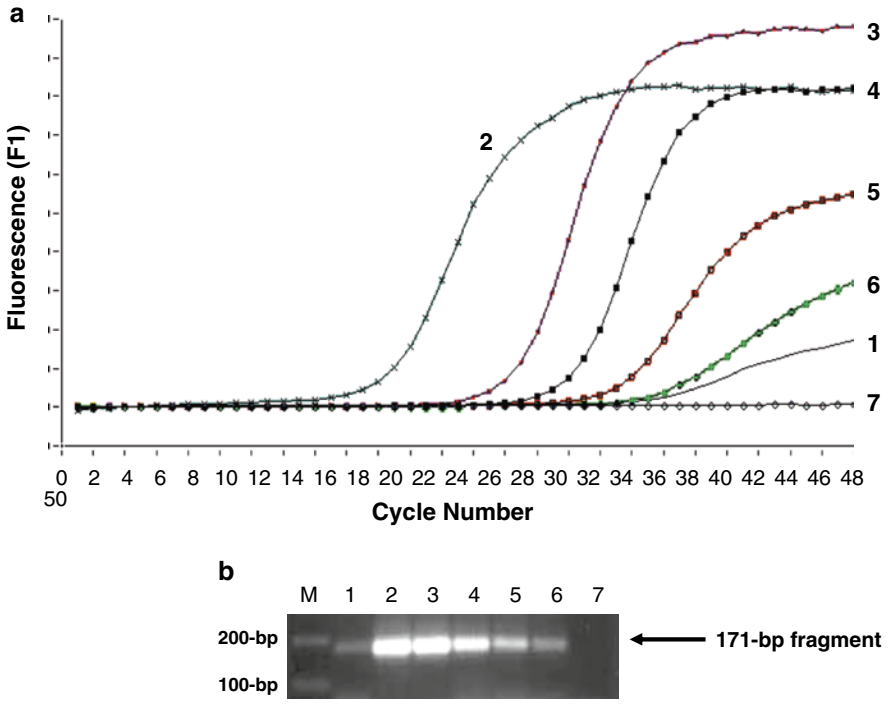

Fig. 16.2 Detection of *Mycobacterium leprae* in skin samples by (**a**) LightCycler real-time PCR and (**b**) PCR-agarose gel electrophoresis (AGE). Lane M, molecular size marker; Samples 1–2, *M. leprae* positive controls; Samples 3–6, skin specimens containing *M. leprae*; Sample 7, negative control. 171-bp fragment is specific for *M. leprae* on 2% AGE (Courtesy of Dr. Benjawan Phetsuksiri, Mycobacteria Laboratory, National Institute of Health, Nonthaburi, Thailand)

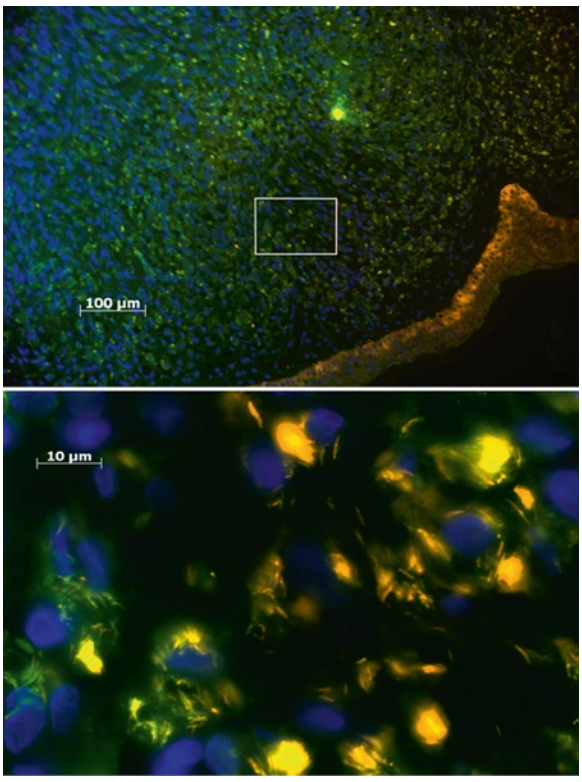

Fig. 16.3 Detection of *Mycobacterium leprae* in a skin sample by peptide nucleic acid-fluorescence in situ hybridization (PNA-FISH). *Top panel*: The overview shows (i) *green* background fluorescence of the tissue and (ii) *blue* host cell nuclei, stained with the nonspecific nucleic acid stain DAPI (4,6-diamidino-2-phenylindole). *Bottom panel*: At higher magnification of the insert, single *M. leprae* cells are visible (*yellow*), using a specific PNA-FISH probe, $MLEP_{TAMRA}$. (Courtesy of Dr. Annette Moter, Institute for Microbiology and Hygiene, Charité-Universitymedicine, Berlin, Germany; and Dr. Alberto Paniz Mondolfi, Instituto de Biomedicina, Universidad Central de Venezuela/Instituto Venezolano de los Seguros Sociales, Caracas, Venezuela)

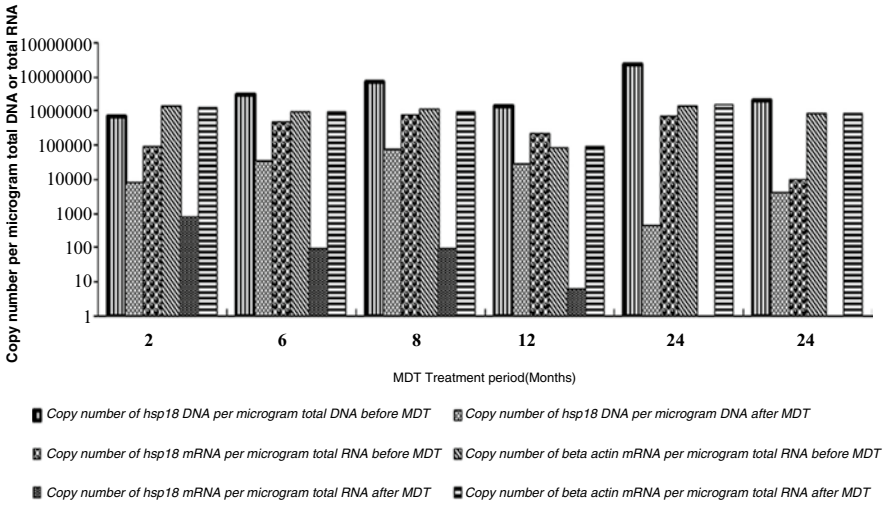

Fig. 16.4 Determination of *Mycobacterium leprae* DNA and mRNA before and after multidrug chemotherapy (MDT). Total DNA and total RNA from six lepromatous leprosy patients were collected before MDT was started and at different time points during treatment. Copy numbers of *hsp18* DNA and mRNA were calculated. Copy number of β-actin mRNA is also shown along with *hsp18* mRNA for comparison. Time points of post-treatment stage (duration in months) at which the samples were collected from each patient are shown on the X-axis. Copy numbers of DNA and mRNA before and after MDT are shown on the Y-axis. Samples from two different patients were analyzed for *hsp18* mRNA at 24 months post-treatment. No detectable RNA could be found at this point in both cases (Courtesy of Dr. Kuppamuthu Dharmalingam, Department of Genetic Engineering, School of Biotechnology, Madurai Kamaraj University, Madurai, Tamil Nadu, India)

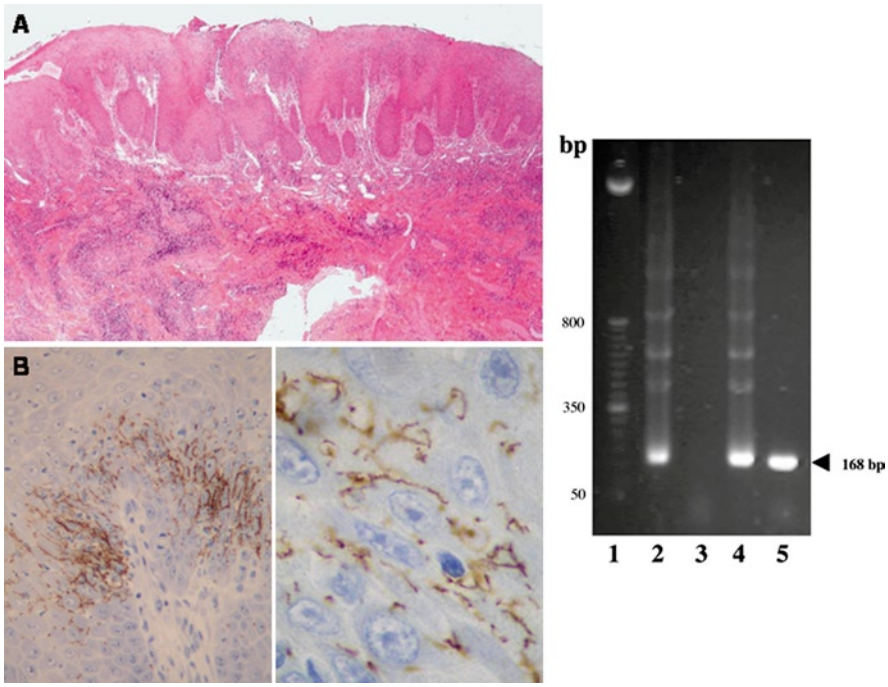

Fig. 16.5 Detection of *Treponema pallidum* in Secondary Syphilis. (**a**) Histopathological features: Irregular epidermal acanthosis with dermal inflammation. (**b**) Immunohistochemistry demonstrating numerous spirochetes within the epidermis. *Right panel*: PCR for *T. pallidum* gene *Tp47* (amplified product is 168-bp). Lane 1, molecular size marker. Lane 2, positive control. Lane 3, negative control. Lane 4, DNA extracted from a swab of a papule from the trunk of a patient with secondary syphilis. Lane 5, DNA extracted from a biopsy of the same papule (Courtesy of Drs. Nicolas Dupin, Philippe Grange, and Françoise Plantier, Departments of Dermatology and Pathology, Hôpital COCHIN, AP-HP and National Reference Center for Syphilis, Paris, France)

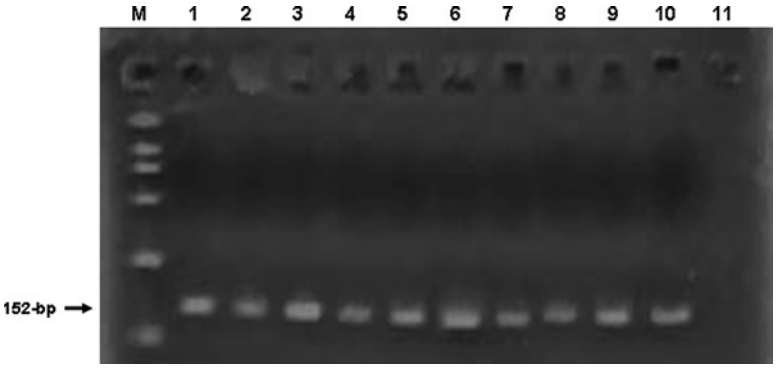

Fig. 16.6 Detection of *Sporothrix schenckii* DNA in nine human lesional skin biopsies by PCR assay. A 152-bp fragment was obtained with the inner primers SS_3 and SS_4. Lane M, molecular size marker ladder DL2000; lane 1, ATCC10268 (positive control); lanes 2–10, skin biopsies; lane 11, distilled water (Courtesy of Dr. Hong-Duo Chen, Dermatology, No.1 Hospital of China Medical University, Shenyang, China)

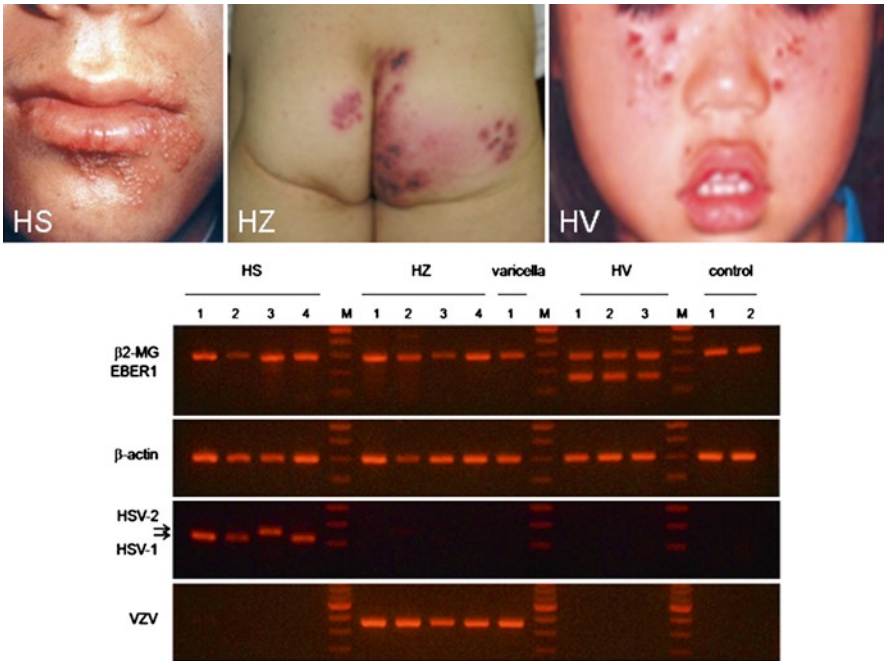

Fig. 16.7 Detection of herpesvirus-encoded transcripts in skin lesions. HS, herpes simplex; HZ, herpes zoster; HV, hydroa vacciniforme; Lane M, molecular size marker; β2-MG, β2-microglobulin; EBER1, *Epstein-Barr virus*-encoded small nuclear RNA-1; HSV, *herpes simplex virus* (HSV-1 UL30, 129-bp; HSV-2 UL30, 163-bp); VZV, *varicella zoster virus* (ORF40, 363-bp) (Courtesy of Drs. Keiji Iwatsuki and Youzi Hirai, Department of Dermatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan)

greatly increasing the signal for each target, and thereby the sensitivity over nonamplified single-probe methods. The latter encompass the hybridization protection assay (HPA), and in situ hybridization (ISH) methodologies, such as FISH and chromogenic in situ hybridization (CISH). In addition, the ViaGram™ staining kit uses fluorescent nucleic acid stains to differentially label many gram-positive and gram-negative bacterial species and, at the same time, distinguish live from dead

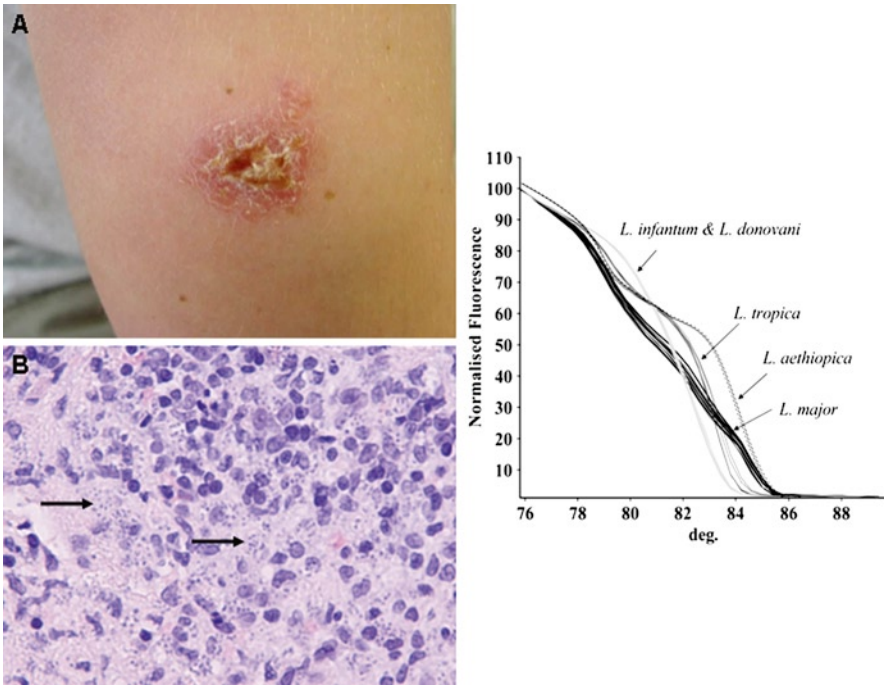

Fig. 16.8 Detection of cutaneous *Leishmaniasis*. (**a**) Clinical signs and (**b**) histopathological features of cutaneous leishmaniasis (*arrows*). *Right panel*: High resolution melting (HRM) curves of the 265–288 bp ITS1-PCR amplicon of Old World *Leishmania* species. Normalized fluorescence is plotted against °C degrees (deg.). The curves include parasites from different hosts and geographic origins, including 7 strains of *L. major*, 5 of *L. aethiopica*, 7 of *L. tropica*, 13 of *L. infantum*, and 2 of *L. donovani* (*Right panel*: Reprinted from Talmi-Frank et al. [87]. Open Access Journal)

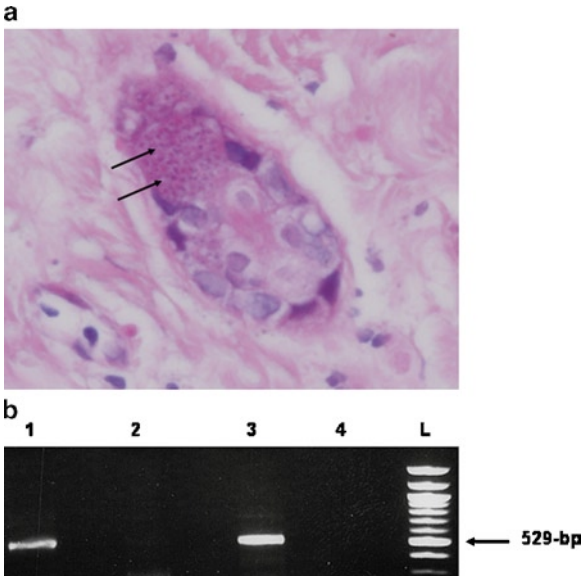

Fig. 16.9 Detection of cutaneous *Toxoplasma gondii*. (**a**) "Cysts" with numerous tiny bradyzoites are seen within the epithelial cells of a sweat gland duct (*arrows*). (**b**) PCR result confirming cutaneous lesion as *T. gondii*. Lane 1: Skin biopsy specimen from *T. gondii*-infected patient; lane 2: skin biopsy specimen from patient with leishmaniasis; lane 3: *T. gondii* positive control; lane 4: negative control; lane L: 100-bp ladder (Courtesy of Drs. Gail Amir and Harold Salant, Departments of Pathology and Parasitology, Hadassah Medical Center and Hebrew University, Jerusalem, Israel)

microbes on the basis of plasma membrane integrity. Although some probes require prior growth in culture for accurate identification and achievement of their reported sensitivities, hybridization technologies can also be used directly on clinical specimens or histologic sections for microbe detection. One advantage of in situ technologies is the capacity to correlate results of molecular studies with the morphological features of the disease (i.e., the location of the pathogen within the tissue can be identified). Of note, the ability to use fluorescent molecular probe technologies (such as FISH and ViaGram™) to not only determine bacterial presence, but also to visually characterize the bacterial communities that exist in cutaneous ulcers is leading to a better understanding of the relationship between particular bacteria and the pathobiology of chronic wounds (Fig. 17.3) [22, 90]. Growing bacteria (and fungi) produce an abundance of ribosomal RNAs (rRNAs) that contain regions of highly conserved, species-specific sequences – ideal targets for detection assays, such as FISH. However, the target sequences are frequently located in highly structured regions of the rRNA that are virtually inaccessible to DNA probes. In addition, bacterial cell walls may hamper DNA probe penetration. The unique properties of PNA probes facilitate access to these sites. A PNA probe is a DNA mimic, in which the negatively charged sugar–phosphate backbone of DNA is replaced with a noncharged polyamide or "peptide" backbone, but still contains the same nucleotide bases as DNA (adenine (A), cytosine (C), guanine (G), and thymine (T)), and follows standard Watson-Crick base-pairing rules upon hybridization to complementary nucleic acid sequences. Because of their uncharged chemical backbone, PNA probes possess unique hybridization characteristics, including rapid and stronger binding to complementary targets compared with traditional DNA probes. PNA-FISH is particularly suited to the fast and accurate identification of bacteria and fungi in tissue sections (Fig. 16.3). For example, FISH performed with a species-specific PNA probe, in combination with a PNA probe that detects all eubacterial species, represents a powerful technique for rapid tissue-based detection of bacteria, and for the determination of their structural organization and spatial distribution in clinical samples, such as chronic wounds. Although chronic wounds are commonly polymicrobial, investigators have used PNA-FISH and ViaGram™ technologies to determine that bacteria often exist in aggregates (usually *P. aeruginosa* or *S. aureus*), which are enclosed in self-produced extracellular polymeric matrices within wounds ("biofilms") (see Chap. 17) [90].

Depending on the clinical setting, different technologies and protocols may be employed. For example, PCR-based assays for bacterial detection commonly investigate for 16S ribosomal DNA sequences ("the universal primer"), found in all bacterial species, but not in eukaryotes. In this setting, post-amplification analyses of amplicons (i.e., sequencing) are relied upon to more accurately identify bacterial subtypes. Alternatively, testing using primers that are specific to a particular bacterium or species may be employed; for example, *hsp65* gene and fragment of the right-arm of the insertion element IS6110 for *mycobacterium* spp. [10, 13], *mtp40* gene for *M. tuberculosis* [10], 172-bp fragment of rRNA and *hsp18* gene for *M. leprae* [17, 18], *nuc* and *orfX* genes for *S. aureus* [21, 22], *oprL* gene for *P. aeruginosa* [21, 22], *htrA* and *gltA* genes for *Bartonella* spp. [34, 37], *ompA* gene for *R. rickettsii* [40], *rpoB*, *capA* and *pagA* genes for *B. anthracis* [41], and *Tp47* gene for *T. pallidum* [47]. The most common targets used for fungal detection by molecular methods are the ribosomal genes, including 5.8S rDNA, 18S rDNA, 28S rDNA, ITS1, and ITS2 regions (see Chap. 15). Preferred target genes are those that are present as multiple copies in the microbe genome, thereby increasing the sensitivity of detection. One important caveat is that assays which rely on DNA analysis can identify both viable and nonviable microbes. Importantly, fragments of DNA from microorganisms that have lysed as a function of antimicrobial therapy and/or host immune response may be incorrectly identified as viable species. It is likely that evolving technologies will increasingly focus on mRNA analysis, facilitating the detection of only viable microbes in the tissue or fluid sample. Another advantage of RNA-directed assays is that the starting number of template molecules is typically much higher (particularly for rRNA) compared to DNA, thereby significantly increasing test sensitivity and decreasing required sample volumes [85].

These technologies are described in greater detail in Chap. 3. A number of kits with US Food and Drug Administration (FDA)-clearance/approval are now commercially available for the detection of common infectious agents. An updated list of approved bacterial, fungal, and viral tests can be found on the Association for Molecular Pathology website (http://www.amp.org/FDATable/FDATable.doc). These assays have been cleared or approved for clinical diagnostic use and require minimal laboratory validation prior to routine testing. However, kits are not available for the identification of all pathogens associated with mucocutaneous disease. Accordingly, laboratories must develop and validate in-house molecular assays (home brew tests) for many infectious conditions.

One area of dermatology where molecular technologies are proving to be invaluable is in the evaluation of chronic wounds, including pressure, diabetic and venous leg ulcers [20–22, 90–92]. There is now a large body of evidence concerning the pathophysiology of aberrant wound healing, and it is known that nonhealing chronic ulcers show microbial, in addition to biochemical and histopathological, differences compared with their healing counterparts. Of note, bacterial loads and the presence of "biofilms" within chronic ulcers may adversely affect healing, even in the absence of clinical signs of infection [20–22, 90–92]. The utilization of molecular assays has demonstrated how culture-based methodologies commonly underestimate the bacteria present, particularly in those ulcers with slow-growing, fastidious, or anaerobic microbes [20–22, 90–92]. In order to improve treatment outcomes of chronic ulcers, it is imperative to determine which microbes are "benign" colonizers and whether the most commonly identified bacteria are the critical causative agents, or if other microbes also contribute to wound persistence. The application of molecular technologies to the management of chronic ulcers is discussed in Chap. 17.

In addition to the detection and identification of infectious agents, molecular methods may also be used to characterize virulence factors and potential drug resistance mechanisms in microbes (Table 16.3). In this regard, both HA-MRSA (healthcare-associated methicillin-resistant *S. aureus*) and CA-MRSA (community-associated methicillin-resistant *S. aureus*) are now becoming major concerns in dermatology outpatient clinics [24, 25]. However, CA-MRSA is distinct from HA-MRSA in a number of respects. HA-MRSA mainly possess the type I-IV staphylococcal cassette chromosome *mec* (SCC*mec*), which confers resistance to both β-lactam and many non-β-lactam antibiotics, including clindamycin, gentamicin, and the fluoroquinolones [25]. In contrast, CA-MRSA predominantly possess SCC*mec*A type IV or V, and are susceptible to most groups of antibiotic agents, but do display variable resistance to a small number of antimicrobials, including fusidic acid (which is associated with the *far-1* gene, coding for a ribosome protection mechanism) [25]. Furthermore, the majority of CA-MRSA possess the Panton-Valentine leukocidin (PVL) gene, which is uncommon in HA-MRSA. PVL is a bicomponent (*luk*S-PV and *luk*F-PV), pore-forming exotoxin which targets inflammatory cells, including neutrophils [25]. PVL-producing strains, including CA-MRSA, are preferentially isolated from particular skin lesions, such as furuncles and cutaneous abscesses. While most PVL-related infections are uncomplicated, there is a risk, albeit small, that severe systemic infection may occur [25]. Dermatologists are ideally positioned to identify the presence of virulent resistant *S. aureus* strains. In this respect, recent studies by Yao et al. [24] and Jappe et al. [25] employed PCR-based methods to determine PVL production and presence of drug resistance genes (*mecA* and *far-1*) in both hospital-acquired and community-acquired *S. aureus* isolates causing skin and soft tissue infections.

Another interesting application of molecular technologies is the monitoring of treatment responses in patients with cutaneous infections and chronic wounds, in an effort to determine drug efficacy and/or identify disease persistence or relapse [17, 19, 43, 92]. Lini et al. [17] evaluated the efficacy of multidrug chemotherapy (MDT) in 47 leprosy patients, using a qPCR-based assay to quantify bacterial DNA and *hsp18* mRNA copy number from paraffin-embedded biopsy samples (Fig. 16.4). A reduction in both DNA and mRNA during MDT was observed, and *hsp18* mRNA could not be identified in patients who had received 2 years of treatment. However, Lini et al. [17] reported that *M. leprae* DNA was still detectable, even after 2 years of MDT. In addition, *hsp18*

mRNA was found in reactional cases, indicating that the treatment regimens of such patients should take into consideration the possibility of live bacilli persistence in reversal cases. Importantly, the correlation between MDT and the decline in gene expression level indicated the utility of this approach for monitoring disease persistence/progression and efficacy of chemotherapy in patients with leprosy. Phetsuksiri et al. [19] reported that a one-step RT-PCR assay was also effective in monitoring bacterial clearance in leprosy patients during chemotherapy. In this study, the persistence of *M. leprae* 16S rRNA gene positivity in ~32% of patients (16 of 36 with multibacillary disease, 3 of 24 with paucibacillary disease), following MDT for 6 months, indicated the need for longer courses of treatment [19]. In addition, Pícha et al. [43] noted that a decrease in PCR positivity correlated with the clinical effect of antibacterial therapy in patients with Lyme borreliosis. Finally, Price et al. [92] used 16S rRNA gene-based pyrosequencing analysis to determine that chronic wound bacterial flora from antibiotic-treated patients was significantly different from untreated patients, and characterized by *Pseudomonas*-dominated communities. Interestingly, antibiotic use may select for biofilm-producing microbes, such as *Pseudomonas*, and retard rather than expedite wound healing [92]. In addition, chronic wounds in diabetic patients are associated with significant *Streptococcus* colonization, and antibiotic therapy is found to reduce this finding [92].

Molecular testing has also been used to discover and characterize microbes that play a role in the pathophysiology of a number of benign and malignant mucocutaneous proliferations and tumors. These include *human papillomavirus* (HPV) in proliferative epithelial lesions; *human herpesvirus 8* (HHV-8) in Kaposi's sarcoma; *Merkel cell polyomavirus* (MCPyV) in Merkel cell carcinoma (Fig. 3.1); *Epstein-Barr virus* (EBV) in oral hairy leukoplakia, lymphoepithelioma-like carcinoma of the skin, and extranodal NK/T-cell lymphoma (Fig. 11.3); and *human T-cell lymphotropic virus type 1* (HTLV-1) in adult T-cell leukemia/lymphoma (Fig. 11.6). In some instances, these discoveries have provided an opportunity to utilize more widely available laboratory technologies for the diagnosis of tumor-associated microbes; for example, the recently developed monoclonal antibody CM2B4 can be used for immunohistochemical-based identification of MCPyV [95]. Interestingly, the detection of MCPyV may have both diagnostic and prognostic implications in the setting of Merkel cell carcinoma, in addition to identifying mechanisms of tumor pathogenesis [82]. The HPVs represent a large and diverse group of viruses (>100 types) which infect keratinocytes at mucocutaneous surfaces [60–69]. There are five major HPV genera (*alpha*, *beta*, *gamma*, *mu*, and *nu*), and cutaneous types are found in otherwise normal-appearing skin, benign skin warts (verrucae), epidermodysplasia verruciformis, actinic keratoses, and non-melanoma skin cancer (NMSC), including cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC) [60–69]. Of note, a higher prevalence of HPV infection is reported for immunosuppressed patients compared with immunocompetent individuals. HPV infection is also associated with genital condylomata, cervical carcinoma, and other mucosal cancers [60–69]. The frequency of HPV DNA detection in primary and metastatic cSCC ranges from 9% to 60%, and includes alpha-HPV (#18) and beta-HPV (#15, 20, 22, 23, 24, 35) types [60–69]. However, HPV integration into the host genome is rare in cSCC, and most NMSC contain only very low viral loads, usually less than 1 copy/1,000 cells. Occasional NMSC may demonstrate very high levels of virus (up to 1.3×10^6 copies/cell) [60–69]. The exact role of HPV infection in NMSC remains unclear. The search for new HPV types in NMSC continues, and as yet unidentified HPV types could be present in such lesions. In addition, the recent development of preventive vaccines for HPV types associated with cervical cancer may spur on further research for preventive and/or therapeutic vaccines against HPV-associated cutaneous proliferative epithelial lesions, particularly in immunocompromised individuals (i.e., organ transplant recipients). Of course, the development of such vaccines will require a greater understanding of the natural history of HPV types that are associated with cutaneous warts and NMSC.

Based on test volume data, the detection and characterization of infectious diseases is the most commonly used clinical application of molecular diagnostic testing [96]. Importantly, these methods improve patient care by reducing test turn-around times (relative to conventional testing strategies),

in addition to confirming diagnoses that are based on clinical signs and symptoms. The trend in microbial molecular testing is from single- to multiple-pathogen detection assays, and from centralized laboratory-based to point-of-care (POC) tests [97, 98]. Many molecular (i.e., target amplification) assays consist essentially of three steps: extraction and purification of nucleic acid, amplification of the target sequence, and detection of the amplified product. The goal of POC testing is to streamline and miniaturize these processes in order to develop hand-held devices for "bed-side" testing in resource-limited settings (i.e., developing countries) and/or in the workup of those conditions (i.e., life-threatening infections) where rapid diagnosis is necessary for initiation of appropriate therapy. In the future, it is envisioned that protein/antibody and cDNA/oligonucleotide microarrays, whole genome sequencing, mass spectrometry (for both nucleic acid and proteome analysis), and nano/microfluidic technologies will be increasingly employed in clinical settings. Instead of detecting the infectious agent itself, new strategies could evaluate for either the host immune response or an individual's background genetic variation [99]. It is believed that routine use of these technologies will enable us to more accurately diagnose infections, analyze virulence factors/resistance determinants, and specifically tailor treatment strategies for individual patients. Of note, a commercially available microarray-based HPV test (PapilloCheck HPV Screening Test, Greiner Bio-one GmbH) recently showed comparable results to PCR-based assays [60]. In addition, these technologies may provide a means to evaluate host-specific responses and host-pathogen interactions, and facilitate broader epidemiological and public health investigations [1–5, 97, 98]. While almost all of the currently employed molecular tests rely on *a priori* genomic knowledge of the pathogen to be investigated, these new strategies could be used to characterize previously unsuspected or unknown microbes, and should promote the development of novel therapeutic strategies (i.e., drugs and vaccines). While cost-prohibitive at present, it is likely that the speed of technological advancement will make these forms of testing affordable and more widely available in the future. As we move forward, more microbe genomes and target genes will be sequenced, costs associated with equipment and reagents will decrease, greater test standardization and validation data will be achieved, and more kits will be granted FDA approval. The diagnostic applications of these technologies in the setting of cutaneous microbiology will continue to grow, and their general acceptance and broader implementation in the practice of dermatology and dermatopathology will be assured.

References

1. Bravo LT, Procop GW. Recent advances in diagnostic microbiology. Semin Hematol. 2009;46:248–58.
2. Muldrew KL. Molecular diagnostics of infectious diseases. Curr Opin Pediatr. 2009;21:102–11.
3. Procop GW. Molecular diagnostics for the detection and characterization of microbial pathogens. Clin Infect Dis. 2007;45 Suppl 2:S99–111.
4. Weile J, Knabbe C. Current applications and future trends of molecular diagnostics in clinical bacteriology. Anal Bioanal Chem. 2009;394:731–42.
5. Miller MB, Tang YW. Basic concepts of microarrays and potential applications in clinical microbiology. Clin Microbiol Rev. 2009;22:611–33.
6. Sra KK, Torres G, Rady P, et al. Molecular diagnosis of infectious diseases in dermatology. J Am Acad Dermatol. 2005;53:749–65.
7. Payne DA, Vander Straten M, Carrasco D, et al. Molecular diagnosis of skin-associated infectious agents. Arch Dermatol. 2001;137:1497–502.
8. Balasingham SV, Davidsen T, Szpinda I, et al. Molecular diagnostics in tuberculosis: basis and implications for therapy. Mol Diagn Ther. 2009;13:137–51.
9. Palomino JC. Molecular detection, identification and drug resistance detection in Mycobacterium tuberculosis. FEMS Immunol Med Microbiol. 2009;56:103–11.
10. Welsh O, Vera-Cabrera L, Fernández-Reyes M, et al. Cutaneous tuberculosis confirmed by PCR in three patients with biopsy and culture negative for mycobacterium tuberculosis. Int J Dermatol. 2007;46:734–5.

11. Abdalla CM, de Oliveira ZN, Sotto MN, et al. Polymerase chain reaction compared to other laboratory findings and to clinical evaluation in the diagnosis of cutaneous tuberculosis and atypical mycobacteria skin infection. Int J Dermatol. 2009;48:27–35.
12. Chemlal K, Portaels F. Molecular diagnosis of nontuberculous mycobacteria. Curr Opin Infect Dis. 2003;16:77–83.
13. Da Mata O, Pérez Alfonzo R, Natera I, et al. The diagnosis of two cases of cutaneous ulcer caused by infection with Mycobacterium haemophilum: direct identification in a clinical sample by polymerase chain reaction-restriction endonuclease analysis. Int J Dermatol. 2008;47:820–3.
14. van Coppenraet LS, Smit VT, Templeton KE, et al. Application of real-time PCR to recognize atypical mycobacteria in archival skin biopsies: high prevalence of Mycobacterium haemophilum. Diagn Mol Pathol. 2007;16:81–6.
15. Tigges F, Bauer A, Hochauf K, et al. Sporotrichoid atypical cutaneous infection caused by Mycobacterium marinum. Acta Dermatovenerol Alp Panonica Adriat. 2009;18:31–4.
16. Lefmann M, Schweickert B, Buchholz P, et al. Evaluation of peptide nucleic acid-fluorescence in situ hybridization for identification of clinically relevant mycobacteria in clinical specimens and tissue sections. J Clin Microbiol. 2006;44:3760–7.
17. Lini N, Shankernarayan NP, Dharmalingam K. Quantitative real-time PCR analysis of Mycobacterium leprae DNA and mRNA in human biopsy material from leprosy and reactional cases. J Med Microbiol. 2009;58:753–9.
18. Bang PD, Suzuki K, le Phuong T, et al. Evaluation of polymerase chain reaction-based detection of Mycobacterium leprae for the diagnosis of leprosy. J Dermatol. 2009;36:269–76.
19. Phetsuksiri B, Rudeeaneksin J, Supapkul P, et al. A simplified reverse transcriptase PCR for rapid detection of Mycobacterium leprae in skin specimens. FEMS Immunol Med Microbiol. 2006;48:319–28.
20. Frankel YM, Melendez JH, Wang NY, et al. Defining wound microbial flora: molecular microbiology opening new horizons. Arch Dermatol. 2009;145:1193–5.
21. Melendez J, Frankel Y, An A, et al. Real-time PCR assays compared to culture-based approaches for identification of aerobic bacteria in chronic wounds. Clin Microbiol Infect. 2010;16:1762–9.
22. Thomsen TR, Aasholm MS, Rudkjøbing VB, et al. The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. Wound Repair Regen. 2010;18:38–49.
23. Gao Z, Tseng CH, Pei Z, et al. Molecular analysis of human forearm superficial skin bacterial biota. Proc Natl Acad Sci USA. 2007;104:2927–32.
24. Yao D, Yu FY, Qin ZQ, et al. Molecular characterization of Staphylococcus aureus isolates causing skin and soft tissue infections (SSTIs). BMC Infect Dis. 2010;10:133.
25. Jappe U, Heuck D, Strommenger B, et al. Staphylococcus aureus in dermatology outpatients with special emphasis on community-associated methicillin-resistant strains. J Invest Dermatol. 2008;128:2655–64.
26. Gilpin DF, Tunney MM, Funston C, et al. Rapid detection of MRSA in a routine diagnostic laboratory using a real-time PCR assay. J Hosp Infect. 2007;67:97–9.
27. De Vos D, Lim Jr A, Pirnay JP, et al. Direct detection and identification of Pseudomonas aeruginosa in clinical samples such as skin biopsy specimens and expectorations by multiplex PCR based on two outer membrane lipoprotein genes, oprI and oprL. J Clin Microbiol. 1997;35:1295–9.
28. Mashouf RY, Zamani A, Farahani HS. Diagnostic multiplex polymerase chain reaction assay for the identification of Pseudomonas aeruginosa from the skin biopsy specimens in burn wound infections and detection of antibiotic susceptibility. Saudi Med J. 2008;29:1109–14.
29. Currie BJ. Group A streptococcal infections of the skin: molecular advances but limited therapeutic progress. Curr Opin Infect Dis. 2006;19:132–8.
30. Lee SH, Vigliotti VS, Pappu S. DNA sequencing validation of Chlamydia trachomatis and Neisseria gonorrhoeae nucleic acid tests. Am J Clin Pathol. 2008;129:852–9.
31. Geertsen R, Friderich P, Dobec M, et al. Evaluation of an automated extraction method for the detection of Chlamydia trachomatis and Neisseria gonorrhoeae by Cobas Amplicor PCR from different sample materials. Scand J Infect Dis. 2007;39:405–8.
32. Koenig MG, Kosha SL, Doty BL, et al. Direct comparison of the BD ProbeTec ET system with in-house LightCycler PCR assays for detection of Chlamydia trachomatis and Neisseria gonorrhoeae from clinical specimens. J Clin Microbiol. 2004;42:5751–6.
33. Palmer HM, Mallinson H, Wood RL, et al. Evaluation of the specificities of five DNA amplification methods for the detection of Neisseria gonorrhoeae. J Clin Microbiol. 2003;41:835–7.
34. Hansmann Y, DeMartino S, Piémont Y, et al. Diagnosis of cat scratch disease with detection of Bartonella henselae by PCR: a study of patients with lymph node enlargement. J Clin Microbiol. 2005;43:3800–6.
35. Lange D, Oeder C, Waltermann K, et al. Bacillary angiomatosis. J Dtsch Dermatol Ges. 2009;7:767–9.
36. Amsbaugh S, Huiras E, Wang NS, et al. Bacillary angiomatosis associated with pseudoepitheliomatous hyperplasia. Am J Dermatopathol. 2006;28:32–5.

37. Sala M, Font B, Sanfeliu I, et al. Bacillary angiomatosis caused by Bartonella quintana. Ann N Y Acad Sci. 2005;1063:302–7.
38. Paddock CD, Fernandez S, Echenique GA, et al. Rocky Mountain spotted fever in Argentina. Am J Trop Med Hyg. 2008;78:687–92.
39. Eremeeva ME, Bosserman E, Zambrano M, et al. Molecular typing of novel Rickettsia rickettsii isolates from Arizona. Ann N Y Acad Sci. 2006;1078:573–7.
40. Paddock CD, Zaki SR, Koss T, et al. Rickettsialpox in New York City: a persistent urban zoonosis. Ann NY Acad Sci. 2003;990:36–44.
41. Tatti KM, Greer P, White E, et al. Morphologic, immunologic, and molecular methods to detect bacillus anthracis in formalin-fixed tissues. Appl Immunohistochem Mol Morphol. 2006;14:234–43.
42. Hulínská D, Votýpka J, Vanousová D, et al. Identification of Anaplasma phagocytophilum and Borrelia burgdorferi sensu lato in patients with erythema migrans. Folia Microbiol (Praha). 2009;54:246–56.
43. Pícha D, Moravcová L, Holecková D, et al. Examination of specific DNA by PCR in patients with different forms of Lyme borreliosis. Int J Dermatol. 2008;47:1004–10.
44. Cerar T, Ruzić-Sabljić E, Glinsek U, et al. Comparison of PCR methods and culture for the detection of Borrelia spp. in patients with erythema migrans. Clin Microbiol Infect. 2008;14:653–8.
45. Behrhof W, Springer E, Brauninger W, et al. PCR testing for Treponema pallidum in paraffin-embedded skin biopsy specimens: test design and impact on the diagnosis of syphilis. J Clin Pathol. 2008;61:390–5.
46. Šmajs D, Matejková P, Woznicová V. Diagnosis of syphilis by polymerase chain reaction and molecular typing of Treponema pallidum. Rev Med Microbiol. 2006;17:93–9.
47. Buffet M, Grange PA, Gerhardt P, et al. Diagnosing Treponema pallidum in secondary syphilis by PCR and immunohistochemistry. J Invest Dermatol. 2007;127:2345–50.
48. Vollmer T, Störmer M, Kleesiek K, et al. Evaluation of novel broad-range real-time PCR assay for rapid detection of human pathogenic fungi in various clinical specimens. J Clin Microbiol. 2008;46:1919–26.
49. Turin L, Riva F, Galbiati G, et al. Fast, simple and highly sensitive double-rounded polymerase chain reaction assay to detect medically relevant fungi in dermatological specimens. Eur J Clin Invest. 2000;30:511–8.
50. Sato T, Takayanagi A, Nagao K, et al. Simple PCR-based DNA microarray system to identify human pathogenic fungi in skin. J Clin Microbiol. 2010;48:2357–64.
51. Forrest GN, Mankes K, Jabra-Rizk MA, et al. Peptide nucleic acid fluorescence in situ hybridization-based identification of Candida albicans and its impact on mortality and antifungal therapy costs. J Clin Microbiol. 2006;44:3381–3.
52. Goldani LZ, Aquino VR, Lunardi LW, et al. Two specific strains of Histoplasma capsulatum causing mucocutaneous manifestations of histoplasmosis: preliminary analysis of a frequent manifestation of histoplasmosis in southern Brazil. Mycopathologia. 2009;167:181–6.
53. Meece JK, Anderson JL, Klein BS, et al. Genetic diversity in Blastomyces dermatitidis: implications for PCR detection in clinical and environmental samples. Med Mycol. 2010;48:285–90.
54. Bialek R, González GM, Begerow D, et al. Coccidioidomycosis and blastomycosis: advances in molecular diagnosis. FEMS Immunol Med Microbiol. 2005;45:355–60.
55. Castañón-Olivares LR, Güereña-Elizalde D, González-Martínez MR, et al. Molecular identification of coccidioides isolates from Mexican patients. Ann N Y Acad Sci. 2007;1111:326–35.
56. Segner S, Jouret F, Durant JF, et al. Cutaneous infection by Alternaria infectoria in a renal transplant patient. Transpl Infect Dis. 2009;11:330–2.
57. Lo Cascio G, Ligozzi M, Maccacaro L, et al. Utility of molecular identification in opportunistic mycotic infections: a case of cutaneous Alternaria infectoria infection in a cardiac transplant recipient. J Clin Microbiol. 2004;42:5334–6.
58. Xu TH, Lin JP, Gao XH, et al. Identification of Sporothix schenckii of various mtDNA types by nested PCR assay. Med Mycol. 2010;48:161–5.
59. Hu S, Chung WH, Hung SI, et al. Detection of Sporothrix schenckii in clinical samples by a nested PCR assay. J Clin Microbiol. 2003;41:1414–8.
60. Dalstein V, Merlin S, Bali C, et al. Analytical evaluation of the PapilloCheck test, a new commercial DNA chip for detection and genotyping of human papillomavirus. J Virol Methods. 2009;156:77–83.
61. Schenk T, Brandstetter T, Zur Hausen A, et al. Performance of a polymer-based DNA chip platform in detection and genotyping of human papillomavirus in clinical samples. J Clin Microbiol. 2009;47:1428–35.
62. de Koning M, Struijk L, Feltkamp M, et al. HPV DNA detection and typing in inapparent cutaneous infections and premalignant lesions. Methods Mol Med. 2005;119:115–27.
63. Lei YJ, Gao C, An R, et al. Development of a multiplex PCR method for detecting and typing human papillomaviruses in verrucae vulgaris. J Virol Methods. 2008;147:72–7.
64. de Koning MN, ter Schegget J, Eekhof JA, et al. Evaluation of a novel broad-spectrum PCR-multiplex genotyping assay for identification of cutaneous wart-associated human papillomavirus types. J Clin Microbiol. 2010;48:1706–11.

65. Küsters-Vandevelde HV, de Koning MN, Melchers WJ, et al. Expression of p14ARF, p16INK4a and p53 in relation to HPV in (pre-)malignant squamous skin tumours. J Cell Mol Med. 2009;13:2148–57.
66. Küsters-Vandevelde HV, Van Leeuwen A, Verdijk MA, et al. CDKN2A but not TP53 mutations nor HPV presence predict poor outcome in metastatic squamous cell carcinoma of the skin. Int J Cancer. 2010;126:2123–32.
67. Feltkamp MC, de Koning MN, Bavinck JN, et al. Betapapillomaviruses: innocent bystanders or causes of skin cancer. J Clin Virol. 2008;43:353–60.
68. Ekström J, Forslund O, Dillner J. Three novel papillomaviruses (HPV109, HPV112 and HPV114) and their presence in cutaneous and mucosal samples. Virology. 2010;397:331–6.
69. Zaravinos A, Kanellou P, Spandidos DA. Viral DNA detection and RAS mutations in actinic keratosis and nonmelanoma skin cancers. Br J Dermatol. 2010;162:325–31.
70. Engelmann I, Petzold DR, Kosinska A, et al. Rapid quantitative PCR assays for the simultaneous detection of herpes simplex virus, varicella zoster virus, cytomegalovirus, Epstein-Barr virus, and human herpesvirus 6 DNA in blood and other clinical specimens. J Med Virol. 2008;80:467–77.
71. Vrioni G, Kalogeropoulos C, Gartzonika C, et al. Usefulness of Herpes Consensus PCR methodology to routine diagnostic testing for herpesviruses infections in clinical specimens. Virol J. 2007;4:59.
72. Yamamoto T, Yamada A, Tsuji K, et al. Tracing of the molecular remnants of herpes virus infections in necrotic skin tissue. Eur J Dermatol. 2008;18:499–503.
73. Abraham AM, Babu M, Kavitha S, et al. A molecular method for typing Herpes simplex virus isolates as an alternative to immunofluorescence methods. Indian J Med Microbiol. 2009;27:22–6.
74. Gulley ML, Tang W. Laboratory assays for Epstein-Barr virus-related disease. J Mol Diagn. 2008;10:279–92.
75. Ko YH, Cho EY, Kim JE, et al. NK and NK-like T-cell lymphoma in extranasal sites: a comparative clinicopathological study according to site and EBV status. Histopathology. 2004;44:480–9.
76. Tsai KS, Hsieh HJ, Chow KC, et al. Detection of cytomegalovirus infection in a patient with febrile ulceronecrotic Mucha-Habermann's disease. Int J Dermatol. 2001;40:694–8.
77. Galan A, McNiff JM, Choi JN, et al. Fatal HHV6 infection in an immunocompromised patient presenting with skin involvement. J Cutan Pathol. 2010;37:277–81.
78. Pak F, Mwakigonja AR, Kokhaei P, et al. Kaposi's sarcoma herpesvirus load in biopsies of cutaneous and oral Kaposi's sarcoma lesions. Eur J Cancer. 2007;43:1877–82.
79. Pak F, Pyakural P, Kokhaei P, et al. HHV-8/KSHV during the development of Kaposi's sarcoma: evaluation by polymerase chain reaction and immunohistochemistry. J Cutan Pathol. 2005;32:17–21.
80. Bonvicini F, La Placa M, Manaresi E, et al. Parvovirus b19 DNA is commonly harboured in human skin. Dermatology. 2010;220:138–42.
81. Sastre-Garau X, Peter M, Avril MF, et al. Merkel cell carcinoma of the skin: pathological and molecular evidence for a causative role of MCV in oncogenesis. J Pathol. 2009;218:48–56.
82. Bhatia K, Goedert JJ, Modali R, et al. Merkel cell carcinoma subgroups by merkel cell polyomavirus DNA relative abundance and oncogene expression. Int J Cancer. 2010;126:2240–6.
83. Shimamoto Y, Suga K, Shibata K, et al. Clinical importance of extraordinary integration patterns of human T-cell lymphotropic virus type I proviral DNA in adult T-cell leukemia/lymphoma. Blood. 1994;84:853–8.
84. Tsukasaki K, Tsushima H, Yamamura M, et al. Integration patterns of HTLV-I provirus in relation to the clinical course of ATL: frequent clonal change at crisis from indolent disease. Blood. 1997;89:948–56.
85. Reithinger R, Dujardin JC. Molecular diagnosis of leishmaniasis: current status and future applications. J Clin Microbiol. 2007;45:21–5.
86. Tordini G, Giaccherini R, Pacenti L, et al. Cutaneous leishmaniasis: usefulness of PCR on paraffin-embedded skin biopsies as part of routine investigation. Ann Trop Med Parasitol. 2007;101:745–9.
87. Talmi-Frank D, Nasereddin A, Schnur LF, et al. Detection and identification of old world Leishmania by high resolution melt analysis. PLoS Negl Trop Dis. 2010;4:e581.
88. Amir G, Salant H, Resnick IB, et al. Cutaneous toxoplasmosis after bone marrow transplantation with molecular confirmation. J Am Acad Dermatol. 2008;59:781–4.
89. Fukuyama S, Nishimura T, Yotsumoto H, et al. Diagnostic usefulness of a nested PCR assay for detecting Sarcoptes scabiei DNA in skin scrapings from clinically suspected scabies. Br J Dermatol. 2010;163:892–4.
90. Burmølle M, Thomsen TR, Fazli M, et al. Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. FEMS Immunol Med Microbiol. 2010;59:324–36.
91. Martin JM, Zenilman JM, Lazarus GS. Molecular microbiology: new dimensions for cutaneous biology and wound healing. J Invest Dermatol. 2010;130:38–48.
92. Price LB, Liu CM, Melendez JH, et al. Community analysis of chronic wound bacteria using 16S rRNA gene-based pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. PLoS One. 2009;4:e6462.
93. Alexander BD, Ashley ED, Reller LB, et al. Cost savings with implementation of PNA FISH testing for identification of Candida albicans in blood cultures. Diagn Microbiol Infect Dis. 2006;54:277–82.

94. Ramers C, Billman G, Hartin M, et al. Impact of a diagnostic cerebrospinal fluid enterovirus polymerase chain reaction test on patient management. JAMA. 2000;283:2680–5.
95. Busam KJ, Jungbluth AA, Rekthman N, et al. Merkel cell polyomavirus expression in Merkel cell carcinomas and its absence in combined tumors and pulmonary neuroendocrine carcinomas. Am J Surg Pathol. 2009;33:1378–85.
96. Murg S, Terry M, editors. Business strategies for molecular diagnostics in the lab 2009, including state of the market report. 2nd ed. Newark, NJ: Washington G-2 Reports; 2009.
97. Lee WG, Kim YG, Chung BG, et al. Nano/Microfluidics for diagnosis of infectious diseases in developing countries. Adv Drug Deliv Rev. 2010;62:449–57.
98. Kaittanis C, Santra S, Perez JM. Emerging nanotechnology-based strategies for the identification of microbial pathogenesis. Adv Drug Deliv Rev. 2010;62:408–23.
99. te Witt R, van Belkum A, van Leeuwen WB. Molecular diagnostics and genotyping of MRSA: an update. Expert Rev Mol Diagn. 2010;10:375–80.

Chapter 17
Wound Healing Disorders: Chronic Wounds and Keloids

Michael J. Murphy

The normal wound healing response can be divided into (1) inflammatory, (2) proliferative, and (3) tissue remodeling (i.e., fibroplasia and maturation) phases that involve complex interactions between various cutaneous-derived and inflammatory cells, cytokines, and the extracellular matrix (ECM) [1–6]. Numerous studies continue to uncover the genetic, epigenetic (i.e., microRNA), cellular (including stem cells), molecular, and biochemical mechanisms underlying this process [1–9]. An in-depth review of normal wound healing principles is beyond the scope of this chapter. However, it is important to point out that a tightly regulated balance between ECM production and degradation is required for normal scar formation. Any disturbances in these opposing processes can lead to wound healing disorders, such as chronic nonhealing ulcers (i.e., ↓ production and ↑ degradation of ECM) or keloids (↑ production and ↓ degradation of ECM) [1–9]. It is envisioned that efforts to improve our current understanding of the mechanisms and pathways that underpin the pathobiology of these disorders may also lead to the development of predictive and/or diagnostic molecular tests that are clinically useful in the management of patients with these conditions, as well as to the discovery of novel therapeutic targets. In this regard, gene and stem-cell therapy is emerging as a promising approach to cutaneous wound treatment [5, 8]

Chronic Wounds

Chronic wounds, including venous leg ulcers (VLU), pressure ulcers, and diabetic ulcers, are a major health care problem and among the most common conditions seen in dermatology clinics. The annual cost of chronic wound care in the USA exceeds $10 billion, representing >50% of the total expenditure for all skin diseases [10]. The prevalence of VLU is ~1% in the USA, affecting more than one million individuals, and accounting for >50% of all lower extremity ulcerations [1, 2]. Importantly, the treatment of VLU places a significant burden on the USA health care system, with management expenditures for this subtype of chronic wound estimated at $1–$2.5 billion per annum [1, 3]. For European countries, wound treatment has been estimated to cost the equivalent of $360 million per year in Denmark, and represents ~1.5–2% of the annual healthcare budget in France,

M.J. Murphy (✉)
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, 21 South Rd., Farmington, CT 06030, USA
e-mail: drmichaelmurphy@netscape.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_17,

Germany, and the UK [11]. In addition to cost considerations, chronic wounds can lead to disability and decreased quality of life, with further psychosocial implications. VLU is a complex multifactorial disease, with both predisposing and exacerbating factors, that include increasing age, female gender, chronic venous disease (CVD), diabetes mellitus, trauma, and skin cancers [3]. Unfortunately, minimal evidence exists with regard to the efficacy of currently available therapies for chronic wounds, and patients with seemingly similar disease phenotypes and undergoing comparable therapeutic modalities can show significantly different outcomes [10]. Only 55–66% of VLU patients respond to the current standard of care (i.e., compression bandaging) [1]. A number of measurable signs, such as initial wound size, wound duration, and change in wound area with treatment, appear to have some clinical utility in identifying nonresponsive cases, but are associated with only ~75% accuracy in predicting healing outcome [1, 2].

There is now a large body of evidence in the research literature on the pathophysiology of aberrant wound healing. It is known that nonhealing chronic ulcers show biochemical and histopathological differences compared with their healing counterparts [1–5]. For example, poor wound healing is associated with the variable expression of cytokines (↑ TNF-α), proteases (↑ MMP-2, ↑ MMP-9) and their inhibitors (↓ TIMPs), fibroblast senescence markers, and oxidative stress markers (↑ iron). Tissue microbiology status is also a major factor (i.e., presence of pathogenic bacteria, ↑ polymicrobial populations), with bacterial loads in chronic wounds adversely affecting healing, even in the absence of clinical signs of infection [1, 2, 10–19]. However, all wounds are colonized by bacteria, and the differentiation of benign colonizers from invading pathogenic microbes is difficult. Common methods employed to determine bacterial populations in chronic wounds are outlined in Table 17.1. Of note, metagenomics, the most recent development in this field, can be used to characterize the entire genetic composition of all bacterial populations in a particular environment, including those present in low numbers or in a dormant metabolic state [10]. Interestingly, chronic wounds show diverse polymicrobial populations, with variable proportions of aerobic and anerobic bacteria; the latter appearing to be dependent on the underlying wound etiology (Fig. 17.1). With culture-based assays, the most frequently observed bacteria in VLU are *Staphylococcus aureus, Pseudomonas aeruginosa*, and *Enterococcus faecalis* [11, 12]. Unfortunately, routine culturing procedures are time-consuming and often lack sensitivity. The increasing use of molecular technologies, in concert with improved sampling techniques, has demonstrated how culture-dependent methods commonly underestimate the bacteria present in wounds, particularly in those ulcers with slow-growing, fastidious, or anaerobic microbes [11, 12]. In a recent study, up to 40% of "cultivable" organisms identified from VLU by molecular methods could not be identified by culture [13]. In fact, robust nucleic acid-based techniques have now demonstrated that only a minority (~1%) of environmental and human host bacteria are detectable by culture methods [10].

In order to improve the treatment of VLU and other chronic wounds, it is imperative to determine if the most commonly identified bacteria are the critical causative agents, or if other microbes also contribute to wound persistence. To date, SeptiFast® (Roche Diagnostics), a multispecies PCR-based test developed for the detection and identification of bacterial and fungal bloodstream infections, has not been applied to the study of chronic wounds [15]. Accordingly, a number of groups have utilized their own laboratory-developed molecular panels to investigate chronic wound flora, employing quantitative polymerase chain reaction (PCR)-, fluorescence in situ hybridization (FISH)-, fingerprinting-, 16S rRNA gene-, pyrosequencing- and metagenomics-based methodologies [10–19]. For example, Melendez et al. [15] have assessed the utility of real-time PCR (qPCR) to determine microbial diversity in chronic wounds, and demonstrated that this approach can be used for rapid detection of the most common, clinically relevant, aerobic organisms. qPCR is faster (~4–6 h) than traditional PCR, and does not require a post-amplification step for microbial identification (Fig. 17.2). The sensitivity of qPCR is comparable with results from tissue culture studies, and nonculturable organisms may even be identified in some instances [15]. In addition, qPCR can be used to distinguish methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus*

Table 17.1 Techniques employed to characterize bacterial flora in chronic wounds (Modified from Martin et al. [10])

Method	Technique description	Processing time and costs	Advantages	Disadvantages	Practice implications
Qualitative culture	Swab culture of wound; Standard bacteriology culture	1 h for Gram stain; 1–3 days for culture results; inexpensive	Inexpensive, widely available	Low sensitivity, not quantitative	Rapid and widely available, used to screen for methicillin-resistant *S. aureus* or *Pseudomonas*
Quantitative culture	Tissue specimen homogenized and organisms identified and quantified	3–4 days for speciation and quantification; expensive	Accurate and reproducible	Labor intensive, requires specialized facilities and not widely available. Long lag time	Widely used in Burn units, but clinical correlation not well defined
Nucleic acid amplification-PCR	DNA is extracted from specimens and amplified, using organism-specific primers	4–6 h; moderate cost	Rapid turnaround, highly sensitive. Equipment increasingly available	Requires testing for known organisms. Not useful for identifying "unknowns", not clinically standardized	Research settings largely. Used for methicillin-resistant *S. aureus* surveillance
PNA-FISH and ViaGram™	In situ fluorescent molecular probe technologies; DNA extraction not required	1–3 h; moderate cost	Correlation of microbial presence/load with tissue morphological features. Distinction of live versus dead bacteria, and gram-positive versus gram-negative bacteria	Requires specialized equipment	Research settings largely
Metagenomic methods	DNA is extracted from wound specimens, amplified and sequenced. All bacterial clones in specimen are sequenced	Several days; requires dedicated facility and personnel; expensive	Identifies all bacterial sequences within a specimen, including fastidious organisms and organisms in low numbers	Expensive, long turnaround time, not standardized. Sequences identified may include surface contaminants and nonviable organisms	Research settings at present, needs clinical correlation

PCR polymerase chain reaction, *PNA-FISH* peptide nucleic acid-fluorescence in situ hybridization

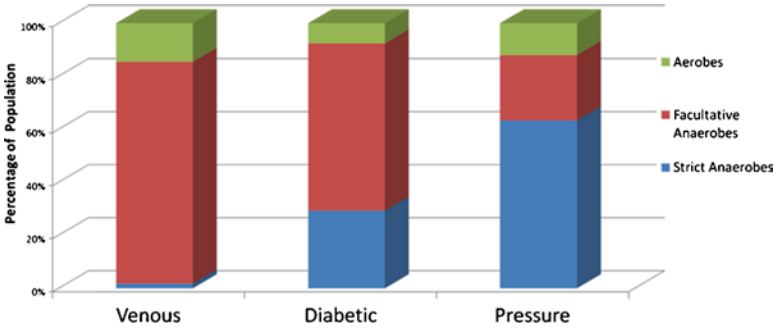

Fig. 17.1 *Distribution of bacterial populations in chronic wounds in relation to aerotolerance*. Diabetic, venous, or pressure ulcer types were analyzed separately using pyrosequencing and the resulting populations grouped into three categories based upon their suggested aerotolerance. This figure graphically illustrates the relative distribution of these functional categories among the wound types (Reprinted from Dowd et al. [18], Open access article)

(MRSA) in chronic wounds [14, 15]. "*Following the development of additional assays targeting other clinically-relevant aerobic and anaerobic organisms*," Melendez et al. [15] report that they "*look forward to developing a rapid, cost-effective, clinically-applicable molecular diagnostic panel to serve in the diagnosis and care of chronic wounds*." In another study, Thomsen et al. [11] used a combination of molecular technologies, including rRNA sequencing, fingerprinting, qPCR, and peptide nucleic acid (PNA)-FISH, to also investigate the bacterial flora in chronic VLU. They noted that each wound contained an average of 5.4 species, but that the actual species varied between wounds [11]. In addition, the numbers of particular bacterial species varied greatly between samples taken at different locations and depths in the same ulcer; emphasizing the need for multiple samplings in order to obtain an accurate representation of the bacterial composition of the entire wound [11, 12]. Of note, molecular analyses have identified a nonrandom distribution pattern of bacterial wound colonization, where *S. aureus* is primarily located close to the wound surface, while *P. aeruginosa* is primarily located deeper in the wound bed [11, 12]. This finding may explain the underrepresentation of *P. aeruginosa* and overrepresentation of *S. aureus* in chronic wounds by conventional cultivation of wound swab samples, which detects the bacteria that are associated with the wound surface, but may not identify the bacteria that are located inside the wound bed [12]. With the use of fluorescent molecular probe technologies (such as PNA-FISH and ViaGram™), bacteria are commonly detected in chronic wounds as small, highly organized niches or microcolonies, also known as "biofilms" (Fig. 17.3) [11, 12, 16, 17]. These are densely aggregated colonies of bacteria often surrounded by an extracellular polymeric substance matrix; a finding that may explain both how bacteria survive within wound beds and the lack of efficacy of some antimicrobial therapies [11, 12, 16, 17]. Evidence suggests that biofilms play a major role in the chronicity of wounds and aberrant wound healing. A recent study by James et al. [16] demonstrated that 60% of chronic wounds contain biofilms compared with only 6% of their acute counterparts. Biofilm-associated bacteria are known to be difficult to culture, respond poorly to antimicrobials, and are resistant to host defenses [10]. In another study by Price et al. [19], large-scale genomic sequencing was used to further enhance our understanding of the pathobiology of chronic wounds. Employing 16S rRNA gene-based pyrosequencing analysis, an average of ten different bacterial families (~4× more than that estimated by culture), as well as anaerobic predominance, can be found in chronic wounds [19]. In addition, Price et al. [19] determined that chronic wound bacterial flora from antibiotic-treated patients was significantly different from that found in untreated patients, and characterized by *Pseudomonas*-dominated communities. Interestingly, antibiotic use may select for biofilm-producing microbes, such as *Pseudomonas*, and delay rather than expedite wound healing [19].

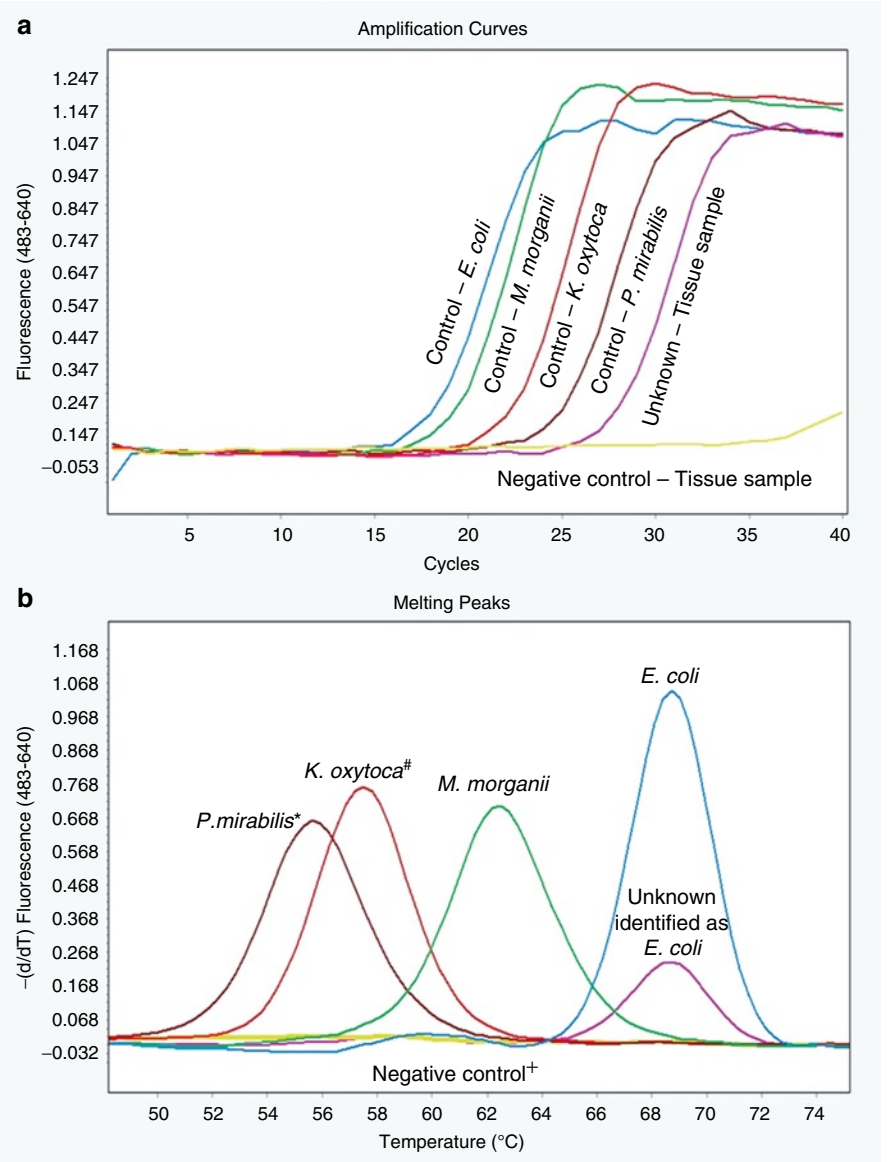

Fig. 17.2 *Real-time PCR-based detection of bacteria directly from chronic wound tissue samples*. (**a**) Representative amplification plots obtained from DNA extracted from bacterial cultures and tissue samples. (**b**) Melt curve profile used for speciation of species. No melt curve was observed for the negative control (*yellow line*). **Proteus mirabilis* and *Proteus vulgaris* have identical melt curves. #Melt curve profile of *Klebsiella oxytoca* is identical to that of *K. pneumoniae.* +Negative control = Tissue sample negative for bacterial DNA. *M. morganii*, *Morganella morganii*; *E. coli*, *Escherichia coli* (Courtesy of Dr. Johan Melendez, Infectious Diseases, Johns Hopkins Medical Institutions, Baltimore, USA)

Of note, chronic wounds in diabetic patients are reported to be associated with significant *Streptococcus* colonization, and antibiotic use is noted to reduce this finding [19].

According to some investigators, the different results obtained with culture-based and molecular-based strategies demonstrate that the use of one approach alone is not sufficient to identify all of the bacteria present in wounds [11]. It is important to consider that molecular technologies,

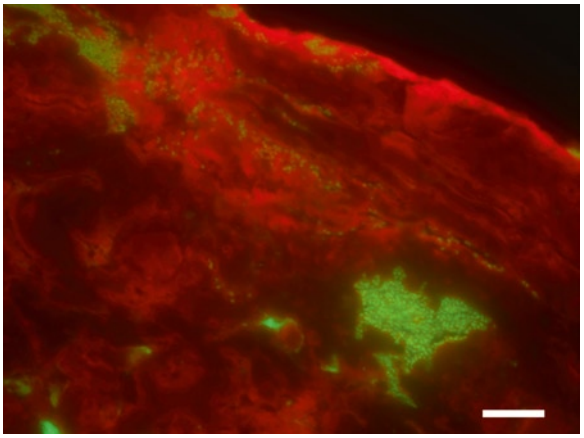

Fig. 17.3 *Biofilm in a venous leg ulcer* (VLU). Epifluorescence micrograph from a thin section (5 μm) of a VLU specimen stained with ViaGram™. The bacteria appear *green* and the host (human) tissue *red*. Note the biofilm in the lower right hand quadrant. Microcolonies are visible in the upper left hand corner and top of the image. Individual bacteria are also present. The scale bar is 10-μm long (Courtesy of Drs. Garth James and Kelly Kirker, Center for Biofilm Engineering, Montana State University, Bozeman, MT)

similar to culture-based methods, have their limitations and biases. These include unknown extracted DNA quantity/quality, the amplification of naked DNA (from dead/nonviable bacteria), differential amplification due to PCR primer bias, and heterogeneity and comigration of amplicon bands using gel-based analyses (i.e., DGGE fingerprinting) [11]. It is also important to consider tissue sampling techniques. Superficial swabs often isolate only noninvasive benign colonizers on the wound surface and demonstrate low yield for anaerobic microbes [10]. Deep tissue swab/biopsy or curettage for a combined approach of conventional cultivation (including quantitative cultures), microscopic review (with Gram staining), and molecular analysis may provide a broader picture of the bacterial species that reside in chronic wounds [10, 12]. Data could be correlated with clinical outcomes in order to establish testable hypotheses and evidence-based algorithms for the management of these conditions [10].

A number of recent papers in the literature highlight the search for predictive, diagnostic, and prognostic biomarkers in VLU [1–3, 9]. The ultimate goal is the translation of this information from bench-to-bedside with the development of wound care strategies which incorporate objective parameters that predict clinical course into wound assessment systems. This will allow for healing response monitoring and early identification of those VLU that are likely to fail standard care, in addition to potentially uncovering novel therapeutic targets. It is conceivable that these will include measurable biomarkers that can be quantified in the peripheral blood and/or wound microenvironment (i.e., tissue samples and wound exudates). For example, protease levels in wound exudates (i.e., uPA, MMP-9) have been proposed as potential biomarkers of wound healing status [2]. On light microscopy, disordered regulation of the normal healing process is reflected in distinct histopathological findings (i.e., chronic nonresolving inflammation, ↑ mast cells). In addition, phenotypic changes, such as aberrant expression and/or localization of wound healing factors (i.e., ↓ TGF-β, ↑ TNF-α, ↑ uPA), can be determined by immunohistochemistry [2, 9]. Eming et al. [20] recently reported a mass spectrometry- and immunohistochemistry-based comparative proteomic study of both exudates and tissue samples obtained from normal healing and nonhealing (VLU) chronic wounds. Of note, a number of proteins were found to be unique to the healing ($n = 23$) and nonhealing ($n = 26$) groups [20]. In addition, proteins in particular categories (i.e., ECM, proteinases, inhibitors, and immune modulators) showed differential distribution between the two wound

types. Whereas healing wounds were characterized by mediators that promote tissue growth and mitigate against inflammation-induced tissue damage, nonhealing wounds were associated with a chronic inflammatory response phenotype [20]. Novel proteins with yet unknown functions in skin repair were also identified [20].

A putative role for genetic factors in chronic ulcer predisposition, development, response to treatment, and ultimate outcome has been proposed. A number of studies, using DNA microarray technology for high-throughput analysis of gene expression, have uncovered novel transcripts that appear to be associated with poor/delayed healing [1]. A gene expression profiling study on lesional tissue by Charles et al. [1] reported that healing and nonhealing VLU are characterized by differential expression of transcripts encoding for inflammatory mediators (i.e., selectin-E), transcription factors (i.e., SFRP4), apoptosis regulators (i.e., clusterin), and structural epidermal proteins (i.e., KRT16). Other studies have proposed that a chronic wound is comprised of a central wound bed and a peripheral wound boundary, with the latter further subdividable into two biologically distinct areas: (1) a more central nonhealing edge and (2) a more peripheral healing edge [21]. In the nonhealing wound edge, there is deregulation of TGF-β signaling with decreased expression of TGF-β receptors (TGF-βRI, TGF-βRII, TGF-βRIII), suppression of TGF-β-inducible transcription factors (GADD45β, ATF3, ZFP36L1), and inactivation of the SMAD signaling cascade (absent pSMAD2, ↓ SMAD7), leading to tissue hyperproliferation [22]. Inhibition of keratinocyte differentiation/migration and suppression of re-epithelialization in chronic wounds, even in the presence of adequate granulation tissue, may be due to activation of the β-catenin/c-myc pathway with repression of KRT6/16 keratins, inhibition of epidermal growth factor (EGF) effects, and upregulation of the glucocorticoid pathway [23]. This is evidenced by the presence of nuclear β-catenin and increased c-myc protein expression in keratinocytes at the nonhealing wound edge of chronic ulcers [23]. In contrast, keratinocytes in the healing edge demonstrate normal capacity for proliferation and migration in response to wound healing signals [21]. Therefore, it has been proposed that the latter could be mobilized, following debridement of the stalled nonhealing edge, in an effort to improve treatment outcomes for chronic ulcers [21]. Accordingly, molecular diagnostic strategies to distinguish the nonhealing and healing edges of these lesions have been investigated [21]. Of note, the two edges of chronic wounds show different transcriptomic profiles, with variable expression of multiple genes, including KRT2A and SPRR3 [24]. Therefore, bioassays, such as (1) immunohistochemistry to identify β-catenin+ and c-myc+ ulcerogenic cells and/or (2) microarray technologies to develop transcriptional maps, could be used in the clinical setting to promote wound healing and enhance therapeutic potential. Theoretically, results of such tests would guide the extent of surgical debridement required to remove the nonhealing edge and expose its lagging healing counterpart to the wound bed/granulation tissue (concept of "molecular surgery," as proposed by Brem and Tomic-Canic) [21, 25].

Recently, Gemmati et al. [3] have proposed that the routine use of DNA microarray technology, in order to identify specific single nucleotide polymorphisms (SNPs) in peripheral blood samples, could have a role in the clinical management of patients with VLU and CVD (Table 17.2). Preliminary findings suggest that the simultaneous evaluation of a limited set of SNPs ($n = 5$) can provide a predictive and prognostic assessment for VLU vis-à-vis susceptibility (HFE, FPN1, MMP-12), healing time (FXIII), ulcer size (MMP-12, FXIII), and response to surgery (FXIII) [3]. Therefore, the potential exists for both VLU prevention and treatment programs to be influenced by the results of molecular diagnostic testing. According to Gemmati et al. [3], "*one example is given by HFE and FPN1 SNPs. A positive test for one or both gene variants would suggest indication and priority for surgical correction of superficial venous insufficiency,* so that *primary varicose veins could be treated more appropriately before any lesion appears in those patients with a critical gene haplotype*." Validation of these findings, as well as the possible identification of other disease-associated SNPs, requires additional study.

In the future, chronic wound/VLU management will likely incorporate molecular diagnostic tools that employ a panel of clinically validated biomarkers. This should facilitate stratification based on healing potential and provide a rationale for effective treatments in all patients.

Table 17.2 SNP markers for venous leg ulcer risk and prognosis (Adapted from Gemmati et al. [3])

SNPs	VLU risk (primary CVD)	VLU onset (primary CVD)	VLU size	Healing time (after surgery)
HFE C282Y	Y-allele ↑ risk (×6–7)	–	–	No effect after correction
HFE H65D	–	D-allele ↓ age of onset (~10 years)	–	–
FPN1-8CG	GG-genotype ↑ risk (~×5)	–	GG-genotype ↑ size (NS)	–
MMP-12-82AG	AA-genotype ↑ risk (~×2)	–	GG-genotype ↓ size (~×2)	–
FXIII V34L	–	–	L-allele ↓ size (~×3.5)	VV-genotype ↑ HT (~×2)
HFE/FPN1	Y/G-carriers ↑ risk (NS)	–	–	–
FXIII/MMP-12	–	–	L/G-carriers ↓ size (NS)	–

SNP single nucleotide polymorphism, *VLU* venous leg ulcer, *CVD* chronic venous disease, *NS* not significant, *HT* healing time

Keloids

Keloids represent a form of abnormal wound healing in predisposed individuals; typically arising between the ages of 10 and 30 years, and following local (even minor) trauma [6, 7, 26, 27]. These locally aggressive benign fibroproliferative scars may be functionally disabling and/or associated with disturbing symptoms (i.e., intense pruritus, pain) or cosmetic concerns, resulting in impaired quality of life and causing psychological distress. Their incidence is higher in darkly pigmented individuals (4–16%) compared with Caucasians (<1%) [6, 7, 26, 27]. In addition to surgical excision, other therapeutic modalities include intralesional steroid injections, laser and radiation treatment, and compression/occlusive dressings [26]. However, no satisfactory treatment for keloids has been found to date, and recurrence rates are high (55–100%) [26, 27]. A greater understanding of the pathogenesis of this disease is necessary in order to develop predictive/prognostic models and more effective therapeutic strategies [27].

A number of clinical and light microscopic criteria can be used to definitively diagnose keloids and distinguish them from other fibrosing cutaneous lesions [6, 7, 26, 27]. By definition, keloids are raised, extend beyond the boundaries of the original wound site, grow continuously and rarely regress. They demonstrate larger, thicker collagen fibers, an increased type I/III collagen ratio, and scant α-smooth muscle actin-reactive cells. In contrast, hypertrophic scars, while also raised, remain within the confines of the original wound area, and after a period of growth generally undergo spontaneous regression. In addition, fine collagen fibers and increased α-smooth muscle actin-expressing myofibroblasts are identified in the latter [6, 7, 26, 27].

Research continues to identify the genetic, cellular, immunological, and biochemical pathways, in addition to environmental factors, responsible for the development of keloids (Fig. 17.4). There appear to be: (1) variable inherited predisposition to their formation (autosomal dominant, autosomal recessive, and X-linked recessive); (2) possible linkage to loci at chromosomes 2q23 and 7p11; (3) an association with several human leukocyte antigen (HLA) alleles (HLA-DRB1*15, HLA-DQA1*0104, DQ-B1*0501, and DQB1*0503); (4) an association with gene polymorphisms and mutations (TP53, RUNX3, and TNFRII); and (5) aberrant expression of ~25 genes (reported to date), including TGF-βRIII, POSTN, ANXA1, and a number of collagen types [6, 7, 26, 27]. Gene expression studies in keloids have utilized high-throughput DNA microarray technology and evaluated

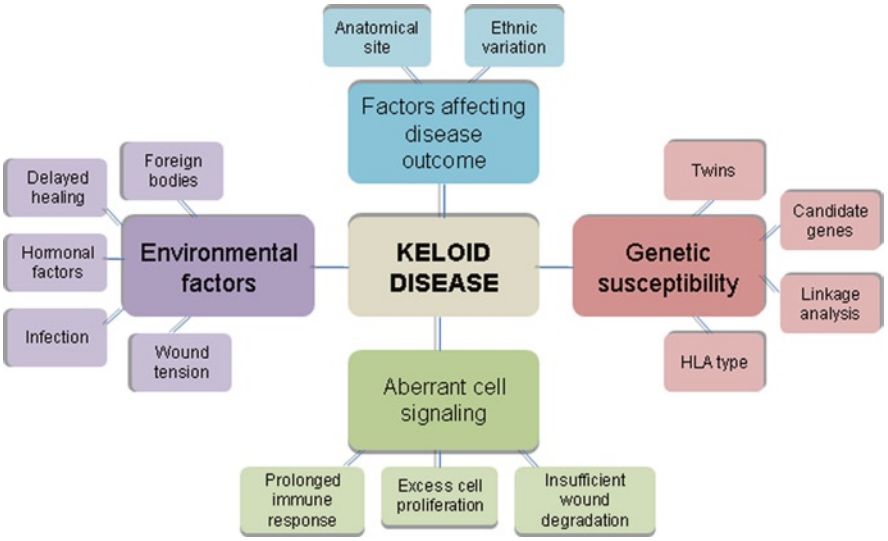

Fig. 17.4 Putative etiological factors in the pathogenesis of keloids (Adapted from Shih et al. [7])

transcript levels in tissue biopsies, keloid-derived fibroblast cultures, or both [6, 7, 26, 27]. However, few overlapping gene expression patterns have been identified. While a number of factors may contribute to these discordant findings, such as the site and age of keloid analyzed, prior therapy, and/or family history, it has also been shown that transcriptomic results generated from cultures do not consistently correlate to their biopsy equivalents [27]. Importantly, a number of molecules known to play a role in normal wound healing have been found to show increased or decreased expression in keloids [7]. In fact, the development and rationale for use of a number of novel therapies have been based on these genomic discoveries, including IFN-γ, imiquimod, recombinant IL-10, and anti-TGF-β1 [6]. Other associations (↓ SMAD7, ↑ IL-6) may be targets for future therapeutic strategies in keloids. A recent study demonstrated a set of genes that are significantly upregulated in biopsy samples of keloid margins compared with adjacent normal skin [27]. These identified genes, including ACAN, ASPN, C5ORF13, EGFR, HDGF, HIF1A, IGFBP7, INHBA, LGALS1, PTN, SERPINH1, and TNFAIP6, may serve as potentially important biomarkers for keloidal disease [27]. Interestingly, unique mRNA transcript expression profiles are also noted in different areas within a keloid (i.e., center *vs.* margin) [28]. Although no definitive keloid-causing gene has been identified, the pathogenesis of these lesions appears to involve a complex interaction between abnormal keloid-derived fibroblasts and ECM remodeling, with deregulation of apoptosis, mitogen-activated protein kinase (MAPK), protease, cytokine, and growth factor signaling pathways [6, 7]. Comparative mass spectrometry-based proteomic analysis of keloids and normal skin has also been accomplished [29]. With this approach, differentially expressed proteins specific to keloids have been identified, including ↑ stratifin, ↑ galectin-1, ↑ maspin, ↑ asporin, and ↑ PEDF. Again, these may also represent potential targets for therapeutic intervention [29]. To date, however, a predictive, diagnostic or prognostic role for genomic or proteomic testing in this disorder is yet to be established.

References

1. Charles CA, Tomic-Canic M, Vincek V, et al. A gene signature of nonhealing venous ulcers: potential diagnostic markers. J Am Acad Dermatol. 2008;59:758–71.
2. Moore K, Huddleston E, Stacey MC, et al. Venous leg ulcers – the search for a prognostic indicator. Int Wound J. 2007;4:163–72.

3. Gemmati D, Federici F, Catozzi L, et al. DNA-array of gene variants in venous leg ulcers: detection of prognostic indicators. J Vasc Surg. 2009;50:1444–51.
4. Sand M, Gambichler T, Sand D, et al. MicroRNAs and the skin: tiny players in the body's largest organ. J Dermatol Sci. 2009;53:169–75.
5. Lau K, Paus R, Tiede S, et al. Exploring the role of stem cells in cutaneous wound healing. Exp Dermatol. 2009;18:921–33.
6. Seifert O, Mrowietz U. Keloid scarring: bench and bedside. Arch Dermatol Res. 2009;301:259–72.
7. Shih B, Garside E, McGrouther DA, et al. Molecular dissection of abnormal wound healing processes resulting in keloid disease. Wound Repair Regen. 2010;18:139–53.
8. Branski LK, Gauglitz GG, Herndon DN, et al. A review of gene and stem cell therapy in cutaneous wound healing. Burns. 2009;35:171–80.
9. Charles CA, Romanelli P, Martinez ZB, et al. Tumor necrosis factor-alfa in nonhealing venous leg ulcers. J Am Acad Dermatol. 2009;60:951–5.
10. Martin JM, Zenilman JM, Lazarus GS. Molecular microbiology: new dimensions for cutaneous biology and wound healing. J Invest Dermatol. 2010;130:38–48.
11. Thomsen TR, Aasholm MS, Rudkjøbing VB, et al. The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. Wound Repair Regen. 2010;18:38–49.
12. Burmølle M, Thomsen TR, Fazli M, et al. Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections. FEMS Immunol Med Microbiol. 2010;59:324–36.
13. Davies CE, Hill KE, Wilson MJ, et al. Use of 16S ribosomal DNA PCR and denaturing gradient gel electrophoresis for analysis of the microfloras of healing and nonhealing chronic venous leg ulcers. J Clin Microbiol. 2004;42:3549–57.
14. Frankel YM, Melendez JH, Wang NY, et al. Defining wound microbial flora: molecular microbiology opening new horizons. Arch Dermatol. 2009;145:1193–5.
15. Melendez JH, Frankel YM, An AT, et al. Real-time PCR assays compared to culture-based approaches for identification of aerobic bacteria in chronic wounds. Clin Microbiol Infect. 2010;16:1762–9.
16. James GA, Swogger E, Wolcott R, et al. Biofilms in chronic wounds. Wound Repair Regen. 2008;16:37–44.
17. Kirketerp-Møller K, Jensen PØ, Fazli M, et al. Distribution, organization, and ecology of bacteria in chronic wounds. J Clin Microbiol. 2008;46:2717–22.
18. Dowd SE, Sun Y, Secor PR, et al. Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiol. 2008;8:43.
19. Price LB, Liu CM, Melendez JH, et al. Community analysis of chronic wound bacteria using 16S rRNA gene-basedt pyrosequencing: impact of diabetes and antibiotics on chronic wound microbiota. PLoS ONE. 2009;4:e6462.
20. Eming SA, Koch M, Krieger A, et al. Differential proteomic analysis distinguishes tissue repair biomarker signatures in wound exudates obtained from normal healing and chronic wounds. J Proteome Res. 2010;9:4758–66.
21. Tomic-Canic M, Ayello EA, Stojadinovic O, et al. Using gene transcription patterns (bar coding scans) to guide wound debridement and healing. Adv Skin Wound Care. 2008;21:487–92.
22. Pastar I, Stojadinovic O, Krzyzanowska A, et al. Attenuation of the transforming growth factor beta-signaling pathway in chronic venous ulcers. Mol Med. 2010;16:92–101.
23. Stojadinovic O, Brem H, Vouthounis C, et al. Molecular pathogenesis of chronic wounds: the role of beta-catenin and c-myc in the inhibition of epithelialization and wound healing. Am J Pathol. 2005;167:59–69.
24. Brem H, Stojadinovic O, Diegelmann RF, et al. Molecular markers in patients with chronic wounds to guide surgical debridement. Mol Med. 2007;13:30–9.
25. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest. 2007;117:1219–22.
26. Shih B, Bayat A. Genetics of keloid scarring. Arch Dermatol Res. 2010;302:319–39.
27. Shih B, McGrouther DA, Bayat A. Identification of novel keloid biomarkers through profiling of tissue biopsies versus cell cultures in keloid margin specimens compared to adjacent normal skin. Eplasty. 2010;10:e24.
28. Seifert O, Bayat A, Geffers R, et al. Identification of unique gene expression patterns within different lesional sites of keloids. Wound Repair Regen. 2008;16:254–65.
29. Ong CT, Khoo YT, Mukhopadhyay A, et al. Comparative proteomic analysis between normal skin and keloid scar. Br J Dermatol. 2010;162:1302–15.

Chapter 18
Alopecias

Diane M. Hoss and Michael J. Murphy

Primary alopecias of the scalp are divided into scarring and nonscarring types [1]. Scarring alopecias include: (a) lymphocytic (discoid lupus erythematosus [DLE], lichen planopilaris [LPP], pseudopelade of Brocq [PPB], central centrifugal cicatricial alopecia [CCCA]); (b) neutrophilic (dissecting folliculitis/cellulitis, folliculitis decalvans); and (c) combined (acne keloidalis) subtypes. Nonscarring alopecias include alopecia areata (AA), telogen effluvium, trichotillomania, traction alopecia, and androgenetic alopecia (AGA) [1]. The etiology of many of these disorders is unclear; although, a combination of genetic and environmental factors appears to contribute to the pathogenesis of several types of alopecia [2]. In addition, hair loss may also occur secondary to inflammatory dermatoses, infections, neoplastic disorders (i.e., follicular mycosis fungoides [MF]), drug eruptions, trauma, and genodermatoses (as discussed in Chap. 19) [3]. The diagnosis and classification of alopecias is currently based on the correlation of clinical findings with histopathological changes on scalp biopsy. A number of these disorders can show significantly overlapping morphological features, and a definitive distinction may be difficult in some instances (i.e., LPP *vs.* PPB). Additional tests, such as direct immunofluorescence studies for immmunoreactant deposition (i.e., IgG, C3), may be helpful for particular conditions (i.e., DLE) [1]. As in other skin diseases, a number of molecular technologies have been employed to elucidate the pathophysiology of these disorders, and have provided some understanding of deregulated pathways underlying disease development [4, 5]. Interestingly, hair-specific cDNA microarrays, designed for gene expression profiling studies, have even been developed [6]. From a clinical perspective, it is conceivable that molecular testing could supplement current subjective morphology-based classification systems of alopecia and play a role in daily practice. Data from gene expression profiling studies may provide a basis for a more objective and accurate diagnosis of these disorders, identify biomarkers for disease activity and clinical end-points, and uncover potential drug targets. Other molecular tests, such as PCR-based T-cell receptor gene rearrangement (TCR-GR) analysis, may be useful in distinguishing primary lymphocyte-mediated alopecia (i.e., polyclonal result) from secondary alopecia due to follicular MF (i.e., monoclonal result) [7]. However, with regard to TCR-GR studies, it is important to consider that autoimmune dermatoses such as DLE, can also demonstrate monoclonal/restricted oligoclonal T-cell expansions.

D.M. Hoss (✉)
Caris Life Sciences, Dermatopathology Division, 406 Farmington Ave.,
Farmington, CT 06032, USA
e-mail: dianehoss@att.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_18,

Alopecia Areata

Alopecia areata (AA) is one of the most prevalent autoimmune diseases, affecting ~5.3 million individuals in the USA, and associated with a lifetime risk of 1.7% [8]. In a recent genome-wide association study (GWAS) of >1,000 AA cases, Petukhova et al. [8] identified 139 single nucleotide polymorphisms (SNPs) that are significantly associated with this disease (Table 18.1). Of note, several risk loci common to other types of autoimmunity, including systemic lupus erythematosus, psoriasis, rheumatoid arthritis, and generalized vitiligo, were found; supporting the proposed common-cause hypothesis of human autoimmune disorders. The authors reported that both innate and adaptive immunity, in addition to upregulation of ULBP3 (a NK-cell activating ligand) within the hair follicle dermal sheath/papilla, are involved in the pathogenesis of AA [8]. Results of a study by Dudda Subramanya et al. [4], using gene expression profiling technology, have also implicated immune response, as well as cell cycle control and apoptosis-related genes, in the development of this disease. In another cDNA microarray-based study, Carroll et al. [5] demonstrated 95 genes that were differentially regulated (31 upregulated; 64 downregulated) in biopsies of chronic nonresponsive AA compared with control groups. Identified upregulated genes (i.e., IL-2R1, STAT-1, granzyme A, IFI27) again highlighted the putative role of immune cell-mediated mechanisms in the pathogenesis of this disease [5]. More recently, Lueking et al. [9] utilized a protein biochip which was purported to facilitate (a) the detection of potential autoantigens in AA and (b) the discrimination of AA from other inflammatory skin disorders. By profiling the autoantibody repertoire of sera from AA patients against a human high-density array containing 37,200 redundant recombinant human proteins, a set of 8 putative autoantigens was identified, and subsequently verified by Western blot analysis. These proteins included GLCDAC05, NOL8, α-endosulfine, signal recognition particle subunit 14, FGFR3, endemic pemphigus foliaceus autoantigen, dematin, and SCG10 [9].

Table 18.1 Genes significantly associated with alopecia areata (Adapted from Petukhova et al. [8])

Region	Gene	Function	Association with other autoimmune disorders with skin manifestations
2q33.2	CTLA4	Costimulatory family	SLE, RA
	ICOS	Costimulatory family	
4q27	IL-21/IL-2	T-, B-, and NK-cell proliferation	PS, RA
6q25.1	ULBP6	NKG2D-activating ligand	None
	ULBP3	NKG2D-activating ligand	None
9q31.1	STX17	Premature hair graying	None
10p15.1	IL-2RA	T-cell proliferation	GV
11q13	PRDX5	Antioxidant enzyme	None
12q13	Eos (IKZF4)	Treg transcription factor	SLE
	ERBB3	Epidermal growth factor receptor	
6p21.32	MICA	NKG2D-activating ligand	PS, SLE, RA
(HLA)	NOTCH4	Hematopoietic differentiation	RA
	C6orf10	Unknown	PS, GV, RA
	BTNL2	Costimulatory family	SLE, GV, RA
	HLA-DRA	Antigen presentation	GV, RA
	HLA-DQA1	Antigen presentation	SLE, PS, RA
	HLA-DQA2	Antigen presentation	RA
	HLA-DQB2	Antigen presentation	RA

SLE systemic lupus erythematosus, *RA* rheumatoid arthritis, *PS* psoriasis, *GV* generalized vitiligo

Except for FGFR3, which is already known to be strongly related to hair disorders and expressed in the suprabasal/inner layers of hair follicles and precuticle cells of the hair bulb, the possible pathophysiological roles of the other detected proteins in AA remain to be defined. According to the study, "these (eight) autoantigens were arrayed on protein microarrays to generate a disease-associated protein chip that may be suitable for fast diagnosis [9]." Using this disease-associated protein chip, the authors reported accurate identification of AA in 90% of cases, when compared with sera from patients with psoriasis or hand-and-foot eczematous dermatitis [9]. Finally, Coda et al. [10] suggested that gene expression profiling of the peripheral blood of AA patients could determine transcriptional signatures related to: (a) genetic susceptibility to the disease (3,750 differentially regulated genes, including ↑LRCH4, ↑BCL3, ↓ADAM15, ↓HOXB8); (b) phenotypic expression of the disease (882 differentially expressed genes, including ↑PADI4, ↑FGD4, ↓EPYC, ↓SREBF1); and (c) disease severity (464 differentially expressed genes, including ↑GPR3, ↑CARD11, ↓ANXA3, ↓CX3CR1). It is likely that future studies will lead to the identification of clinically applicable biomarkers of AA that are relevant to disease prediction, classification, prognosis, and treatment response.

Lichen Planopilaris and Pseudopelade of Brocq

Yu et al. [11] have utilized DNA microarray technology to investigate LPP and PPB; two scarring alopecias that often exhibit similar clinical and microscopic features, and considered by some physicians to represent variants of the same disorder [1, 11]. In addition to proposing mechanisms for disease pathogenesis, gene expression profiles in this study suggested that LPP and PPB are in fact distinct conditions. This was based on the finding of 504 differentially expressed genes, including 479 upregulated and 25 downregulated transcripts, in LPP as compared to PPB [11]. Of note, a number of specific genes (MMP-11, TNFSF13B, and APOL2) showed significantly different levels of both mRNA and protein expression, by DNA microarray, quantitative polymerase chain reaction (PCR), and immunohistochemical analysis, for both conditions (Fig. 18.1).

Androgenetic Alopecia

Patterned hair loss in men, also known as androgenetic alopecia (AGA) and male balding, is a common condition, affecting up to 50% of men by age 50 [12–14], and up to 90% of men by age 90 [15, 16]. This type of hair loss has been shown to be both androgen-dependent and inherited [17]; thus the term AGA is an apt one. Patterned hair loss in women, initially known as female AGA, but now termed female pattern hair loss (FPHL), is somewhat less common, affecting up to 25% of women under age 50 [18], and up to 40% of women at age 70 [19].

The mode of inheritance of AGA remains controversial. Initially, the condition was felt to be autosomal dominant in men, and autosomal recessive in women, based on a study of 22 families, published by Osborn in 1916 [20]. However, recent work favors a polygenic inheritance, based on several observations, including: (a) the high prevalence of AGA (i.e., single gene traits rarely occur with a frequency greater than 1 in 1,000); (b) the association between an increased risk of becoming bald and increasing numbers of affected relatives; (c) the gradual distribution of balding patterns in the general population, ranging from minimal to severe balding, falls along a gaussian curve, as seen in other polygenic traits; and (d) the results of the largest twin study to date [16, 21–23]. In addition, it has been proposed that microRNAs, a recently discovered class of noncoding RNAs, may have a role in the pathogenesis of AGA [24].

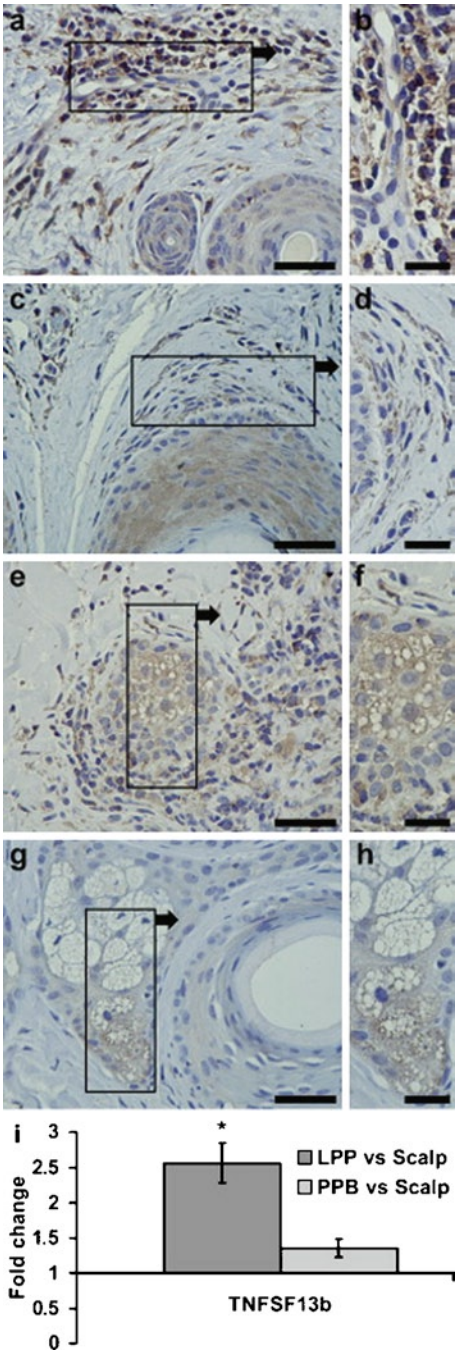

Fig. 18.1 TNFSF13B expression in lichen planopilaris (*LPP*), pseudopelade of Brocq (*PPB*), and normal scalp skin. Inflammatory infiltrate cells in LPP expressed TNFSF13B (**a**, **b**). Little or no positive expression was found in PPB infiltrating leukocytes (**c**–**f**). In LPP, limited expression of TNFSF13B was present in follicular keratinocytes (**a**), while PPB keratinocytes more readily expressed TNFSF13B (**c**). Normal hair follicle keratinocytes were consistently negative for TNFSF13B (**g**). TNFSF13B was found in the normal sebaceous gland (**g** and **h**) and was also detected in the inflammatory infiltrated sebaceous gland (**e** and **f**). Bar = 50 μm (**a**, **c**, **e**, and **g**); 25 μm (**b**, **d**, **f**, and **h**). Quantitative PCR confirmed increased expression in *TNFSF13B* (**i**). (From Yu et al. [11]. Reprinted with permission from Elsevier, Copyright © 2010)

The association between androgens and male balding was first noted more than 2,000 years ago by Hippocrates, who observed that children and eunuchs never developed patterned baldness [25]. In 1942, Hamilton [17] observed 54 men who never became bald, and were noted either to have been castrated before puberty ($n = 10$) or in early adolescence ($n = 34$), or to have severe testicular

insufficiency ($n = 10$). However, administration of exogenous testosterone to four of these men, all of whom had a strong family history of AGA, caused male pattern baldness [17]. The relationship of androgens to the development of FPHL is more complex than that in male AGA. While women with hyperandrogenism certainly have a very high incidence of FPHL (up to 86%), the vast majority of women with FPHL have normal low levels of serum androgens [26]. In fact, FPHL has even been reported in a woman with no circulating androgens [27].

Androgens are sex steroids that are required in utero for normal development of the male fetus, and which result at puberty in the development of primary (libido and potency) and secondary (pubic, axillary, and facial hair; male muscle mass) male sexual characteristics. Androgens can affect hair follicles in both a stimulatory capacity (i.e., beard growth after puberty) and an inhibitory one (i.e., AGA) [28]. There is no significant difference in circulating testosterone levels in men with and without AGA [29, 30]. Testosterone, the main circulating androgen in men, is metabolized to dihydrotestosterone (DHT) in tissues, by the enzyme 5α-reductase, which has two isozymes. The type 1 isozyme is found in the outer root sheath of scalp hair follicles, dermal papillae, sebaceous glands, liver, adrenal gland, and kidney; while the type 2 isozyme is localized in the inner and outer root sheaths of scalp hair follicles, dermal papillae, beard, liver, prostate, testes, and seminal vesicles. However, localization studies often employ different methodologies and show some conflicting results [31–34]. Both men and women with AGA have higher levels of 5α-reductase types 1 and 2 in frontal hair follicles (balding area) than in occipital hair follicles (nonbalding area) [34]. The role of DHT in male balding is demonstrated by male pseudohermaphrodites, who lack 5α-reductase and are born with ambiguous genitalia and undescended testes. At puberty, their testes descend and produce testosterone, with resultant development of normal primary male sexual characteristics and increased muscle mass. However, beard growth remains scanty and AGA does not occur [35]. Finasteride, an inhibitor of 5α-reductase type 2, has been found to be effective in some men with AGA, when given at a dose of 1 mg/day [36]. The same dose of finasteride showed a lack of efficacy in postmenopausal women with FPHL [37]. However, four women with hyperandrogenism and FPHL did respond to finasteride therapy at 1.25 mg/day [38]. An uncontrolled study has also shown higher doses of finasteride (2.5 mg/day) to be beneficial for premenopausal women with FPHL [39].

Both testosterone and its metabolites, like all steroid hormones, exert their effects by binding to a nuclear receptor, thereby forming a hormone/receptor complex that modulates transcription and processing of proteins through its interaction with DNA [32]. Androgen receptors (AR) in the skin are localized in sebaceous glands (sebocytes), keratinocytes of the pilosebaceous ducts, and in the dermal papillae of hair follicles [40]; the latter are composed of mesenchymal cells that regulate the cyclic regeneration of hair follicles during the hair cycle [41]. In a study by Choudhry et al. [40], using skin samples from the scalp, face, limb, and genitalia of men and women that were discarded after surgery, AR were noted to be absent in the hair germinative matrix, outer and inner root sheaths, and hair shafts. In contrast, Sawaya and Price [34] noted the presence of AR in the outer root sheaths of hair follicles from both men and women with AGA. Of note, higher AR levels have been demonstrated in balding scalp compared with nonbalding scalp [34, 42, 43].

Given the genetic predisposition of male and female pattern hair loss, and the role of androgens in its pathogenesis, several genes involved in androgen function have been studied. Genetic association studies of the genes encoding for both isotypes of 5α-reductase (SRD5A1 on chromosome 5 and SRD5A2 on chromosome 2), using dimorphic intragenic restriction fragment length polymorphisms (RFLP), found no significant differences in allele, genotype, or haplotype frequencies between young bald men and older nonbald male controls [22]. These results suggest that the genes encoding for the two 5α-reductase isozymes are not associated with male pattern baldness [22]. Attention has turned to the AR gene that is located on the X chromosome (Xq11–12). The amino-terminal domain (exon 1), required for transcriptional activation, contains two triplet-repeat polymorphisms that are functional variants; a polyglutamine repeat (CAG) and a polyglycine repeat (GGC or GGN) [44]. In humans, the AR gene is polymorphic, and can include a synonymous SNP

in exon 1, known as S*tu1* (rs6152, RFLP, E211 G>A), in addition to variations in the number of CAG repeats lying proximal and GGC repeats lying distal to S*tu1*. Several groups have shown that polymorphisms of the AR gene are associated with the development of male AGA [44–48]. The strongest association is seen with the *Stu1* (rs6152) restriction site [42, 45, 46]. Ellis et al. [44] found the *Stu1* marker to be present in 98% of young bald men (aged 18–30), in 92% of older bald men (50 years and older), and in 77% of nonbald controls. Since such a large proportion of nonbald men carry this marker, they hypothesize that these men must lack other necessary causes of AGA, thus supporting a polygenic pathogenesis for this condition [44]. Based on the results of this study, the chance of developing baldness in the absence of this marker is extremely low [44]. Therefore, a negative *Stu1* test might be useful for identifying men who are highly unlikely to develop AGA. The findings of another recent study suggest that AR *Stu1* polymorphism does not serve as a biomarker for FPHL predisposition, as it does for male AGA [49].

Studies have found shorter CAG triplet-repeats to be associated with the development of male AGA [44, 45], and both pre- and postmenopausal FPHL [45, 50]. There are conflicting studies regarding the role of polyglycine repeats in AGA. One study suggests an association between shorter GGN repeats and male AGA [46]. However, Ellis et al. [48] found that the AR GGN repeat polymorphism does not independently confer AGA susceptibility, and they postulate that the association is due to linkage disequilibrium between this and more relevant (yet to be defined) AR gene variants. In addition, Wakisaka et al. [51] have noted that the lower the sum of the CAG plus GGC repeats, the greater the severity of baldness.

Several other genes are found to be associated with AGA, in keeping with the probable polygenic transmission of this condition. The EDA2R gene, located close to the AR gene on the X chromosome, is expressed in cells of the hair bulb and hair matrix. A nonsynonymous SNP rs1385699 on the EDA2R gene has been shown to be strongly associated with the development of AGA [52]. Recent genome-wide association studies found a significant correlation between five SNPs on chromosome 20p11 and the development of early onset male pattern baldness in a group of German and Austrian men [53]. An AGA susceptibility locus has also been mapped to chromosome 3q26 in a population of German men [54].

A postulated genetic basis for the variable response to finasteride therapy for male AGA has also been investigated. The gene expression of several cytokines that are believed to regulate hair growth was analyzed in follicular dermal papillae, before and after finasteride therapy, in a small group of nine men with AGA [55]. Increased expression of insulin-like growth factor 1 mRNA was associated with a positive response to finasteride therapy [55]. In addition, a possible association between AR CAG/GGC triplet-repeats and response to finasteride has been proposed. In a study of 488 Japanese men, a sum of ≤40 CAG plus GGC triplet-repeats in the AR gene was associated with improved response to finasteride, despite this group presenting with more severe AGA [51]. These results were verified in a second study of 178 men: 70% of men with a marked response to finasteride had CAG repeat lengths <22, while 70% of those with only minimal drug response had CAG repeat lengths >22 [56, 57]. A recent unpublished study, measuring the sum of CAG and GGC repeats in 842 balding men, confirms that lower combined repeat scores (≤40) are associated with a higher chance of developing male AGA, but paradoxically predict a better response to finasteride therapy (Fig. 18.2) (N. Wakisaka, personal communication, 2010). The difference in response rates between the short (≤40) and long (≥41) repeat groups was greatest in older patients (Fig. 18.2).

The results of these molecular studies are now being incorporated into the clinical evaluation and treatment of male AGA and FPHL [58]. One objective would be the development of a screening test that would allow for earlier identification of AGA, as long-term treatment with finasteride has been shown to decrease the likelihood of developing further visible hair loss [59]. In this regard, a genetic screening test for male AGA that analyzes several AGA-related markers, including the S*tu1* SNP, is now being marketed by a California-based company (A. Goren, HairDx LLC, personal communication, 2010 and [57]). This company estimates that the test, performed on a cheek swab, has a positive

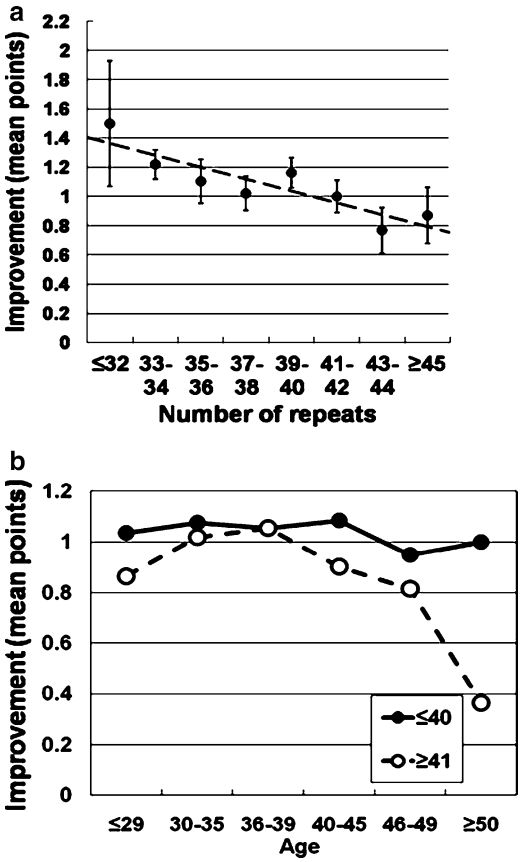

Fig. 18.2 (**a**) Relationship between number of triplet-repeats and effect of finasteride therapy in male androgenetic alopecia (*AGA*). Patients ($n=842$) with different numbers of CAG+GGC repeats in the androgen receptor (*AR*) gene were treated with finasteride for 6 months (total dose of 50 mg or more). Severity and pattern of AGA before and after therapy was judged according to the modified Hamilton–Norwood scale. For statistical analysis, AGA patterns I–VII were converted to numerical values, 1–9. Effectiveness of finasteride therapy was expressed as the degree of improvement (i.e., difference between the pre-treatment and post-treatment severity value). The number of triplet-repeats was plotted against the improvement (expressed in mean points). *Dashed line* = regression line, *vertical bar* = standard error of the mean. Fitness was demonstrated by regression analysis (at $\alpha=1\%$, $p=0.007$). Decline ($\mu 1 \geq \mu i$) was proved by Kruskal–Wallis test ($\alpha=5\%$). (**b**) Finasteride response in male AGA, in relationship to short or long triplet-repeats and patient age. Patients were divided into two groups: short repeat group (number of repeats ≤40, *closed circles*) and long repeat group (number of repeats ≥41, *open circles*), and treated with finasteride for 6 months. The drug was more effective for patients in the short repeat group than for those in the long repeat group. This difference was greatest in older patients. Significant difference between these two groups was demonstrated by ANOVA ($\alpha=1\%$, $p=0.0023$) (Courtesy of Dr. Nagaoki Wakisaka, NPO, Future Medical Laboratory, Tokyo, Japan)

predictive value of 80% and a negative predictive value of 90% (A. Goren, HairDx LLC, personal communication, 2010 and [57]). A second genetic test, which measures the number of CAG repeats in the AR gene, is used to predict a woman's risk of developing FPHL [57]. The company cites the 1998 study by Sawaya and Shalita ($n=60$), which reported that less than 2% of women with >23 CAG repeats developed AGA, while the vast majority of women (97.7%) with <16 CAG repeats had AGA [45, 57]. In women with intermediate numbers of CAG repeats (between 16 and 23), lower repeat scores tended to be associated with FPHL; however, there was overlap in repeat scores between women with and without FPHL in this intermediate range [45]. Therefore, this commercial test could be used to reassure a woman whose CAG repeats are >23 that she is very unlikely to develop FPHL. Likewise, a woman with <16 CAG repeats might strongly consider initiating therapy for FPHL.

Another objective would be to determine subgroups of patients predicted to show either high or low response rates to available therapy. A third test, marketed by the same company as the "RxR genetic test for finasteride response" (in male AGA), also measures the number of CAG repeat lengths in the AR gene. It is advertised that the resulting score would allow the "patient and his physician to compare the results to the current scientific literature", helping them in their decision as to whether finasteride therapy would be beneficial [57].

In conclusion, molecular diagnostic tests may have a role in the prediction and diagnosis of hair loss disorders, in identifying subsets of patients expected to show either good or poor response(s) to therapy, and in uncovering candidate targets for novel therapeutic approaches.

Acknowledgment We would like to thank Andy Goren, HairDx, LLC, for his helpful discussions.

References

1. Stefanato CM. Histopathology of alopecia: a clinicopathological approach to diagnosis. Histopathology. 2010;56:24–38.
2. Alkhalifah A, Alsantali A, Wang E, et al. Alopecia areata update. Part I. Clinical picture, histopathology, and pathogenesis. J Am Acad Dermatol. 2010;62:177–88.
3. Finner AM, Otberg N, Shapiro J. Secondary cicatricial and other permanent alopecias. Dermatol Ther. 2008;21:279–94.
4. Dudda Subramanya RLK, Xiang Z, King H, et al. Microarray based gene expression profiling in alopecia areata implicates immune response, cell cycle control, and apoptosis related genes in disease pathogenesis [abstract]. J Invest Dermatol. 2003;121:219.
5. Carroll JM, McElwee KJ, King LE, et al. Gene array profiling and immunomodulation studies define a cell-mediated immune response underlying the pathogenesis of alopecia areata in a mouse model and humans. J Invest Dermatol. 2002;119:392–402.
6. Moonkyu K, Young-Hee K, Sang-Ku H, et al. Gene expression profile in dermal papilla cells and construction of hair specific cDNA microarrays. Proceedings of the Third International Meeting of Hair Research Societies, Chinzan-so, Tokyo; 2001. p. 29.
7. Boone SL, Guitart J, Gerami P. Follicular mycosis fungoides: a histopathologic, immunohistochemical, and genotypic review. G Ital Dermatol Venereol. 2008;143:409–14.
8. Petukhova L, Duvic M, Hordinsky M, et al. Genome-wide association study in alopecia areata implicates both innate and adaptive immunity. Nature. 2010;466:113–7.
9. Lueking A, Huber O, Wirths C, et al. Profiling of alopecia areata autoantigens based on protein microarray technology. Mol Cell Proteomics. 2005;4:1382–90.
10. Coda AB, Hysa VQ, Seiffert-Sinha K, et al. Peripheral blood gene expression in alopecia areata reveals molecular pathways distinguishing heritability, disease and severity. Genes Immun. 2010;11:531–41.
11. Yu M, Bell RH, Ross EK, et al. Lichen planopilaris and pseudopelade of Brocq involve distinct disease associated gene expression patterns by microarray. J Dermatol Sci. 2010;57:27–36.
12. Hamilton JB. Patterned loss of hair in man; types and incidence. Ann NY Acad Sci. 1951;53:708–28.
13. Rhodes T, Girman CJ, Savin RC, et al. Prevalence of male pattern hair loss in 18–49 year old men. Dermatol Surg. 1998;24:1330–2.
14. Severi G, Sinclair R, Hopper JL, et al. Androgenetic alopecia in men aged 40–69 years: prevalence and risk factors. Br J Dermatol. 2003;149:1207–13.
15. Norwood OT. Male pattern baldness: classification and incidence. South Med J. 1975;68:1359–65.
16. Nyholt DR, Gillespie NA, Heath AC, et al. Genetic basis of male pattern baldness. J Invest Dermatol. 2003;121:1561–4.
17. Hamilton JB. Male hormone stimulation is prerequisite and an incitant in common baldness. Am J Anat. 1942;71:451–80.
18. Olsen EA. Female pattern hair loss. J Am Acad Dermatol. 2001;45:S70–80.
19. Birch MP, Lalla SC, Messenger AG. Female pattern hair loss. Clin Exp Dermatol. 2002;27:383–8.
20. Osborn D. Inheritance of baldness. J Hered. 1916;7:345–55.
21. Küster W, Happle R. The inheritance of common baldness: two B or not two B? J Am Acad Dermatol. 1984;11(5 Pt 1):921–6.

22. Ellis JA, Stebbing M, Harrap SB. Genetic analysis of male pattern baldness and the 5alpha-reductase genes. J Invest Dermatol. 1998;110:849–53.
23. Birch MP, Messenger AG. Genetic factors predispose to balding and non-balding in men. Eur J Dermatol. 2001;11:309–14.
24. Goodarzi HR, Abbasi A, Saffari M, et al. MicroRNAs take part in pathophysiology and pathogenesis of male pattern baldness. Mol Biol Rep. 2010;37:2959–65.
25. Kligman AM, Freeman B. History of baldness. From magic to medicine. Clin Dermatol. 1988;6:83–8.
26. Futterweit W, Dunaif A, Yeh HC, et al. The prevalence of hyperandrogenism in 109 consecutive female patients with diffuse alopecia. J Am Acad Dermatol. 1988;19(5 Pt 1):831–6.
27. Orme S, Cullen DR, Messenger AG. Diffuse female hair loss: are androgens necessary? Br J Dermatol. 1999;141:521–3.
28. Randall VA, Hibberts NA, Thornton MJ, et al. The hair follicle: a paradoxical androgen target organ. Horm Res. 2000;54:243–50.
29. Burton JL, Halim MM, Meyrick G, et al. Male-pattern alopecia and masculinity. Br J Dermatol. 1979;100:567–71.
30. Phillipou G, Kirk J. Significance of steroid measurements in male pattern alopecia. Clin Exp Dermatol. 1981;6:53–6.
31. Eicheler W, Dreher M, Hoffmann R, et al. Immunohistochemical evidence for differential distribution of 5 alpha-reductase isoenzymes in human skin. Br J Dermatol. 1995;133:371–6.
32. Kaufman KD. Androgen metabolism as it affects hair growth in androgenetic alopecia. Dermatol Clin. 1996;14:697–711.
33. Bayne EK, Flanagan J, Einstein M, et al. Immunohistochemical localization of types 1 and 2 5alpha-reductase in human scalp. Br J Dermatol. 1999;141:481–91.
34. Sawaya ME, Price VH. Different levels of 5alpha-reductase type I and II, aromatase, and androgen receptor in hair follicles of women and men with androgenetic alopecia. J Invest Dermatol. 1997;109:296–300.
35. Imperato-McGinley J, Guerrero L, Gautier T, et al. Steroid 5α-reductase deficiency in man: an inherited form of male pseudohermaphroditism. Science. 1974;186:1213–5.
36. Kaufman KD, Olsen EA, Whiting D, et al. Finasteride in the treatment of men with androgenetic alopecia. Finasteride Male Pattern Hair Loss Study Group. J Am Acad Dermatol. 1998;39(4 Pt 1):578–89.
37. Price VH, Roberts JL, Hordinsky M, et al. Lack of efficacy of finasteride in postmenopausal women with androgenetic alopecia. J Am Acad Dermatol. 2000;43(5 Pt 1):768–76.
38. Shum KW, Cullen DR, Messenger AG. Hair loss in women with hyperandrogenism: four cases responding to finasteride. J Am Acad Dermatol. 2002;47:733–9.
39. Iorizzo M, Vincenzi C, Voudouris S, et al. Finasteride treatment of female pattern hair loss. Arch Dermatol. 2006;142:298–302.
40. Choudhry R, Hodgins MB, Van der Kwast TH, et al. Localization of androgen receptors in human skin by immunohistochemistry: implications for the hormonal regulation of hair growth, sebaceous glands and sweat glands. J Endocrinol. 1992;133:467–75.
41. Randall VA. Androgens and hair growth. Dermatol Ther. 2008;21:314–28.
42. Randall VA, Thornton MJ, Messenger AG. Cultured dermal papilla cells from androgen-dependent human hair follicles (e.g. beard) contain more androgen receptors than those from non-balding areas of scalp. J Endocrinol. 1992;133:141–7.
43. Hibberts NA, Howell AE, Randall VA. Balding hair follicle dermal papilla cells contain higher levels of androgen receptors than those from non-balding scalp. J Endocrinol. 1998;156:59–65.
44. Ellis JA, Stebbing M, Harrap SB. Polymorphism of the androgen receptor gene is associated with male pattern baldness. J Invest Dermatol. 2001;116:452–5.
45. Sawaya ME, Shalita AR. Androgen receptor polymorphisms (CAG repeat lengths) in androgenetic alopecia, hirsutism, and acne. J Cutan Med Surg. 1998;3:9–15.
46. Hillmer AM, Hanneken S, Ritzmann S, et al. Genetic variation in the human androgen receptor gene is the major determinant of common early-onset androgenetic alopecia. Am J Hum Genet. 2005;77:140–8.
47. Levy-Nissenbaum E, Bar-Natan M, Frydman M, et al. Confirmation of the association between male pattern baldness and the androgen receptor gene. Eur J Dermatol. 2005;15:339–40.
48. Ellis JA, Scurrah KJ, Cobb JE, et al. Baldness and the androgen receptor: the AR polyglycine repeat polymorphism does not confer susceptibility to androgenetic alopecia. Hum Genet. 2007;121:451–7.
49. el-Samahy MH, Shaheen MA, Saddik DE, et al. Evaluation of androgen receptor gene as a candidate gene in female androgenetic alopecia. Int J Dermatol. 2009;48:584–7.
50. Ali I, Dawber RPR, Wojnarowska FT. The role of the androgen receptor gene CAG repeat polymorphism and X-chromosome inactivation pattern in postmenopausal female pattern hair loss [abstract]. Br J Dermatol. 2008;159 Suppl 1:8–9.

51. Wakisaka N, Taira Y, Ishikawa M, et al. Effectiveness of finasteride on patients with male pattern baldness who have different androgen receptor gene polymorphism. J Investig Dermatol Symp Proc. 2005;10:293–4.
52. Prodi DA, Pirastu N, Maninchedda G, et al. EDA2R is associated with androgenetic alopecia. J Invest Dermatol. 2008;128:2268–70.
53. Hillmer AM, Brockschmidt FF, Hanneken S, et al. Susceptibility variants for male-pattern baldness on chromosome 20p11. Nat Genet. 2008;40:1279–81.
54. Hillmer AM, Flaquer A, Hanneken S, et al. Genome-wide scan and fine-mapping linkage study of androgenetic alopecia reveals a locus on chromosome 3q26. Am J Hum Genet. 2008;82:737–43.
55. Tang L, Bernardo O, Bolduc C, et al. The expression of insulin-like growth factor 1 in follicular dermal papillae correlates with therapeutic efficacy of finasteride in androgenetic alopecia. J Am Acad Dermatol. 2003;49:229–33.
56. Sato A, Arima Y, Kojima Y, et al. Correlation between polymorphic CAG-repeats in the androgen receptor gene and therapeutic efficacy of finasteride in androgenetic alopecia. Skin Surg. 2008;17:80–6.
57. HairDx. Available at http://www.hairdx.com. Accessed Jan 10, 2010.
58. Goh C, Zippin JH. Androgenetic alopecia: diagnosis and treatment with a focus on recent genetic implications. J Drugs Dermatol. 2009;8:185–92.
59. Kaufman KD, Rotonda J, Shah AK, et al. Long-term treatment with finasteride 1 mg decreases the likelihood of developing further visible hair loss in men with androgenetic alopecia (male pattern hair loss). Eur J Dermatol. 2008;18:400–6.

Chapter 19
Genodermatoses: Inherited Diseases of the Skin

Frances J.D. Smith and W.H. Irwin McLean

This chapter discusses the molecular basis of inherited diseases in which the primary changes are manifest in the skin and its appendages (nails, hair, sweat glands, and sebaceous glands). The disorders have been subdivided into sections according to their clinical presentation and molecular basis, but this is not a fixed classification, as many diseases will fit into more than one category. With increasing knowledge of the identities and functions of the genes involved in these inherited skin conditions, many of these disorders are being reclassified, as has occurred recently for epidermolysis bullosa (EB) and the ectodermal dysplasias. Molecular-based diagnostic criteria should help streamline the archaic and sometimes confusing classification systems currently in use. Knowledge of the precise molecular defect(s) underlying hereditary skin diseases will be a necessary step in the development and application of "personalized medicine" approaches to treatment, so that strategies targeting specific genes, pathways, or even mutant alleles may be possible in the near future.

Genodermatoses

The term "genodermatoses" covers a range of inherited skin disorders that are due to heritable defects in one or more genes whose function is essential for normal skin physiology. The underlying genetic lesions fall into three broad categories: (a) single gene disorders (the vast majority of genodermatoses); (b) chromosomal disorders; and (c) complex traits. In the single gene disorders (also known as monogenic or Mendelian traits), inheritance patterns are relatively simple (usually dominant, recessive, autosomal or X-linked). Chromosomal abnormalities are often sporadic – whole or partial chromosomes, and therefore, many genes may be deleted, duplicated, or rearranged. In complex traits (also known as polygenic or multifactorial disorders), a number of genes, usually scattered throughout the genome, act in concert with environmental or behavioral factors to promote disease. Skin diseases with complex traits, such as eczema, psoriasis, acne, and alopecia, affect as many as one in four individuals in some ethnic populations. Monogenic genodermatoses are individually rare, each affecting ~1 in 10,000 or fewer, but collectively these skin disorders are not uncommon. Due to the large number of single gene disorders that exclusively or primarily affect the skin and its appendages, this chapter will mainly focus on these genodermatoses.

W.H.I. McLean (✉)
Division of Molecular Medicine, Colleges of Life Sciences and Medicine, Dentistry and Nursing, MSI/WTB/JBC Complex, University of Dundee, Dundee DD1 5EH, Scotland, UK
e-mail: w.h.i.mclean@dundee.ac.uk

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_19,

Inheritance Patterns and Risk in Genodermatoses

In the monogenic genodermatoses, inheritance is normally in an autosomal dominant or autosomal recessive pattern, but there are also a small number of X-linked dominant and X-linked recessive disorders. Identifying the inheritance pattern of a disorder within a family is important so that the risk to any future offspring is determined and appropriate genetic counseling can be provided. Commonly encountered inheritance patterns are shown in Fig. 19.1.

In autosomal dominant disorders, affected individuals are heterozygous carriers of a mutant allele which is inherited from an affected parent, except in those cases of "de novo" mutations, as outlined below. Both males and females are equally affected. An important indication of autosomal dominant inheritance is male-to-male transmission of the condition within the pedigree, excluding the possibility of X-linked inheritance, since the Y-chromosome passes from the male parent to all male offspring. In an autosomal dominant condition, each offspring of an affected individual has a 50% chance of inheriting the disorder. Examples of autosomal dominant skin disorders include epidermolysis bullosa simplex (EBS) caused by mutations in the keratin genes *KRT5* or *KRT14*, and hereditary hemorrhagic telangiectasia (HHT) due to mutations in *ENG, ACVRLI,* or *SMAD4* genes.

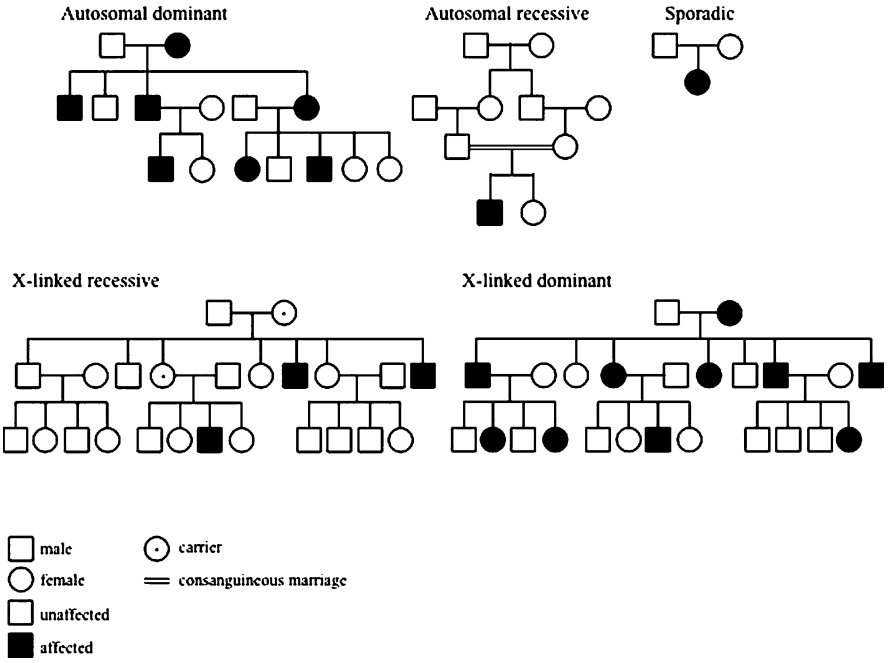

Fig. 19.1 Inheritance patterns in genodermatoses. Example pedigrees showing classical inheritance patterns commonly encountered in clinical practice. *Squares* indicate males; *circles*, females; *filled symbols*, disease phenotype; *open symbols*, normal individuals. Autosomal dominant inheritance can be distinguished by male-to-male transmission, both males and females with disease, and persons in each subsequent generation affected. In autosomal recessive conditions, the parents are phenotypically normal carriers. In-breeding (consanguinity) is often, but not universally, associated with a recessive inheritance pattern. In X-linked recessive diseases, only males are affected, born to unaffected carrier females. In X-linked dominant diseases, affected females transmit the disorder to both male and female offspring, whereas affected males transmit the condition to all their daughters, but not their sons. Other more complex patterns of inheritance also occur (not shown here). Sporadic cases are difficult to diagnose, and can represent recessive; de novo dominant; X-linked, particularly if the affected individual is male; or complex trait patterns of inheritance. In these isolated cases, reference to the literature and resources like Online Mendelian Inheritance in Man (OMIM) are essential to make the diagnosis and counsel the family appropriately

"De novo" mutations can occur in autosomal dominant disorders (Fig. 19.1). In these cases, the parents are unaffected, and a new mutation has arisen in their offspring within the gene associated with that specific disorder. In a smaller number of these sporadic or spontaneous cases, one of the parents may be the carrier of a germline mosaic mutation (i.e., the mutation arose during embryogenesis in such a way that the gonadal tissue carries the mutation, but not the skin). In such instances, there is a significant risk of having a second affected child.

In dermatology, genetic mosaicism is much more easily observed than in other medical specialties, as the skin is readily visualized. Epidermal nevi (or birthmarks) are instances of genetic mosaicism, where the molecular defect occurs at a stage of embryogenesis and allows for the formation of clinically evident lesions within a background of normal-appearing skin. Such lesions tend to follow the lines of Blaschko – areas within adult skin that correspond to ectodermal cell migration patterns in the embryo. An archetypal epidermal nevus is nevoid epidermolytic hyperkeratosis, which results from mutations in the keratin genes *KRT1* and/or *KRT10* [1, 2]. In many instances, mosaicism may affect only internal tissues and remain unknown, until perhaps an affected person has children with obvious cutaneous lesions.

In autosomal recessive disorders, there is a requirement for two copies of the mutant allele to be present, in order for a person to be affected by the disease (Fig. 19.1). Affected individuals may have the same mutation on each allele (homozygous) or different mutations on each allele (compound heterozygous). Recessive disorders are usually transmitted by unaffected carrier (heterozygous) parents. In addition, recessive diseases are commonly encountered when there is consanguinity (in-breeding) within the pedigree, as each parent is likely to carry the same mutant allele inherited from a shared ancestor in this instance. With each pregnancy of carrier parents, there is a 25% chance of inheriting the disorder. Autosomal recessive disorders are often severe; for example, harlequin ichthyosis, recessive dystrophic epidermolysis bullosa (RDEB), and oculocutaneous albinism (OCA). In rare cases of recessive disorders, only one of the parents is a heterozygous carrier and the second genetic event arises in their offspring due to a "de novo" mutation on the other allele of the same gene. Examples of this phenomenon have been seen in EBS with muscular dystrophy (EBS-MD), which is due to mutations in the plectin gene (*PLEC1*) [3].

X-linked disorders are those in which the mutant gene(s) is present on the X chromosome (Fig. 19.1). Similar to autosomal diseases, these can be inherited as either dominant or recessive traits, depending on the function of the mutant gene. In X-linked recessive cases, affected individuals are usually male. An example is X-linked ichthyosis (XLI), one of the first inherited skin disorders to have its gene identified [4]. Half the male offspring of an unaffected female carrier will develop XLI, and half the daughters of a female carrier will also be carriers. All daughters of an affected male will be carriers of the mutant gene, and therefore at risk of having an affected male child. There is no male-to-male transmission observed in X-linked conditions, allowing these to be readily separated from autosomal disorders. In X-linked dominant disorders, both males and females are affected. A male with an X-linked dominant disorder can transmit only the mutant allele to his daughters, while affected females can transmit the disorder to both their sons and daughters. Very often in X-linked dominant disorders, the affected males do not survive, as they have no compensatory normal X chromosome. This is seen in the X-linked dominant genodermatosis, incontinentia pigmenti (IP) [5].

Conditions associated with chromosomal abnormalities may be due to: (a) gain or loss of a chromosome(s) or (b) structural rearrangement due to chromosomal breakage. These conditions may present with multiple congenital signs, including minor skin defects; for example, deletion of the long arm of chromosome 18 leads to multiple developmental abnormalities, in addition to an increased risk of eczema/atopic dermatitis [6].

Rare inheritance patterns include: (a) disorders due to mutations in mitochondrial DNA (i.e., palmoplantar keratoderma with deafness) and (b) those where uniparental disomy occurs. In the

latter cases, the affected individual receives both copies of a chromosome or part of a chromosome from one parent and no copies from the other parent. This phenomenon has been observed in unusual cases of recessive EB [7].

In contrast to some of the very common skin disorders (i.e., atopic dermatitis), most genodermatoses are rare "orphan" disorders with the result that many general practice physicians and dermatologists will only very occasionally come across such cases during their careers. In addition, affected patients and their families can feel very isolated. However, there are specialist clinics for a number of these disorders to which patients can be referred; for example, there are several hospital centers in the USA and UK which run clinics for individuals with EB and can also provide information about any relevant patient support groups/charities.

Genotypic and Phenotypic Variation and Penetrance

Among inherited disorders of the skin there is a great degree of both clinical and genetic heterogeneity. There may be variable expression of the clinical signs of genodermatoses, both between and within families, ranging from mild to severe disease. It is likely that this variability is due to modifier genes and/or environmental factors. Examples include severe cases of XLI which, in addition to a predicted mutation in the steroid sulphatase (*STS*) gene, also contain a mutation in the filaggrin (*FLG*) gene, resulting in more prominent scaling of the skin [8]. Another example is RDEB, where a functional single nucleotide polymorphism (SNP) in the matrix metalloproteinase (MMP1) promoter has been reported to be a modifier of the disease, resulting in a more severe clinical phenotype. This range of severity has been noted within members of one family and also in a cohort of unrelated cases [9]. Genetic heterogeneity may be found in: (a) some of the keratin disorders, where the same clinical phenotype can result from mutations in either one of a pair of functionally linked keratin genes [10]; and (b) xeroderma pigmentosum (XP), where at least seven different genes are involved, all of which have a role in DNA excision repair [11]. In contrast, different mutations in the gene encoding desmoplakin (DSP), an epithelial cell junction protein, can give rise to very heterogeneous clinical phenotypes [12]. The spectrum of clinical features associated with this molecular defect depends on: (a) the type of mutation present (i.e., missense, nonsense, splice site, or frameshift); and/or (b) the mechanisms by which these mutations act at the protein level (including dominant–negative interference, loss-of-function, gain-of-function, or haploinsufficiency). In some cases, the position of the mutation within a particular gene is important. Some genetic abnormalities are also due to large genomic deletions that may involve one or more exons of a gene, an entire gene, or even groups of genes. The consequences of any genetic mutation are strongly influenced by the function of the resultant protein (i.e., it may result in abnormal protein structure and function; loss of a specific protein or enzyme; or a defect in a specific step within a biological pathway).

The penetrance of a disorder is defined as the percentage of individuals with the mutant allele(s) who demonstrate clinical signs of the disorder. Complete penetrance is when all individuals have the mutant allele and a clinical phenotype. Incomplete/reduced penetrance is when some individuals carry the mutant allele, but do not show clinical evidence of disease. In general terms, penetrance tends to be complete or close to 100% in monogenic disorders, whereas penetrance in complex traits is generally low. This means that inheritance patterns are readily determined in monogenic diseases, and risks for future pregnancies can be estimated with high certainty. In contrast, for complex traits, one tends to observe clustering of affected individuals within extended kindreds, but the inheritance pattern may be unclear. Therefore, the risk to future offspring can be difficult to estimate with any degree of certainty in these disorders.

Databases

The Online Mendelian Inheritance in Man (OMIM) website (www.ncbi.nlm.nih.gov/omim) provides details on all genetic disorders and includes clinical findings together with information on the specific genes/mutations involved. Another useful database is "*A current and online genodermatosis database*" that was recently published [13]. For skin disorders associated with intermediate filaments, particularly the many keratin genes expressed in the epidermis and its appendages, a valuable online resource is the Human Intermediate Filament Database (www.interfil.org), which records all published mutations in genes encoding intermediate filament proteins, together with DNA sequence, protein changes, and clinical data [14].

Molecular Diagnosis of Genodermatoses

The genetic defect for many inherited diseases of the skin is now known, and diagnosis at the molecular level using a variety of techniques is routinely performed to confirm clinical findings. Most of these molecular tests utilize polymerase chain reaction (PCR) and direct sequencing of known disease-associated gene(s), in order to identify the specific pathogenic mutation(s). Some disorders have known mutation hotspots within a gene and these regions may be sequenced first; for example, pachyonychia congenita (PC) is due to mutations in the keratin genes *KRT6A, KRT6B, KRT16,* or *KRT17* [15]. The majority of mutations in PC patients are found within DNA sequences that encode the highly conserved helix initiation and termination motif at either end of the rod domain of a keratin protein. To date, these aberrations are all either missense or small insertion/deletion mutations, inherited in an autosomal dominant manner. Mutational analysis can be performed on genomic DNA derived from either a blood or saliva sample. Oragene®•DNA saliva kits (DNA Genotek Inc., Ontario, Canada) are particularly useful for young children as they are less invasive than a blood sample. Such kits are also useful when samples cannot be sent immediately for DNA extraction. Once samples are collected with such kits, they are stable at room temperature for several weeks and are also safe and easy to ship to diagnostic laboratories.

In some disorders, one specific mutation may account for nearly all cases of the disease (i.e., in EBS with mottled pigmentation, almost all cases carry the p.P25L mutation in the keratin gene *KRT5*). As an alternative to sequencing, other technologies may be used to detect the predicted mutation, such as restriction digest/allelic discrimination Taqman assays or mass spectrometry genotyping methods. Some of these methods are particularly useful for high-throughput screening of large numbers of samples and are therefore widely used in complex trait genetics.

However, there are still a number of genetic skin conditions which remain to be elucidated at the molecular level. In some instances, pathogenic mutations are not identified in the candidate genes and/or the clinical features may not exactly fit a known disorder. In these cases, further investigations are necessary to definitively identify the disease and genetic defect. Firstly, the mode of inheritance should be established (Fig. 19.1). If the disorder is inherited in an autosomal dominant manner and genomic DNA is available from a number of family members, genetic linkage can be performed for candidate genes. From even a relatively small pedigree, it may be possible by linkage analysis to exclude some candidate genes and therefore reduce the number of potential markers to be sequenced. If all candidate genes are excluded by genetic linkage analysis or direct DNA sequencing, then the next step would be to perform genome-wide linkage analysis to identify the gene involved. This is achieved using microsatellite markers, at intervals corresponding to five million base pairs of DNA or less, across the entire genome. Linkage of a disorder to certain chromosomal regions can be further defined using markers close together within these regions. Depending on how narrow the interval is

(i.e., it usually gets smaller with increasing number of samples/families analyzed), a number of candidate genes may be identified for further investigation. The expression profiles of these genes can be used to determine if they are likely to be candidate markers. Sometimes a number of genes may be sequenced before the correct gene and mutation is identified.

Homozygosity mapping is a method employed to identify the gene(s) involved in a recessively inherited disorder. Again, DNA samples are required from as many family members (and families) as possible. This technique was used to determine the genes involved in both Kindler syndrome [16] and laryngo-onycho-cutaneous (LOC) syndrome [17], with the respective mutations subsequently confirmed by DNA sequencing.

For both genetic linkage and homozygosity mapping studies, procurement of samples from several families with the same phenotypic features increases the chance of identifying the region of the genome and/or the actual gene involved in the disorder(s). But it is important that careful clinical examination be carried out on all affected members to ensure that they indeed have the same disease.

Identification of susceptibility loci/genes for complex traits is very different from analyses on monogenic disorders. The current state-of-the-art technology for genome analysis in complex traits will be discussed at the end of this chapter.

Molecular Basis of Genodermatoses

In this chapter, the genodermatoses have been subdivided into sections according to their clinical presentation and molecular basis [13, 18]. Those disorders with known causative genes are listed in the Tables. Those diseases for which a locus or loci, but not a specific gene, has been identified, have been excluded in the interest of brevity. Due to overlap in clinical features, the classification of some of these disorders is complex, as they may fit into more than one group.

Disorders of Keratinization

Ichthyoses

The molecular basis for a number of clinically distinct ichthyoses has now been identified (Table 19.1). Inheritance patterns include autosomal dominant, semidominant, recessive, and X-linked (both dominant and recessive). Mutations have been identified in a number of genes, including filaggrin (*FLG*), transglutaminase-1 (*TGM1*), connexin 26 (*GJB2*), and several of the suprabasally expressed keratin genes (*KRT1, KRT2, KRT10*).

Ichthyosis Vulgaris

The genetic defect in patients with the most commonly inherited keratinizing disorder, ichthyosis vulgaris, was for many years predicted by biochemical assays to be caused by mutations in the *FLG* gene (Fig. 19.2). However, due to the large and highly repetitive structure of this gene, and the difficulty in designing specific primers for PCR and DNA sequence analysis, its role was only recently confirmed at the molecular level with the identification of nonsense or frameshift mutations in a number of ichthyosis vulgaris families [19]. On close examination of a number of large pedigrees, it has been established that ichthyosis vulgaris is inherited as a semidominant trait. Homozygous or compound heterozygous individuals present with more severe clinical phenotypes compared with those who are classic heterozygotes. In the original families analyzed, two mutations were identified, R501X and 2282del4, which have since been found to be the prevalent mutations in white

Table 19.1 Ichthyoses

Name	OMIM number	Inheritance	Gene
Ichthyosis vulgaris (Also atopic dermatitis 2, susceptibility ATOD2)	146700 (605803)	Semidominant (Complex trait)	*FLG*
X-linked recessive ichthyosis (XLI)	308100	XLR	*STS*
Ichthyosis lamellar 1 (LI1)	242300	AR	*TGM1*
Ichthyosis lamellar 2 (LI2)	601277	AR	*ABCA12*
Ichthyosis lamellar 3 (LI3)	604777	AR	*CYP4F22*
Ichthyosis, leukocyte vacuoles, alopecia, and sclerosing cholangitis	607626	AR	*CLDN1*
Bullous congenital ichthyosiform erythroderma (BCIE)/ Epidermolytic hyperkeratosis (EHK)	113800	AD	*KRT1, KRT10*
Ichthyosis, cyclic with epidermolytic hyperkeratosis (CIEHK)	607602	AD	*KRT1, KRT10*
Ichthyosis bullosa of Siemens (IBS)	146800	AD	*KRT2*
Ichthyosis hystrix (Curth Macklin) (IHCM)	146590	AD	*KRT1*
Ichthyosis hystrix-like with deafness (HID)	602540	AD	*GJB2*
Ichthyosiform erythroderma, congenital, nonbullous (NCIE)	242100	AR	*ALOXE3, ALOX12B, TGM1, ABHD5*
Ichthyosis harlequin	242500	AR	*ABCA12*
Ichthyosis, congenital, autosomal recessive, Ichthyin-related (ARCI)	612281	AR	*ICHTHYIN*
Netherton syndrome (NETH)	256500	AR	*SPINK5*
Ichthyotic neutral lipid storage disease (NLSD)/ Chanarin-Dorfam disease	275630	AR	*CGI58 (ABHD5)*
Chondrodysplasia punctata 1 (CDPX1)	302950	XLR	*ARSE*
Chondrodysplasia punctata 2 (Conradi-Hünerman syndrome) (CDPX2)	302960	XLD	*EBP*
Chondrodysplasia punctata, rhizomelic form (CDPR)	215100	AR	*PEX7*
Congenital hemidysplasia with ichthyosiform erythroderma and limb defects (CHILD)	308050	XLD	*NSDHL*
Keratitis-Ichthyosis-Deafness (KID) syndrome	148210	AD	*GJB2*
Sjögren-Larsson syndrome (SLS)	270200	AR	*FALDH (ALDH3A2)*
Refsum syndrome	266500	AR	*PEX7, PHYH*
Gaucher disease type I (GDI)	230800	AR	*GBA*
Gaucher disease type II (GDII)	230900	AR	*GBA*

AD autosomal dominant, *AR* autosomal recessive, *XLD* X-linked dominant, *XLR* X-linked recessive

populations of European ancestry. Many more mutations have now been found throughout the *FLG* gene, some of which are prevalent in the general population and others that are either very rare or even family-specific [20]. Studies of ichthyosis vulgaris patients from different ethnic backgrounds have identified further common and rare mutations in different ancestral groups [21, 22]. Shortly after the discovery of *FLG* mutations in ichthyosis vulgaris, it was also determined that mutations in this gene are very strongly associated with atopic dermatitis [23], as discussed in detail below.

X-Linked Ichthyosis

XLI, another common disorder of keratinization, was the first keratinizing disorder to be elucidated at the molecular level, in 1987 [4]. Affected individuals present with dark, regular, and adherent scales of skin. Inherited as an X-linked recessive trait, the majority of affected individuals are males, as they inherit the X chromosome carrying the mutant gene from their unaffected carrier mother. Only rarely do females present with the disease. In such an instance, the female would have inherited two mutant X alleles: one from her affected father and one from her carrier mother. This disorder is

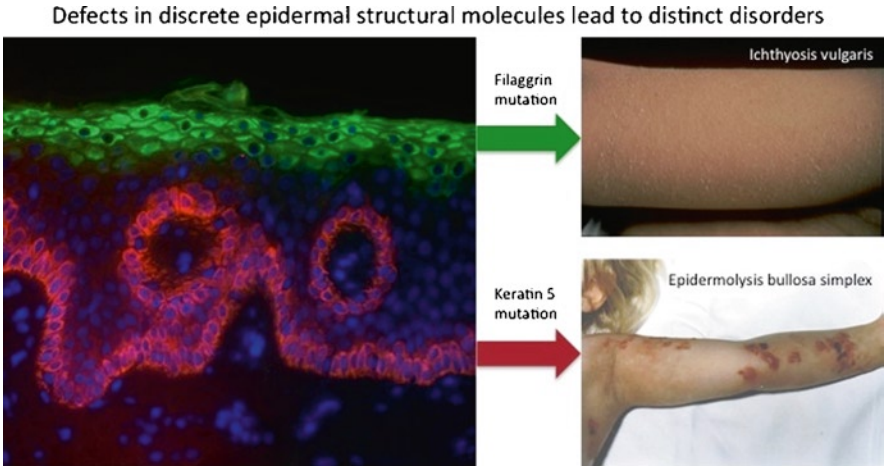

Fig. 19.2 Structural defects causing epidermal diseases. Many epidermal disorders are caused by genetic defects in structural molecules. Two examples are shown here. Keratin K5 (*KRT5*) is a major structural protein of the basal cell layer (*red* staining). Dominant-negative mutations in the *KRT5* gene can lead to epidermolysis bullosa simplex (*EBS*) – an inherited bullous disease. The granular layers of the epidermis are rich in profilaggrin (*green* staining). Upon terminal differentiation of the keratinocytes to form squames of the stratum corneum, profilaggrin is processed into filaggrin, which performs key roles in squame biogenesis and hydration. Loss of filaggrin expression due to loss-of-function mutations leads to ichthyosis vulgaris – the most prevalent inherited keratinizing disorder. Filaggrin (*FLG*) gene mutations are carried by up to 10% of certain human populations; also predisposing individuals to atopic dermatitis, in addition to their role in dry, scaly skin phenotypes

caused by loss-of-function mutations in the *STS* gene, most commonly (in 90% of patients) due to a large genomic deletion of the entire gene, which is located at Xp22.3. The condition can be diagnosed by a biochemical assay for STS enzyme activity in the blood. Molecular confirmation is achieved by either fluorescence in situ hybridization (FISH) to detect the common deletion mutation, or PCR-based sequencing to identify the small insertion/deletion mutations or point mutations present in about 10% of cases.

Palmoplantar Keratodermas

The palmoplantar keratodermas include a range of disorders where the predominant clinical feature is thickening and/or blistering of the palms and soles (Table 19.2). A variety of associated distinct clinical features provide clues as to the molecular aberration involved in an affected individual. Mutations have been identified in a number of genes, including several of those encoding keratins, desmosomal proteins, connexins, and loricrin.

Several keratin disorders fall into the category of palmoplantar keratodermas. Keratins belong to the family of intermediate filaments and exist as obligate heteropolymers, consisting of a type I and type II keratin [24]. They are expressed in epithelial cells, in a tissue- and differentiation-specific manner [25]. One of their main functions is to provide a structural cytoskeleton within cells, which if compromised, due to a mutation within one of the keratins, can result in cell fragility. The latter can be seen clinically as skin blistering and/or hyperkeratosis [26]. A number of clinically distinct disorders caused by mutations in different keratin genes have been identified since the early 1990s [10]. These can affect the skin, hair, nails, cornea, mucosal surfaces, and simple epithelial tissues (www.interfil.org). Other keratin disorders of the skin are classified in this chapter under ichthyoses, EB, and/or ectodermal dysplasias [10, 18].

Pachyonychia congenita (PC) is a keratin disorder affecting the skin, nails, and mucosal tissues [15]. Clinically, PC is characterized predominantly by painful palmoplantar keratoderma, hypertrophic

Table 19.2 Palmoplantar keratodermas (PPKs)

Name	OMIM number	Inheritance	Gene
Epidermolytic palmoplantar keratoderma (Vörner: EPPK)	144200	AD	*KRT9, KRT1*
Nonepidermolytic PPK (NEPPK)	600962	AD	*KRT1, KRT16*
Mal de Meleda (MDM)	248300	AR	*SLURP1*
Vohwinkel syndrome (classic variant: KHM)	124500	AD	*GJB2*
Vohwinkel syndrome (ichthyotic variant)	604117	AD	*LOR*
Pachyonychia congenita type 1 (Jadassohn-Lewandowsky syndrome; PC-1)	167200	AD	*KRT6A, KRT16*
Pachyonychia congenita type 2 (Jackson-Lawler syndrome; PC-2)	167210	AD	*KRT6B, KRT17*
PPK striate 1 (PPKS1)	148700	AD	*DSG1*
PPK striate 2 (PPKS2)	612908	AD	*DSP*
PPK striate 3 (PPKS3)	607654	AD	*KRT1*
PPK + deafness	148350	AD/Mitochondrial	*GJB2/MTTS1*
Striate PPK with dilated cardiomyopathy and woolly hair (Carvajal syndrome)	605676	AR	*DSP*
Skin fragility-woolly hair syndrome (SFWHS)	607655	AR	*DSP*
Naxos disease (PPK + woolly hair + cardiac abnormalities)	601214	AR	*JUP*
Papillon-Lefèvre syndrome (PPK + periodontitis:PALS)	245000	AR	*CSTC (DPPI)*
Haim-Munk Syndrome (HMS)	245010	AR	*CSTC (DPPI)*
Oculodentodigital dysplasia (ODDD)	164200	AD/AR	*GJA1*
Richner-Hanhart syndrome (tyrosinemia)	276600	AR	*TAT*
Knuckle pads, leukonychia, and sensorineural deafness (Bart-Pumphrey syndrome)	149200	AD	*GJB2*
Cerebral dysgenesis, neuropathy, ichthyosis, and PPK (CEDNIK)	609528	AR	*SNAP29*

AD autosomal dominant, *AR* autosomal recessive

nail dystrophy, and oral leukokeratosis. Other findings include palmoplantar blistering, follicular keratoses, cysts, and hyperhidrosis [27]. PC is caused by mutations in one of four keratin genes, *KRT6A, KRT6B, KRT16,* or *KRT17* [28–30]. To date, all cases with identified mutations show autosomal dominant inheritance patterns [15, 31]. Further details about PC can be found on the PC Project website (www.pachyonychia.org), a recently established patient support group that facilitates genetic testing, provides patient information, and organizes both clinical and scientific meetings, as well as funding research into treatments for PC. Recently, dominant mutations in *KRT6C*, which encodes keratin K6c, have been reported in patients with painful focal plantar keratoderma [32]. K6c shows overlap in its distribution with K6a and K6b, but is thought to be expressed at lower levels. Therefore, the disease phenotype associated with *KRT6C* mutations is more restricted than in classic PC, with minimal nail involvement and no other ectodermal features [32].

Another commonly encountered palmoplantar disorder involving keratin mutations is epidermolytic palmoplantar keratoderma (EPPK; also known as Vörner syndrome). EPPK is predominantly caused by dominant-negative mutations in the *KRT9* gene [33]; although, there are also a number of reports of specific mutations in the *KRT1* gene [34]. In this condition and some other keratodermas, the physical findings are generally not associated with debilitating pain, as in classic PC.

Several genetic skin disorders are associated with different proteins located within desmosomes. Desmosomes are cell–cell adhesion junctions that are composed of three families of proteins: (a) armadillo proteins; (b) cadherins; and (c) plakins. Desmosomes are predominantly found in skin and cardiac tissue; therefore, in addition to a skin phenotype, mutations in some of the genes for these proteins can result in forms of cardiomyopathy.

Table 19.3 Other keratinizing disorders

Name	OMIM number	Inheritance	Gene
Darier disease (DD)	124200	AD	*ATP2A2*
Hailey-Hailey disease (HHD)	169600	AD	*ATP2C1*
Acrokeratosis verruciformis (AKV)	101900	AD	*ATP2A2*
Erythrokeratodermia variabilis (EKV)	133200	AD/AR	*GJB3, GJB4*
Keratosis follicularis spinulosa decalvans (KFSDX)	308800	XL	*SAT1*
Infantile systemic hyalinosis (ISH)	236490	AR	*CMG2 (ANTXR2)*
Restrictive dermopathy, lethal	275210	AD/AR	*LMNA, ZMPSTE24*

AD autosomal dominant, *AR* autosomal recessive, *XL* X-linked

DSP belongs to the plakin family of cytoskeletal linker proteins. It is a large desmosomal plaque protein, whose gene is located on chromosome 6p24. A number of different types of mutations have been identified in this gene, and are associated with distinct phenotypes showing skin, hair, and/or heart abnormalities, but with clinical heterogeneity [35]. Autosomal dominant mutations (heterozygous nonsense or splice site mutations) cause striate palmoplantar keratoderma (SPPK), as a result of haploinsufficiency of DSP [36, 37]. Dominant mutations have also been reported in cases presenting with SPPK and woolly hair, a condition associated with cardiomyopathy that can lead to death at an early age [38]. There are also some dominant mutations that demonstrate only cardiac abnormalities, such as arrhythmogenic right or left ventricular cardiomyopathy [39]. As a recessive trait, homozygous or compound heterozygous mutations (nonsense, frameshift resulting in premature termination codons [PTC], or missense mutations) cause keratoderma, woolly hair, and arrhythmyogenic dilated cardiomyopathy which can lead to heart failure in children [40–43]. Other reported cases of compound heterozygous (missense and/or nonsense) mutations have a similar phenotype, but without the cardiac abnormalities; although, these patients should probably be monitored regularly [44]. Interestingly, heterozygous parents are unaffected since some heterozygous mutations result in haploinsufficiency (see above). There is one report of a compound heterozygous individual (resulting in loss of DSP tail domain) who presented with a severe phenotype showing widespread epidermolysis, generalized alopecia, absence of nails, and the presence of neonatal teeth [45]. Another case with bullous dermatosis, plantar keratoderma, alopecia totalis, and childhood lethal cardiomyopathy was found to be due to compound heterozygosity for two nonsense mutations [46]. Thus, DSP is a good example of how different types and/or combinations of mutations in the same gene can lead to a variety of phenotypes with differing severities, in addition to recessive versus dominant inheritance patterns. Other keratinizing disorders are outlined in Table 19.3.

Inherited Bullous Skin Disorders

EB encompasses a number of clinically and genetically distinct disorders that all result in some form of skin blistering, ranging from relatively mild to very severe phenotypes, and affecting many body sites (Table 19.4). Similar to other disorders in this chapter, a provisional diagnosis can often be made on the basis of histopathological features, ultrastructural changes, and/or antigenic staining patterns of skin biopsy specimens [47]. Recently, the classification of EB has been revised on the basis of clinical and molecular findings, and now encompasses several other genetic disorders, such as Kindler syndrome, which are included due to the presence of cutaneous blistering and cell fragility [47]. EB is divided into four main forms that can be distinguished by the level of blistering within the skin: (a) epidermolysis bullosa simplex (EBS; intraepidermal cleavage); (b) junctional EB (JEB; intralamina lucida cleavage); (c) dystrophic EB (DEB; sublamina densa cleavage); and (d) a fourth group in which the level of cleavage is mixed.

Table 19.4 Inherited bullous disorders

Name	OMIM number	Inheritance	Gene
Epidermolysis bullosa simplex – intraepidermal skin cleavage – suprabasal			
Lethal acantholytic EB (EBLA)	609638	AR	*DSP*
Ectodermal dysplasia/skin fragility syndrome (McGrath syndrome)	604536	AR	*PKP1*
Epidermolysis bullosa simplex – intraepidermal skin cleavage – basal			
Epidermolysis bullosa simplex, Dowling Meara (EBS-DM)	131760	AD	*KRT5, KRT14*
EBS, localized (EBS-loc)	131800	AD	*KRT5, KRT14, ITGB4, ITGA6*
EBS, other generalized (EBS, gen-nonDM, EBS)	131900	AD	*KRT5, KRT14*
EBS with mottled pigmentation (EBS-MP)	131960	AD	*KRT5*
EBS, autosomal recessive (EBS-AR)	601001	AR	*KRT14*
EBS with muscular dystrophy (EBS-MD)	226670	AR	*PLEC1*
EBS with pyloric atresia (EBS-PA)	612138	AR	*PLEC1*
EBS-Ogna (EBS-Og)	131950	AD	*PLEC1*
EBS, migratory circinate (EBS-migr)	609352	AD	*KRT5*
Junctional epidermolysis bullosa (JEB) – intralamina lucida skin cleavage			
JEB-Herlitz (JEB-H)	226700	AR	*Laminin-5 (LAMA3, LAMB3, LAMC2)*
JEB, non-Herlitz generalized EB (JEB-nH gen); JEB non-Herlitz localized (JEB-nH loc) and *JEB inversa (JEB-I)*	226650	AR	*COL17A1, Laminin-5 (LAMA3, LAMB3, LAMC2), ITGB4*
JEB with pyloric atresia (JEB-PA)	226730	AR	*ITGB4, ITGA6*
Laryngo-onycho-cutaneous syndrome (LOCS)	245660	AR	*Laminin-5 (LAMA3)*
Dystrophic epidermolysis bullosa (DEB) – sublamina densa skin cleavage			
Dominant DEB, generalized (DDEB-gen)	131750	AD	*COL7A1*
DDEB, pretibial (DDEB-Pt)	131850	AD	*COL7A1*
DDEB pruriginosa (DDEB-Pr), (RDEB-Pr)	604129	AD/AR/ Sporadic	*COL7A1*
DDEB, toenails only (DDEB-na)	607523	AD	*COL7A1*
DDEB, bullous dermolysis of the newborn (DDEB-BDN)	131705	AD	*COL7A1*
Recessive dystrophic epidermolysis bullosa, severe generalized (RDEB-sev gen); RDEB, generalized other (RDEB-O); *RDEB, inversa (RDEB-I)*	226600	AR	*COL7A1*
Kindler syndrome – level of skin cleavage mixed	173650	AR	*KIND1*
Peeling skin syndrome, acral type (APSS)	609796	AR	*TGM5*

Rare subtypes of EB shown in italics

AD autosomal dominant, *AR* autosomal recessive

EBS, where blistering occurs within the basal keratinocytes, is predominantly caused by mutations in the keratin genes *KRT5* or *KRT14*, and normally inherited as a dominant trait [10]. A case of EBS as a result of a *KRT5* mutation is shown in Fig. 19.2. The structure of the *KRT5* gene, which encodes keratin K5, is shown in Fig. 19.3. There are rare cases of recessive EBS due to mutations in *KRT14* [48]. Less commonly, EBS can be associated with mutations in the gene encoding plectin, a linker protein which connects the keratin cytoskeleton to the hemidesmosomes in basal keratinocytes, and also performs a variety of other cytoskeleton cross-linking functions in muscle [49, 50]. Dominant mutations in plectin have been reported in a rare form of EBS, originally described in a large family

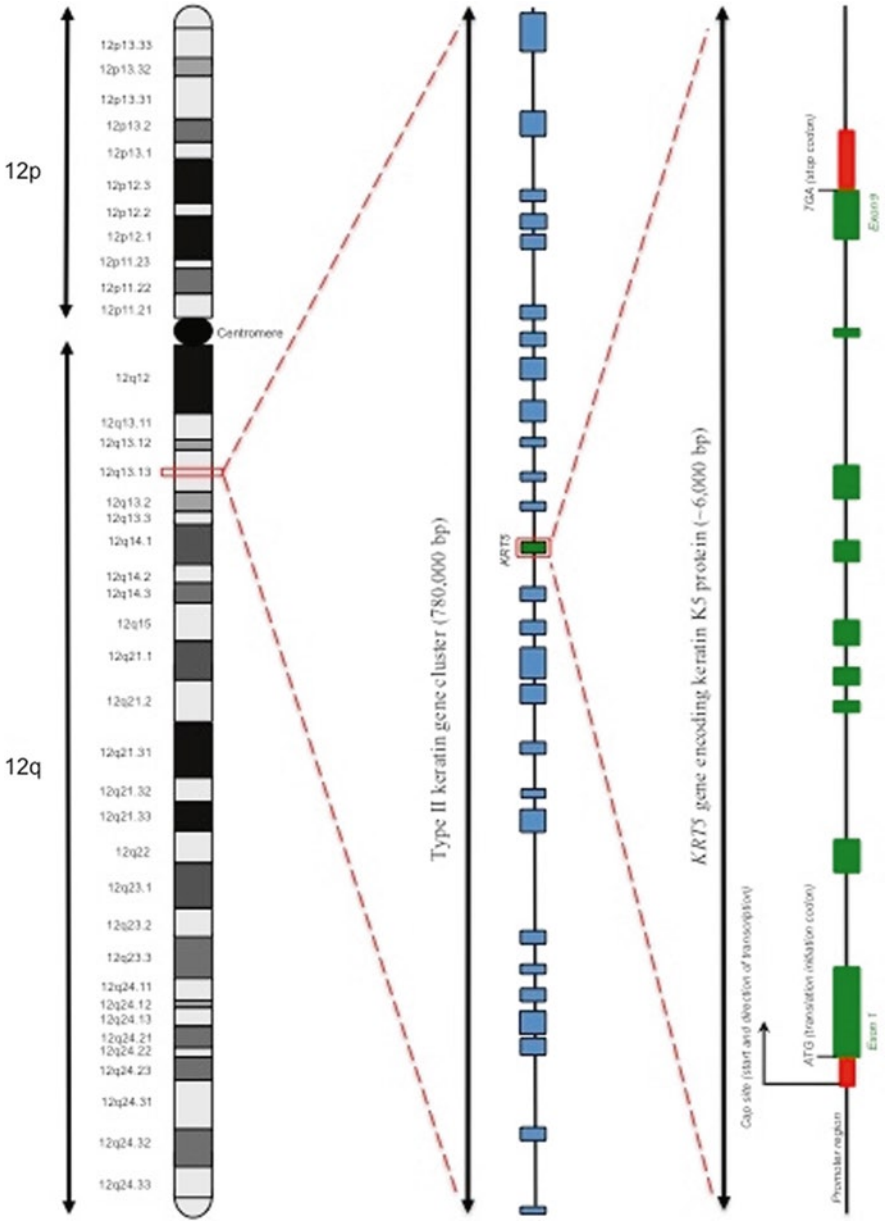

Fig. 19.3 Complexity of the human genome. The human genome consists of approximately three billion base pairs of DNA, packaged into 22 pairs of autosomes (chromosomes 1–22) plus the sex chromosomes, X and Y. There are thought to be ~25,000 genes in the human genome, although new classes of genes are still being discovered. A keratin gene is shown here as an example of typical gene organization. The type II keratin genes are clustered together on chromosome band 12q13.13. There are 27 keratin genes in this cluster, one of which is the *KRT5* gene encoding the K5 protein. This gene consists of nine protein-encoding exons separated by eight introns, and spans about 6,000 base pairs of DNA. These individual exons must be amplified and sequenced to search for mutations in K5-related diseases, such as epidermolysis bullosa simplex (EBS)

on the island of Ogna in Norway [51]. Recessively acting mutations in plectin can cause EBS, either in combination with muscle disease [49, 50] or with pyloric atresia [52].

JEB, where blistering occurs within the lamina lucida of the basement membrane zone, is a result of mutations in: (a) the genes encoding the three subunits of laminin 332 (*LAMA3, LAMB3,*

LAMC2); (b) the two genes encoding α6β4 integrin (*ITGA6* or *ITGB4*); or (c) the collagen XVII gene (*COL17A1)* [47]. The products of these genes are all part of a complex of interconnected molecules required for normal epidermal–dermal adhesion.

The third form is DEB, inherited as either a dominant or recessive trait [47]. The level of blistering is below the basement membrane zone (within the sublamina densa), and is caused by mutations in the collagen VII gene (*COL7A1)* [53].

The fourth group of EB under the current classification system includes Kindler syndrome, in which there are multiple cleavage planes within the skin [16, 54]. Another skin blistering disorder, called acral peeling skin syndrome, shows cleavage high in the epidermis, between the granular layer and the stratum corneum. This autosomal recessive condition is caused by loss-of-function mutations in the transglutaminase-5 gene *(TGM5)* [55]. There are further subtypes of most forms of EB, as described in Table 19.4.

Ectodermal Dysplasias

The ectodermal dysplasias (ED) make up a large and very heterogeneous group of inherited conditions; all of which have a genetic abnormality that affects one or more ectodermal structures and/or epidermal appendages (hair, nails, teeth, and sweat glands) (Table 19.5). The classification of ED

Table 19.5 Ectodermal dysplasias (new classification by Priolo [56])

Name	OMIM number	Inheritance	Gene
Group 1			
Ectodermal dysplasia 1 (ED1)	305100	XLR	*EDA*
Ectodermal dysplasia, hypohidrotic, autosomal dominant (HED)	129490	AD	*EDAR/EDARADD*
Ectodermal dysplasia, anhidrotic	224900	AR	*EDAR/EDARADD*
Incontinentia pigmenti (IP)	308300	XLD	*NEMO (IKBKG)*
Ectodermal dysplasia, hypohidrotic, with immunodeficiency (HED-ID)	300291	XLR	*NEMO (IKBKG)*
Ectodermal dysplasia, anhidrotic, with immunodeficiency, osteopetrosis, and lymphedema (OLEDAID)	300301	XLR	*NEMO (IKBKG)*
Ectodermal dysplasia, anhidrotic, with T-cell immunodeficiency, autosomal dominant	612132	AD	*IKBA*
Ectrodactyly–ectodermal dysplasia – cleft lip/palate syndrome (EEC3)	604292	AD	*TP63*
Anchyloblepharon–ectodermal dysplasia – cleft lip/palate syndrome (AEC)	106260	AD	*TP63*
Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT)	103285	AD	*TP63*
Limb-mammary syndrome (LMS)	603543	AD	*TP63*
Rapp-Hodgkin syndrome (RHS)	129400	AD	*TP63*
Tricho-dento-osseous syndrome (TDO)	190320	AD	*DLX3*
Witkop syndrome (tooth and nail syndrome; TNS)	189500	AD	*MSX1*
Ellis-van Creveld syndrome (EVC)	225500	AR	*EVC, EVC2*
Weyers acrofacial dysostosis	193530	AD	*EVC, EVC2*
Group 2			
Clouston syndrome, hidrotic ectodermal dysplasia	129500	AD	*GJB6*
Cleft lip/palate-ectodermal dysplasia syndrome (CLPED1)	225060	AR	*PVRL1*
Ectodermal dysplasia/skin fragility syndrome (McGrath syndrome)	604536	AR	*PKP1*
Ectodermal dysplasia, ectrodactyly, and macular dystrophy (EEM)	225280	AR	*CDH3*
Odonto-onycho-dermal dysplasia (OODD)	257980	AR	*WNT10A*

AD autosomal dominant, *AR* autosomal recessive, *XLD* X-linked dominant, *XLR* X-linked recessive

Table 19.6 Other ectodermal dysplasias

Name	OMIM number	Inheritance	Gene
Tricho-rhino-phalangeal syndrome type I (TRPS1)	190350	AD	*TRPS1*
Tricho-rhino-phalangeal syndrome type II (Langer-Giedon; TRPS2)	150230	AD	*Micro-deletion syndrome 8q24.11 to 8q24.13 (incl. TRPS1 & EXT1)*
Tricho-rhino-phalangeal syndrome type III (TRPS3)	190351	AD	*TRPS1*
Trichothiodystrophy, photosensitive (TTDP)	601675	AR	*XPD (ERCC2), XPB (ERCC3)*
Steatocystoma multiplex	184500	AD	*KRT17*
Hypotrichosis, congenital, with juvenile macular dystrophy (HJMD)	601553	AR	*CDH3*
T-cell immunodeficiency, congenital alopecia, and nail dystrophy	601705	AR	*FOXN1*
Cartilage-hair hypoplasia (CHH)	250250	AR	*RMRP*
Nail-patella syndrome (NPS)	161200	AD	*LMX1B*
Ectodermal dysplasia, pure hair/nail type	602032	AR	*KRTHB5*
Acrodermatitis enteropathica, zinc-deficiency type (AEZ)	201100	AR	*SLC39A4*

AD autosomal dominant, *AR* autosomal recessive

has historically been difficult and there remains considerable overlap with other non-ED conditions. For example, PC (listed in this chapter under palmoplantar keratoderma) can also be classified as an ED. Another example is plakophilin deficiency, which leads to ectodermal dysplasia-skin fragility syndrome, and which has also been recently classified as a form of EB, due to the presence of skin blistering and fragility.

A new classification system for ED has been recently proposed [56]. Previous classification schemes have been based on clinical features and/or function(s) of associated genes [57–59]. Priolo noted that the genes identified in ED act through two different pathogenetic mechanisms [56]. There are: (a) those genes whose defects involve epithelial–mesenchymal interactions; and (b) those genes whose aberrations affect structural proteins involved in cell–cell adhesion and/or communication (i.e., connexin 30 [*GJB6*]). Some forms of ED are difficult to fit into either category (Table 19.6).

Connective Tissue Disorders

Several of the connective tissue disorders are multisystem conditions (Table 19.7). For example, Marfan syndrome involves the skeletal, ocular, and cardiovascular systems, as a result of mutations in the gene (*FBN1*) encoding the connective tissue protein fibrillin. Pseudoxanthoma elasticum (PXE) is an autosomal recessive condition that affects the skin, eyes, and cardiovascular systems. PXE was initially believed to be inherited as a dominant trait, but careful examination/molecular analyses of a number of families where PXE occurred in two generations has established that this disorder is autosomal recessive (pseudodominance) [60]. Due to the presence of areas of inelastic, leathery skin observed on the neck and flexural areas of patients with PXE, it was originally thought that the disease resulted from a defect in a gene involved in elastic fiber structure/function in the skin. However, mutations were identified in the *ABCC6* gene, which encodes an ABC transporter protein. *ABCC6* is expressed in the liver and kidneys, and only at very low levels, if at all, in the tissues affected in PXE [61]. The mechanisms by which mutations in *ABCC6* result in the mineralization seen in the skin, eyes, and blood vessels of patients with PXE are still under investigation. However, this disease may be regarded primarily as a metabolic disorder with a secondary effect of abnormal calcification of elastic fibers.

Table 19.7 Connective tissue disorders

Name	OMIM number	Inheritance	Gene
Ehlers-Danlos I (gravis; EDSI)	130000	AD	*COL1A1, COL5A1, COL5A2*
Ehlers-Danlos II (mitis, EDS II)	130010	AD	*COL5A1, COL5A2*
Ehlers-Danlos III (benign hypermobile, EDS III)	130020	AD	*COL3A1, TNXB*
Ehlers-Danlos IV (ecchymotic, arterial, EDS IV)	130050	AD	*COL3A1*
Ehlers-Danlos VIA (kyphoscoliotic, EDS VIA)	225400	AR	*PLOD*
Ehlers-Danlos VIIA (arthrochalasia multiplex, EDS VIIA)	130060	AD	*COL1A1*
Ehlers-Danlos VIIB (arthrochalasia multiplex, EDS VIIB)	130060	AD	*COL1A2*
Ehlers-Danlos VIIC (dermatosparaxis, EDS VIIC)	225410	AR	*ADAMTS2*
Ehlers-Danlos, cardiac valvular form	225320	AR	*COL1A2*
Ehlers-Danlos, progeroid form	130070	AR	*B4GALT7*
Ehlers-Danlos variant, heterotopia, periventricular (PVNH4)	300537	XLD	*FLNA*
Ehlers-Danlos-like syndrome due to tenascin X deficiency	606408	AR	*TNXB*
Fabry disease	301500	XLR/XLD	*GLA*
Marfan syndrome (MFS)	154700	AD	*FBN1*
Loeys-Dietz syndrome, type 1A (LDS1A)	609192	AD	*TGFBR1*
Loeys-Dietz syndrome, type 2B (LDS2B)	610380	AD	*TGFBR2*
Overlap connective tissue disease (OCTD)	604308	AD	*FBN1*
Pseudoxanthoma elasticum (PXE)	264800	AR	*ABCC6*
Cutis laxa autosomal dominant	123700	AD	*ELN, FBLN5*
Cutis laxa type I, autosomal recessive	219100	AR	*FBLN4, FBLN5*
Cutis laxa type IIA, autosomal recessive	219200	AR	*ATP6VOA2*
Wrinkly skin syndrome (WSS)	278250	AR	*ATP6VOA2*
Menkes syndrome (MNK)	309400	XLR	*ATP7A*
X-linked cutis laxa	304150	XLR	*ATP7A*
Williams syndrome (WS)	194050	AD	*Contiguous gene deletion at 7q11.23*
Mandibuloacral dysplasia with type A lipodystrophy (MADA)	248370	AR	*LMNA*
Mandibuloacral dysplasia with type B lipodystrophy (MADB)	608612	AR	*ZMPSTE24*
Osteogenesis imperfecta type I (OSI1)	166200	AD	*COL1A1, COL1A2*
Osteogenesis imperfecta type IIA (OSI2A)	166210	AD	*COL1A1, COL1A2*
Osteogenesis imperfecta type IIB (OSI2B)	610854	AR	*CRTAP*
Hypertrophic osteoarthropathy	259100	AR	*HPGD*
Arterial tortuosity syndrome (ATS)	208050	AR	*GLUT10 (SLC2A10)*
Osseous heteroplasia, progressive (POH)	166350	AD	*GNAS*
Shprintzen-Goldberg craniosynostosis syndrome (SGS)	182212	AD/isolated cases	*FBN1*
Mucolipidosis II	252500	AR	*GNPTAB*
Mucolipidosis III	252600	AR	*GNPTAB, GNPTG*
Weill-Marchesani syndrome, autosomal dominant (WMS)	608328	AD	*FBN1*
Weill-Marchesani syndrome, autosomal recessive	277600	AR	*ADAMTS10*
Johanson-Blizzard syndrome (JBS)	243800	AR	*UBR1*
Juvenile hyaline fibromatosis (JHF)	228600	AR	*CMG2 (ANTXR2)*
Systemic lupus erythematosus (SLE) (susceptibility to)	152700	Complex trait	*Multiple genes involved*
Torg-Winchester syndrome	259600	AR	*MMP2*
Melnick-Needles syndrome (MNS)	309350	XLD	*FLNA*
Gangliosidosis, generalized GM1, type 1	230500	AR	*GLB1*
Gangliosidosis, generalized GM1, type 2	230600	AR	*GLB1*

(continued)

Table 19.7 (continued)

Name	OMIM number	Inheritance	Gene
Buschke-Ollendorff syndrome (BOS)	166700	AD	*LEMD3 (MAN1)*
Lipoid Proteinosis	247100	AR	*ECM1*
Progeria syndrome, Hutchinson-Gilford (HGPS)	176670	AD/AR	*LMNA*
Focal dermal hypoplasia (FDH)	305600	XLD	*PORCN*
Congenital disorder of glycosylation type Ia (CDGIa)	212065	AR	*PMM2*

AD autosomal dominant, *AR* autosomal recessive, *XLD* X-linked dominant, *XLR* X-linked recessive

Systemic lupus erythematosus (SLE) is a complex trait disorder affecting a number of organs, including the skin. There are now a number of susceptibility loci associated with the development of SLE (see OMIM); although, definitive causative genes or genetic variants currently await elucidation and confirmation.

Pigmentation Disorders

A number of different genetic defects result in a range of pigmentary skin disorders, some of which are associated with an increased risk of skin cancer (Table 19.8). Inherited pigmentary skin disorders can be subdivided into groups according to the role of the defective gene within the pigmentary system. Disorders such as piebaldism and Waardenburg and Tietz syndromes are due to defects in melanoblast migration from the neural crest to the skin [62]. The characteristic patches of depigmentation of the skin (face, trunk, and extremities) and hair (observed as a white forelock) seen in the autosomal dominant disorder piebaldism, are due to a reduction of melanocytes, rather than loss of melanin pigmentation, at these body sites [63, 64].

On the other hand, the hypopigmentation of the skin, hair, and eyes observed in oculocutaneous albinism (OCA) is due to a reduction or complete lack of melanin biosynthesis by skin melanocytes. There are several types of OCA, and at least four genes are involved in this disorder. Although there is a spectrum of clinical phenotypes, with OCA1A being the most severe, there can be overlap between the subtypes of OCA [65]. Molecular diagnosis is therefore a useful way of confirming the clinical diagnosis of OCA.

Mutations in genes involved in the formation of melanosomes within melanocytes result in the autosomal recessive disorders, Hermansky-Pudlak syndrome and Chediak-Higashi syndrome. Mutations in genes involved in the final stage of the pigmentary pathway (i.e., the transfer of mature melanosomes via the melanocytic dendrites to adjacent keratinocytes) are associated with the rare recessive disorder, Griscelli syndrome, which is characterized by pigmentary dilution of the skin and hair [63, 64].

Vascular Disorders

Several genodermatoses have vascular manifestations (Table 19.9). These include hereditary hemorrhagic telangiectasia 1 and 2 (HHT1, HHT2), which are due to mutations in endoglin (*ENG*) and ALK1 (*ACVRL1*) genes, respectively [66]. Between 1 in 5,000 and 8,000 individuals are affected, and one of the earliest and most common signs of HHT is recurrent nosebleeds in young children [67, 68]. Telangiectasias (small blood vessels) of the skin and mucous membranes can develop early and increase with age. Later in life, individuals may develop pulmonary and hepatic arteriovenous malformations, telangiectasias in the gastrointestinal tract (small intestine), and neurologic problems

Table 19.8 Pigmentation disorders

Name	OMIM number	Inheritance	Gene
Carney complex type I (CNC1)	160980	AD	*PRKAR1A*
Carney complex variant	608837	AD	*MYH8*
Hemochromatosis (HFE)	235200	AR	*HFE*
Hemochromatosis type 2 (HFE2A and HFE2B)	602390	AR	*HJV, HAMP*
Hemochromatosis type 3 (HFE3)	604250	AR	*TFR2*
Hemochromatosis type 4 (HFE4)	606069	AD	*SLC40A1*
Chédiak-Higashi syndrome (CHS)	214500	AR	*LYST*
Griscelli type 1 (GS1)	214450	AR	*MYO5A*
Griscelli type 2 (GS2)	607624	AR	*RAB27A*
Griscelli type 3 (GS3)	609227	AR	*MLPH, MYO5A*
Hermansky-Pudlak type 1 (HPS1)	203300	AR	*HPS1*
Hermansky-Pudlak type 2 (HPS2)	608233	AR	*AP3B1*
Hermansky-Pudlak type 3 (HPS3)	203300	AR	*HPS3*
Hermansky-Pudlak type 4 (HPS4)	203300	AR	*HPS4*
Hermansky-Pudlak type 5 (HPS5)	203300	AR	*HPS5*
Hermansky-Pudlak type 6 (HPS6)	203300	AR	*HPS6*
Hermansky-Pudlak type 7 (HPS7)	203300	AR	*DTNBP1*
Hermansky-Pudlak type 8 (HPS8)	203300	AR	*BLOC1S3*
McCune-Albright syndrome (MAS)	174800	Somatic mosaicism	*GNAS1*
Neurofibromatosis type I (NF1)	162200	AD	*NF1*
Neurofibromatosis type II (NF2)	101000	AD	*NF2*
Neurofibromatosis, familial spinal (FSNF)	162210	AD	*NF1*
Neurofibromatosis-Noonan syndrome (NFNS)	601321	AD	*NF1*
Oculocutaneous albinism type 1A (OCA1A)	203100	AR	*TYR*
Oculocutaneous albinism type 1B (OCA1B)	606952	AR	*TYR*
Oculocutaneous albinism type 2 (OCA2)	203200	AR	*OCA2*
Oculocutaneous albinism type 3 (OCA3)	203290	AR	*TYRP1*
Oculocutaneous albinism type 4 (OCA4)	606574	AR	*MATP (SLC45A2)*
Tietz albinism–deafness syndrome	103500	AD	*MITF*
Peutz-Jeghers (PJS)	175200	AD	*STK11*
Piebaldism (PBT)	172800	AD	*KIT, SNA12 (SLUG)*
Waardenburg syndrome type I (WS1)	193500	AD	*PAX3*
Waardenburg syndrome type IIA (WS2A)	193510	AD	*MITF*
Waardenburg syndrome type IID (WS2D)	608890	AR/sporadic	*SNA12 (SLUG)*
Waardenburg syndrome type IIE (WS2E)	611584	AD	*SOX10*
Waardenburg syndrome type III (WS3)	148820	AD/AR/contiguous gene syndrome	*PAX3*
Waardenburg syndrome type IV (WS4)	277580	AR/AD	*SOX10, EDN3, EDNRB*
Prader-Willi syndrome (PWS)	176270	Isolated cases	*SNRPN, NECDIN, paternal deletions, maternal uniparental disomy, chr 15q11-q13*
Cystinosis, nephropathic (CTNS)	219800	AR	*CTNS*
Phenylketonuria (PKU)	261600	AR	*PAH*
Dermatopathia pigmentosa reticularis (DPR)	125595	AD	*KRT14*
Angelman syndrome (AS)	105830	Isolated cases	*UBE3A, MEPC2, maternal deletions, paternal uniparental disomy, chr 15q11.2-q13, imprinting defects*

(continued)

Table 19.8 (continued)

Name	OMIM number	Inheritance	Gene
Naegeli syndrome (NFJS)	161000	AD	*KRT14*
Dowling-Degos (DDD)	179850	AD	*KRT5*
Adrenal hypoplasia, congenital (AHC)	300200	XL/AR	*NROB1*
LEOPARD syndrome 1	151100	AD	*PTPN11*
Watson syndrome	193520	AD	*NF1*
Yemenite-deaf-blind-hypopigmentation syndrome	601706	AR	*SOX10*
Vitiligo, susceptibility to autoimmune disease (AIS1)	607836	AD	*FOXD3*
Albinism, black lock, cell migration disorder of the neurocytes of the gut and deafness (ABCD)	600501	AR	*EDNRB*
Acanthosis nigricans with Crouzon syndrome	612247	AD	*FGFR3*
Lipodystrophy, familial partial, type 3 (FPLD3)	604367	AD	*PPARG*
Achalasia-addisonianism-alacrima syndrome (AAA)	231550	AR	*AAAS*
Fanconi anemia, types A-G (FANCA-FANCG)	227650	AR	*FANCA-FANCG*

AD autosomal dominant, *AR* autosomal recessive, *XL* X-linked

Table 19.9 Disorders of vascularization

Name	OMIM number	Inheritance	Gene
Cerebral cavernous malformations 1 (CCM1)	116860	AD	*KRIT1*
Cerebral cavernous malformations 2 (CCM2)	603284	AD	*CCM2*
Cerebral cavernous malformations 3 (CCM3)	603285	AD	*PDCD10*
Lymphedema-distichiasis syndrome	153400	AD	*FOXC2*
Lymphedema hereditary, Ia (primary congenital lymphedema; PCL)	153100	AD	*FLT4*
Lymphedema, hereditary II	153200	AD	*FOXC2*
Ataxia-telangiectasia (AT)	208900	AR	*ATM*
Ataxia-telangiectasia-like disorder (ATLD)	604391	AR	*MRE11A*
Hereditary hemorrhagic telangiectasia (HHT1)	187300	AD	*ENG*
Hereditary hemorrhagic telangiectasia (HHT2)	600376	AD	*ACVRL1*
Hereditary hemorrhagic telangiectasia, juvenile polyposis syndrome (JPHT)	175050	AD	*SMAD4*
Hemangioma, capillary infantile (HCI)	602089	AD/Most sporadic	*TEM8 (ANTXR1), VEGFR2 (KDR)*
Glomuvenous malformations (glomangiomas)	138000	AD	*GLMN*
Venous malformations, multiple cutaneous and mucosal (VMCM)	600195	AD	*TIE2 (TEK)*
Microphthalmia with linear skin defects (MLS)	309801	XLD	*HCCS*
Stuve-Wiedemann syndrome (STWS)	601559	AR	*LIFR*
Smith-Lemli-Opitz syndrome (SLOS)	270400	AR	*DHCR7*
Rubinstein-Taybi syndrome (RSTS)	180849	AD	*CREBBP, EP300*
Mulibrey-Nanism	253250	AR	*TRIM37*
Noonan syndrome 1 (NS1)	163950	AD	*PTPN11*
Noonan syndrome 3 (NS3)	609942	AD	*KRAS2*
Noonan syndrome 4 (NS4)	610733	AD	*SOS1*
Congenital disorder of glycosylation type Ie (CDG1E)	608799	AR	*DPM1*
Congenital disorder of glycosylation type If (CDG1F)	609180	AR	*MPDU1*
Apert syndrome (acrocephalosyndactyly type I; ACS1)	101200	AD/most sporadic	*FGFR2*
SC phocomelia syndrome	269000	AR	*ESCO2*

AD autosomal dominant, *AR* autosomal recessive, *XLD* X-linked dominant

[66, 69]. Since this disorder has a number of severe symptoms (such as bleeding and anemia) patients should be regularly monitored. There are a number of HHT self-help websites that are based in different countries, as recently reviewed [66].

Disorders Associated with Malignancy

As listed in Table 19.10, many genodermatoses are associated with an increased risk of malignancy. The genetic defects of many of these diseases are now known. Gorlin syndrome (basal cell nevus syndrome), a rare autosomal dominant disorder, presents clinically with multiple basal cell carcinomas (BCC), hyperkeratosis of palms and soles, and a number of developmental defects [70]. In 1996, mutations were identified in the human ortholog of the Drosophila gene *patched* (*PTCH1*), a tumor suppressor gene on 9q22.32 [71, 72]. Like other tumor suppressor genes, *PTCH1* requires two mutagenic events for tumor formation, as per Knudson's two-hit model of cancer pathogenesis (i.e., a germline mutation is inherited on one allele and a somatic mutation occurs on the second allele) [73, 74]. *PTCH1* is involved in the Sonic hedgehog (SHH) signaling pathway and functions as the SHH receptor. Different types of mutations have now been identified. These include nonsense, frameshift, in-frame deletion, splice site, and missense mutations, in addition to large deletions of the chromosome 9q22 region resulting in haploinsufficiency of *PTCH1* [75]. In 2008, a mutation in patched 2 (*PTCH2*) was reported in a Chinese family with Gorlin syndrome [76].

Another disorder associated with malignancy is xeroderma pigmentosum (XP); an autosomal recessive disorder, characterized by skin photosensitivity and a predisposition to early-onset skin cancers (BCC, squamous cell carcinoma, and cutaneous malignant melanoma [CMM]). XP is a heterogeneous disorder, divided into a number of different subtypes, and due to mutations in at least seven genes involved in nucleotide excision repair or post-replication repair of DNA [77].

A number of susceptibility loci have now been mapped for different forms of familial CMM. In the 1990s, two forms (CMM2 and CMM3) were shown to be associated with mutations in *CDKN2A* and *CDK4*, respectively [78–80]. Both genes are rare high-penetrance CMM alleles, with *CDKN2A* being the highest risk locus identified to date [81, 82]. Several common low-penetrance CMM genes have also been found, including the *MC1R* gene which regulates skin hair/color (see Table 19.13). Specifically, nine MC1R variants associated with an increased risk of CMM have been described [83, 84].

Porphyrias

The porphyrias are a group of seven inherited metabolic disorders that are due to different enzyme deficiencies in the heme synthesis pathway [85]. Of the seven forms, five are inherited as autosomal dominant disorders and two as rare autosomal recessive traits (Table 19.11). Five forms have cutaneous involvement, resulting in skin hyperpigmentation, hypertrichosis, fragility, and photosensitivity leading to blistering, ulceration, and scarring [85, 86].

Disorders Associated with Immunodeficiency

There are several inherited immunodeficiency disorders that can also be classified as genodermatoses as a result of clinical involvement of skin (Table 19.12). The X-linked disorder Wiskott–Aldrich syndrome presents with a wide range of symptoms and signs, including

Table 19.10 Disorders with malignant potential

Name	OMIM number	Inheritance	Gene
Gorlin syndrome (basal cell nevus syndrome; BCNS)	109400	AD	*PTCH1, PTCH2, SUFU*
Xeroderma pigmentosum, group A (XPA)	278700	AR	*XPA*
Xeroderma pigmentosum, group B (XPB)	610651	AR	*ERCC3 (XPB)*
Xeroderma pigmentosum, group C (XPC)	278720	AR	*XPC*
Xeroderma pigmentosum, group D (XPD)	278730	AR	*ERCC2 (XPD)*
Xeroderma pigmentosum, group E (XPE)	278740	AR	*DDB2*
Xeroderma pigmentosum, group F (XPF)	278760	AR	*ERCC4*
Xeroderma pigmentosum, group G (XPG)	278780	AR	*ERCC5*
Xeroderma pigmentosum, variant (XPV)	278750	AR	*POLH*
Xeroderma pigmentosum (De Sanctis-Cacchione syndrome)	278800	AR	*ERCC6*
Cowden syndrome (CS)	158350	AD	*PTEN*
Bannayan-Zonana syndrome (BZS)	153480	AD	*PTEN*
Gardner syndrome (adenomatous polyposis of the colon; APC)	175100	AD	*APC*
Dyskeratosis congenita, X-linked	305000	XLR	*DKC1*
Dyskeratosis congenita, autosomal dominant	127550	AD	*TERC*
Tuberous sclerosis (TS)	191100, 613254	AD	*TSC1, TSC2*
Muir Torre syndrome (MTS)	158320	AD	*MSH2, MLH1*
Costello syndrome (faciocutaneoskeletal syndrome; FCS)	218040	AD	*HRAS*
Werner syndrome (WRN)	277700	AR	*RECQL2*
Cardiofaciocutaneous syndrome (CFC)	115150	AD/isolated cases	*KRAS, BRAF, MEK1, MEK2*
Bloom syndrome (BLM)	210900	AR	*RECQL3*
Androgen insensitivity syndrome (AIS)	300068	XLR	*AR*
Multiple endocrine neoplasia type 1 (MEN1)	131100	AD	*MEN1*
Multiple endocrine neoplasia type IIA (MEN2A)	171400	AD	*RET*
Multiple endocrine neoplasia type IIB (MEN2B)	162300	AD	*RET*
Multiple endocrine neoplasia type IV (MEN4)	610755	AD	*CDKN1B*
Pheochromocytoma	171300	AD	*VHL, RET, SDHD, SDHB, KIF1B, GDNF, TMEM127*
Von Hippel-Lindau syndrome (VHL)	193300	AD	*VHL*
Melanoma, cutaneous malignant, susceptibility to, 2 (CMM2)	155601	AD	*CDKN2A*
Melanoma, cutaneous malignant, susceptibility to, 3 (CMM3)	609048	AD/isolated cases	*CDK4*
Melanoma-astrocytoma syndrome	155755	AD	*CDKN2A*
Melanoma-pancreatic cancer syndrome	606719	AD	*CDKN2A*
Birt-Hogg-Dube syndrome (BHD)	135150	AD	*FLCN*
Beckwith-Wiedemann syndrome (BWS)	130650	AD/isolated cases	*NSD1, mutations or deletions of imprinted genes at 11p15, includes CDKN1C, (p57, KIP2), H19, LIT1*

(continued)

Table 19.10 (continued)

Name	OMIM number	Inheritance	Gene
Turcot syndrome (mismatch repair cancer syndrome)	276300	AD/AR	*MLH1, MSH2, MSH6, PMS2*
Trichoepithelioma (multiple familial 1, MFT1)	601606	AD	*CYLD*
Epidermodysplasia verruciformis (EV)	226400	AR	*EVER1, EVER2*
Neurilemmomatosis, congenital cutaneous (Schwannomatosis)	162091	AD	*SMARCB1, NF2, INI1*
Brooke-Spiegler syndrome (BSS)	605041	AD	*CYLD*
Cylindromatosis, familial	132700	AD	*CYLD*
Leiomyoma (hereditary multiple cutaneous; MCL)	150800	AD	*FH*
Leiomyomatosis and renal cell cancer (LRCC)	605839	AD	*FH*

AD autosomal dominant, *AR* autosomal recessive, *XLR* X-linked recessive

Table 19.11 Porphyrias with skin involvement

Name	OMIM number	Inheritance	Gene
Porphyria (congenital erythropoietic; CEP)	263700	AR	*UROS*
Porphyria cutanea tarda (PCT)	176100	AD	*UROD*
Hereditary coproporphyria (HCP)	121300	AD	*CPOX*
Porphyria variegata (VP)	176200	AD	*PPOX*
Protoporphyria, erythropoietic (EPP)	177000	AD/AR	*FECH*

AD autosomal dominant, *AR* autosomal recessive

Table 19.12 Disorders associated with immunodeficiency

Name	OMIM number	Inheritance	Gene
Wiskott-Aldrich syndrome (WAS)	301000	XLR	*WAS*
Omenn syndrome	603554	AR	*RAG1, RAG2, DCLRE1C*
Nijmegan breakage syndrome (NBS)	251260	AR	*NBS1*
LIG4 syndrome	606593	AR	*LIG4*
Immunodysregulation, polyendocrinopathy, and enteropathy (IPEX)	304790	XLR	*FOXP3*
Chronic granulomatous disease, X linked (CGD)	306400	XLR	*CYBB*
Chronic granulomatous disease, AR cytochrome b negative (CGD)	233690	AR	*CYBA*
Chronic granulomatous disease, AR cytochrome b positive type I (CGD)	233700	AR	*NCF1*
Chronic granulomatous disease, AR cytochrome b positive type II (CGD)	233710	AR	*NCF2*
Hyperimmunoglobulin E recurrent infection syndrome	147060	AD	*STAT3*
Bare lymphocyte syndrome type 1 (BLS)	604571	AR	*TAP1, TAP2, TAPBP*

AD autosomal dominant, *AR* autosomal recessive, *XLR* X-linked recessive

eczematous changes. Omenn syndrome (OS), previously classified as a rare form of severe combined immunodeficiency (SCID), demonstrates severe erythroderma and sometimes loss of eyebrows/eyelashes and scalp alopecia [87]. Recent studies suggest OS is a "leaky" or incomplete form of SCID and represents an inflammatory condition associated with a range of genetic abnormalities [88].

Table 19.13 Hair disorders

Name	OMIM number	Inheritance	Gene
Red hair color (RHC)	266300	AR	*MC1R*
Proopiomelanocortin deficiency (red hair color)	609734	AR	*POMC*
Trichothiodystrophy, nonphotosensitive 1 (TTDN1)	234050	AR	*TTDN1 (C7ORF11)*
Hypotrichosis, localized autosomal recessive (HTL)	607903	AR	*DSG4*
Hypotrichosis-lymphedema-telangiectasia syndrome (HLTS)	607823	AD/AR	*SOX18*
Hypotrichosis, Marie Unna type 1 (MUHH1)	146550	AD	*HR*
Hypotrichosis simplex of the scalp (HTSS)	146520	AD	*CDSN*
Alopecia universalis congenita (ALUNC)	203655	AR	*HR*
Atrichia with papular lesions (APL)	209500	AR	*HR*
Giant axonal neuropathy 1 (GAN1; includes curly/kinky hair)	256850	AR	*GAN*
Monilethrix	158000	AD	*KRT81, KRT83, KRT86*

AD autosomal dominant, *AR* autosomal recessive

Genetic Hair Disorders

The hair follicle is a major appendage of the epidermis. It is one of the most complex epithelial structures in nature, with only the feather having an additional level of complexity [89]. Hair follicles consist of several discrete concentric layers of epithelial cells with varying functions. This mini-organ is highly metabolically active and undergoes complex cycles of growth, regression, rest, and regrowth – the hair cycle. There is extensive body site variation in hair length, diameter, and hair cycle duration. The hair follicle is controlled by a complex network of signaling and developmental pathways. Therefore, there are many genes and gene networks by which genetic variation can lead to hair abnormalities. These include changes in pigmentation, loss of hair, and structural hair defects, in addition to other abnormalities, as listed in Table 19.13. Identification of the genes involved in hair disorders, like those involved in other skin diseases, has shed light on the biological mechanisms at play in this highly specialized system [90].

Miscellaneous Genodermatoses

Other genodermatoses that cannot easily be classified in the above groups are listed in Table 19.14.

Current State-of-the-Art: Genome Analysis in Complex Traits

Recent key technological advances in molecular biology and genomics have led to the development of protocols capable of systemically scanning the entire human genome for susceptibility loci involved in complex traits. In genodermatology, the major complex traits that have been subjected to this kind of analysis are atopic dermatitis [91] and psoriasis [92, 93], with studies planned for other common skin diseases.

The completion of the Human Genome Project in 2003 was the first and major enabling event in complex trait analysis. Thereafter, resequencing of the genomes of different individuals led to the discovery of many millions of SNPs, which were found to occur at a very high density across the genome. Methods were then developed to allow the simultaneous analysis of large numbers of these

Table 19.14 Miscellaneous genodermatoses

Name	OMIM number	Inheritance	Gene
Rapadilono syndrome	266280	AR	*RECQL4*
Popliteal pterygium syndrome (PPS)	119500	AD	*IRF6*
Propionic acidemia	606054	AR	*PCCA, PCCB*
Pseudohypoparathyroidism, type 1A (PHP Ia)	103580	AD	*GNAS*
Glucocorticoid deficiency 1 (GCCD1)	202200	AR	*MC2R (ACTHR)*
Vitamin D-dependent rickets, type II (VDDR II)	277440	AR	*VDR*
Vitamin E, familial isolated deficiency of (VED)	277460	AR	*TTPA*
Vasculopathy, retinal, with cerebral leukodystrophy	192315	AD	*TREX1*
Simpson-Golabi-Behmel syndrome, type 1 (SGBS1)	312870	XLR	*GPC3*
Simpson-Golabi-Behmel syndrome, type 2 (SGBS2)	300209	XLR	*CXORF5*
Sitosterolemia	210250	AR	*ABCG8, ABCG5*
Blau syndrome (synovitis, granulomatous, with uveitis and cranial neuropathy)	186580	AD	*NOD2 (CARD15)*
Pineal hyperplasia, insulin-resistant diabetes mellitus and somatic abnormalities	262190	AR	*INSR*
Warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM)	193670	AD	*CXCR4*
Glucocorticoid deficiency 2 (GCCD2)	607398	AR	*MRAP*
Carboxypeptidase N deficiency	212070	AR	*CPN1*
Weaver (WSS)	277590	AD	*NSD1*
Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)	240300	AR	*AIRE*
Adrenoleukodystrophy (ALD)	300100	XLR	*ABCD1*
Hyperphosphatemic familial tumoral calcinosis (HFTC)	211900	AR	*GALNT3, FGF23, KLOTHO*
Pyogenic sterile arthritis, pyoderma gangrenosum and acne (PAPAS)	604416	AD	*PSTPIP1*
Niemann-Pick disease, type A	257200	AR	*SMPD1*
Neuropathy, hereditary sensory and autonomic, type IIA (HSAN2A)	201300	AR	*WNK1 (HSN2)*
Neuropathy, hereditary sensory and autonomic, type III (HSAN3)	223900	AR	*IKBKAP*
Acromesomelic dysplasia, Maroteaux type (AMDM)	602875	AR	*NPR2*
White sponge nevus of Cannon (WSN)	193900	AD	*KRT4, KRT13*
Wolf-Hirschhorn syndrome (WHS)	194190	Isolated cases	*Partial deletion of short arm of chromosome 4 (4p-)*
Aicardi-Goutieres syndrome 1 (AGS1)	225750	AR	*TREX1*
Angioedema, hereditary (HAE I & II)	106100	AD	*C1NH*
Angioedema, hereditary (HAE III)	610618	AD	*F12*
Congenital insensitivity to pain and anhidrosis (CIPA)	256800	AR	*NTRK1*
Aspartylglucosaminuria (AGU)	208400	AR	*AGA*
Autoimmune lymphoproliferative syndrome types IA and 1B (ALPS)	601859	AD	*FAS, FASL*
Autoimmune lymphoproliferative syndrome type IIA (ALPS2A)	603909	AD	*CASP10*
Cartilage-hair hypoplasia (CHH)	250250	AR	*RMRP*
Mucopolysaccharidosis type IIIA (MPS3A)	252900	AR	*SGSH*

(continued)

Table 19.14 (continued)

Name	OMIM number	Inheritance	Gene
Mucopolysaccharidosis type IIIB (MPS3B)	252920	AR	*NAGLU*
Mucopolysaccharidosis type IIIC (MPS3C)	252930	AR	*HGSNAT*
Multiple sulfatase deficiency (MSD)	272200	AR	*SUMF1*
Myotonic dystrophy 1 (DM1)	160900	AD	*DMPK*
Myotonic dystrophy 2 (DM2)	602668	AD	*ZNF9*
Mannosidosis, alpha B lysosomal	248500	AR	*MAN2B1*
Microcephaly, microphthalmia, ectrodactyly of lower limbs and prognathism (MMEP)	601349	AD/translocation	*SNX3*
Mucolipidosis II (ML II)	252500	AR	*GNPTAB*
Mucolipidosis III complementation group C (ML IIIC)	252605	AR	*GNPTG*
Lipodystrophy, congenital generalized type 1 (CGL1)	608594	AR	*AGPAT2*
Lipodystrophy, congenital generalized type 2 (CGL2)	269700	AR	*BSCL2*
Lipodystrophy, familial partial, type 2 (FPLD2)	151660	AD	*LMNA*
Lipodystrophy, familial partial, type 3 (FPLD3)	604367	AD	*PPARG*
Lowe oculocerebrorenal syndrome (OCRL)	309000	XLR	*OCRL1*
Kanzaki disease (alpha-N-acetylgalactosaminidase deficiency, type II)	609242	AR	*NAGA*
Donohue syndrome (leprechaunism)	246200	AR	*INSR*
Lesch-Nyhan syndrome (LNS)	300322	XLR	*HPRT*
Infantile sialic acid storage disorder (ISSD)	269920	AR	*SLC17A5*
Immunoosseous dysplasia, Schimke type (SIOD)	242900	AR	*SMARCAL1*
Holocarboxylase synthetase deficiency (HLCS)	253270	AR	*HLCS*
Homocystinuria	236200	AR	*CBS*
Hypercholesterolemia, autosomal dominant (FHC)	143890	AD	*LDLR*
Hypercholesterolemia, autosomal dominant type B	144010	AD	*APOB*
Hypercholesterolemia, autosomal recessive (ARH)	603813	AR	*ARH*
Hyperoxaluria, primary, type I (HP1)	259900	AR	*AGXT*
Hyperlipoproteinemia, type I	238600	AR	*LPL*
Hyperlipoproteinemia, type IB (apolipoprotein C-II deficiency)	207750	AR	*APOC2*
Hypertriglyceridemia, familial	145750	AD	*APOA5, LIPI, Polymorphism in RP1*
Hyper-IgD syndrome (HIDS)	260920	AR	*MVK*
Glycogen storage disease 1 (GSD1)	232200	AR	*G6PC*
Glycogen storage disease 1b (GSD1b)	232220	AR	*G6PT1*
Glycogen storage disease 1c (GSD1c)	232240	AR	*G6PT1*
Alport syndrome, X-linked (ATS)	301050	XL	*COL4A5*
Alport syndrome, recessive	203780	AR	*COL4A3, COL4A4*
Alstrom syndrome (ALMS)	203800	AR	*ALMS1*
Neuropathy with pretibial epidermolysis bullosa and deafness	609057	AR	*CD151*
Familial cold autoinflammatory syndrome 1 (FCAS1)	120100	AD	*CIAS1 (NLRP3)*
Familial Mediterranean fever (FMF)	249100	AR	*MEFV*
Faber lipogranulomatosis	228000	AR	*ASAH1*
Fucosidosis	230000	AR	*FUCA1*
Fumarase deficiency	606812	AR	*FH*
Craniofrontonasal syndrome	304110	XLD	*EFNB1*

(continued)

Table 19.14 (continued)

Name	OMIM number	Inheritance	Gene
Cutis gyrata syndrome of Beare and Stevenson	123790	AD	*FGFR2*
Cinca syndrome (chronic neurologic cutaneous and articular syndrome)	607115	AD	*CIAS1*
Cockayne syndrome type A (CSA)	216400	AR	*ERCC8*
Cockayne syndrome type B (CSB)	133540	AR	*ERCC6*
Coffin-Lowry syndrome (CLS)	303600	XLD	*RSK2*
Cold-induced sweating syndrome 1 (CISS1)	272430	AR	*CRLF1*
Familial cold urticaria (FCU)	120100	AD	*CIAS1*
Cornelia de Lange syndrome 1 (CDLS1)	122470	AD/isolated cases	*NIPBL*
Cerebrotendinous xanthomatosis (CTX)	213700	AR	*CYP27A1*
Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy (CADSIL)	125310	AD	*NOTCH3*
Argininosuccinic aciduria	207900	AR	*ASL*
Porokeratosis, disseminated superficial actinic 1 (DSAP1)	175900	AD	*SART3*
Rothmund-Thomson syndrome (RTS)	268400	AR	*RECQL4*
UV-sensitive syndrome (UVS)	600630	AR	*ERCC6*
Hartnup disorder (HND)	234500	AR	*SLC6A19*
Sarcoidosis, early onset (EOS)	609464	AD	*NOD2 (CARD15)*
Seborrhea-like dermatitis with psoriasiform elements	610227	AD	*ZNF750*
Periodic fever, familial (FPF)	142680	AD	*TNFRSF1A*
Amyloidosis, familial visceral	105200	AD	*APOA1, FGA, LYZ*
Amyloidosis V, Finnish type	105120	AD	*GSN*
Biotinidase deficiency	253260	AR	*BTD*

AD autosomal dominant, *AR* autosomal recessive, *XLR* X-linked recessive, *XLD* X-linked recessive, *XL* X-linked

SNPs, predominantly based on array technology – essentially silicon chips consisting of millions of defined DNA probes to which individual DNA samples can be hybridized and analyzed. Currently, it is possible to analyze about two million SNPs in an individual, for a cost of approximately $200–300. Another development was the HapMap – a map of the haplotype structure of the human genome – in other words, identification of blocks of chromosomes that tend to be co-inherited due to the presence of recombination hotspots in the genome.

Genome-wide association studies (GWAS) are the current method of choice to tackle the genetics of complex traits. In GWAS, a case-control study design is used, which ideally consists of 2,000 cases of the disease of interest and 3,000 or more ethnically-matched population controls. The controls are not necessarily screened for the disease phenotype and represent the general population rather than a normal control group. Ethnicity matching is important as it has emerged that the causative defects underlying a complex disease can be highly race-specific, and therefore, mixing ethnicity in a discovery cohort can lead to serious losses of statistical power required to detect a disease association. These 5,000 or so DNA samples are genotyped for typically two million SNPs using an SNP chip or similar method, thereby generating ten billion data points. For each SNP, a statistical test is performed to compare its frequency in the disease cases versus the controls, and a *P* value is generated (i.e., two million chi-square type tests are done). The log of the *P* values is mapped along the chromosome locations of the SNPs to give a "Manhattan" plot, as shown in Fig. 19.4. Where a disease susceptibility locus exists, clusters of strongly associated SNPs are seen. For most statistical analyses in biological research, a *P* value of 0.05 or less is regarded by convention as significant;

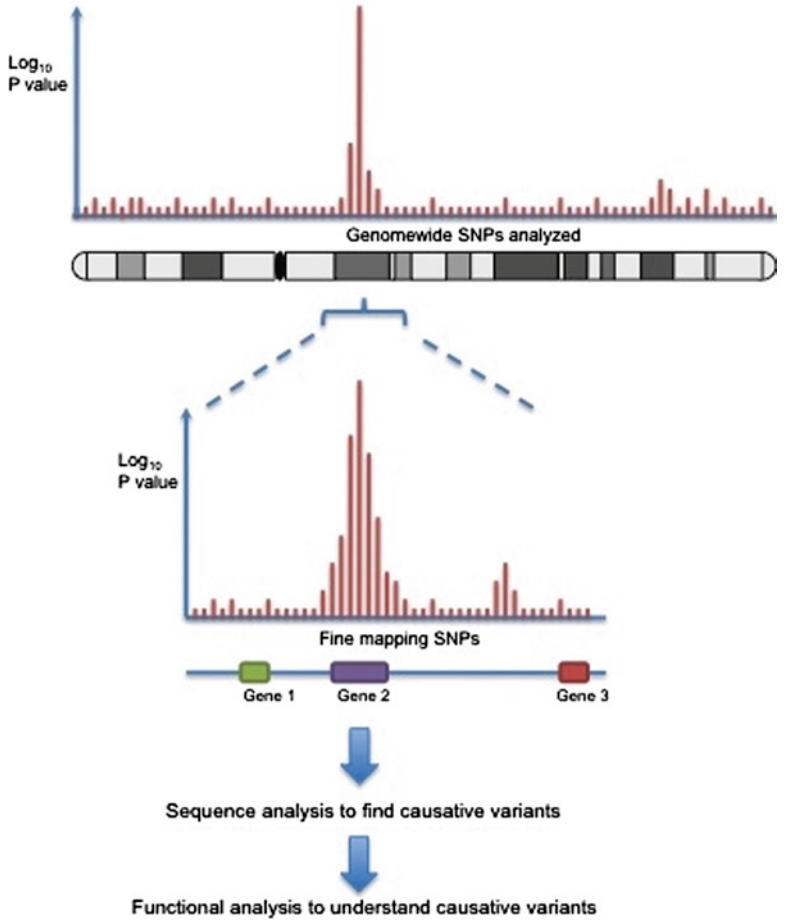

Fig. 19.4 Genome-wide association studies (*GWAS*) in complex trait genetics. It is now possible to genotype individuals for up to two million single nucleotide polymorphisms (*SNPs*) in a single experiment, using gene chip technology. A typical GWAS study would involve 2,000 cases of a complex trait, such as atopic dermatitis, plus 3,000 ethnically-matched controls. All these 5,000 people are genotyped for the two million SNPs. For each SNP, a chi-square-related statistical test is performed to generate a *P* value (i.e., two million tests are performed). This asks the question "is this SNP significantly enriched in the patient group compared to the general population?" for each of the two million SNPs scattered across the genome. If a SNP is linked to a disease susceptibility locus, then that SNP will show significant enrichment in the patient population. The closer the SNP, the better the *P* value will become. This data is plotted as the Log of the *P* value against the chromosome position of the SNP, known as a Manhattan plot, as illustrated (*top*). Peaks of association identified are then subjected to higher density SNP genotyping in order to sublocalize the signal to a smaller region, usually about the size of a single gene or smaller. Sequencing of this region is required to identify the causative variant or variants tagged by the most significantly associated SNPs. These causative variants may require considerable functional analysis to formally identify how they lead to disease susceptibility. As of 2009, most complex traits which have been investigated by GWAS are still at the sequencing/functionality stage of analysis

however, in GWAS, susceptibility loci more typically show *P* values of 10^{-10} or less. Loci detected must be replicated as widely as possible in different study populations, and then the loci are finely mapped using a higher density of SNPs around the initial locus, in order to sublocalize the signal. Once this analysis is complete, the locus is usually limited to a single gene or a comparable small interval. Sequence analysis of this small region is performed in disease cases versus controls, ultimately leading to the discovery of the causative genetic variant or variants at this locus that promote

disease susceptibility. Complete understanding of how these DNA sequence changes lead to disease predisposition may require considerable functional analysis, involving cell biology, biochemistry, and transgenic models. This is particularly true when the locus falls outside of the coding region of a known gene, which, so far in the developing field of complex trait genetics, is a common occurrence. In other words, the causative variants are often difficult to identify and understand, and quite often involve subtle perturbation of gene regulation, rather than an overt or very obvious genetic defect within the protein-encoding regions of a gene.

A very informative example of a complex trait is the recently discovered role of the *FLG* gene in atopic dermatitis. As discussed previously, we have determined that common loss-of-function mutations (all nonsense or frameshift mutations) in this gene are the cause of ichthyosis vulgaris [19]. We were also prompted to investigate the role of the *FLG* gene and its mutations in the pathogenesis of atopic dermatitis due to: (a) the high population frequency of its mutations (carried by up to 10% of some populations); (b) the fact that many ichthyosis vulgaris patients also have atopic dermatitis; and (c) the early mapping of atopic dermatitis susceptibility to the vicinity of the *FLG* gene [23]. By case-control analysis in a very small study of 52 atopic dermatitis cases versus 189 population controls, we were able to obtain a *P* value of 10^{-17}. *FLG* has emerged as the strongest predisposing gene for atopic dermatitis, having been universally and strongly replicated in every population where the causative variants are found [94]. Profilaggrin is highly abundant in the granular layer of the epidermis, where it contributes to the biogenesis of the stratum corneum, the subsequent hydration of the squames, and perhaps other functions [94]. In 2009, we showed that filaggrin-deficient mice have an inherent skin barrier deficiency, as insufficient expression of the *FLG* gene in the skin leads to percutaneous antigen priming, which in turn drives an allergic immune response [95]. Although we identified this gene by essentially a candidate gene approach, a recent GWAS study confirmed that this is indeed a major eczema gene [91]; although, there is another locus of unknown function on chromosome 11, and other loci are likely to be found in future rounds of GWAS. The other complex trait in dermatology that has been successfully subjected to GWAS is psoriasis [92, 93], with several highly significant loci emerging, including possible causative variants in some cases [96].

Future Perspectives

The coming years promise to bring forth further tremendous increases in our understanding of the genodermatoses. Next generation sequencing (NGS) is now being applied to the study of skin diseases. This new technology allows rapid analysis of hundreds of genes in a single experiment; for example, it was recently used to identify a gene for cutis laxa, where more than 80 genes were sequenced simultaneously [97]. This type of technology has the potential to revolutionize genetic testing. For example, one can envision the use of NGS to sequence all the exons of all the EB genes in a single run, rather than performing PCR and sequencing for each of the hundreds of individual exons.

So-called future generation sequencing (FGS) is also under development, and is expected to be in common use within the next few years. The aim is to sequence essentially the entire genome in one run, for a very low cost, perhaps as little as $100. This would further expand genetic testing, including the study of complex traits. For example, it may be less expensive to sequence the genomes of a case-control collection than to genotype high-density SNPs, with the added advantage of being able to identify the causative variants at the same time. Similarly, it would be feasible to sequence the whole genome in a patient with a skin disorder and evaluate only the relevant genes, rather than use currently available technologies to look at one gene, or even NGS to assess a subset of genes.

Of course, these technological advances raise new ethical issues that must be carefully considered. For example, a patient might present with a genodermatosis and have their whole genome sequenced to determine the causative gene and confirm the diagnosis. Elsewhere in their individual genome sequence data, there might be a variant predisposing to a late-onset disease of another organ system (i.e., colon cancer or type II diabetes mellitus). Should they also be counseled for this genetic defect? These issues will require close collaboration and dialogue between clinical specialists, geneticists, and ethicists in order to responsibly apply these rapidly evolving technologies in the clinical setting.

In conclusion, our understanding of the pathogenesis of genodermatoses has evolved enormously since the advent of molecular genetics in the late 1980s and early 1990s. This knowledge base will undoubtedly increase exponentially in the upcoming years, and with this understanding will hopefully come improvements in diagnosis, genetic counseling, patient management, treatment, and perhaps a cure for some of these devastating illnesses.

References

1. Moss C, Jones DO, Blight A, et al. Birthmark due to cutaneous mosaicism for keratin 10 mutation. Lancet. 1996;345:596.
2. Paller AS, Syder AJ, Chan YM, et al. Genetic and clinical mosaicism in a type of epidermal nevus. New Engl J Med. 1994;331:1408–15.
3. Rouan F, Pulkkinen L, Meneguzzi G, et al. Epidermolysis bullosa: novel and de novo premature termination codon and deletion mutations in the plectin gene predict late-onset muscular dystrophy. J Invest Dermatol. 2000;114:381–7.
4. Bonifas JM, Morley BJ, Oakey RE, et al. Cloning of a cDNA for steroid sulfatase: frequent occurrence of gene deletions in patients with recessive X chromosome-linked ichthyosis. Proc Natl Acad Sci USA. 1987;84:9248–51.
5. Ehrenreich M, Tarlow MM, Godlewska-Janusz E, et al. Incontinentia pigmenti (Bloch-Sulzberger syndrome): a systemic disorder. Cutis. 2007;79:355–62.
6. Nakayama T, Sakakihara Y, Hanaoka S, et al. Calcification of basal ganglia in a patient with partial trisomy 5q and partial monosomy 18q. Acta Paediatr Jpn. 1993;35:340–4.
7. Takizawa Y, Pulkkinen L, Chao SC, et al. Mutation report: complete paternal uniparental isodisomy of chromosome 1: a novel mechanism for Herlitz junctional epidermolysis bullosa. J Invest Dermatol. 2000;115:307–11.
8. Liao H, Waters AJ, Goudie DR, et al. Filaggrin mutations are genetic modifying factors exacerbating X-linked ichthyosis. J Invest Dermatol. 2007;127:2795–8.
9. Titeux M, Pendaries V, Tonasso L, et al. A frequent functional SNP in the MMP1 promoter is associated with higher disease severity in recessive dystrophic epidermolysis bullosa. Hum Mutat. 2008;29:267–76.
10. Irvine AD, McLean WHI. Human keratin diseases: the increasing spectrum of disease and subtlety of the phenotype-genotype correlation. Br J Dermatol. 1999;140:815–28.
11. Gratchev A. The nucleotide excision repair of DNA in human cells and its association with xeroderma pigmentosum. Adv Exp Med Biol. 2008;637:113–9.
12. Lai Cheong JE, Wessagowit V, McGrath JA. Molecular abnormalities of the desmosomal protein desmoplakin in human disease. Clin Exp Dermatol. 2005;30:261–6.
13. Leech SN, Moss C. A current and online genodermatosis database. Br J Dermatol. 2007;156:1115–48.
14. Szeverenyi I, Cassidy AJ, Chung CW, et al. The human intermediate filament database: comprehensive information on a gene family involved in many human diseases. Hum Mutat. 2008;29:351–60.
15. Smith FJD, Liao H, Cassidy AJ, et al. The genetic basis of pachyonychia congenita. J Investig Dermatol Symp Proc. 2005;10:21–30.
16. Siegel DH, Ashton GH, Penagos HG, et al. Loss of kindlin-1, a human homolog of the Caenorhabditis elegans actin-extracellular-matrix linker protein UNC-112, causes Kindler syndrome. Am J Hum Genet. 2003;73:174–87.
17. McLean WH, Irvine AD, Hamill KJ, et al. An unusual N-terminal deletion of the laminin alpha3a isoform leads to the chronic granulation tissue disorder laryngo-onycho-cutaneous syndrome. Hum Mol Genet. 2003;12:2395–409.
18. Irvine AD, McLean WHI. The molecular genetics of the genodermatoses: progress to date and future directions. Br J Dermatol. 2003;148:1–13.

19. Smith FJD, Irvine AD, Terron-Kwiatkowski A, et al. Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. Nat Genet. 2006;38:337–42.
20. Sandilands A, Terron-Kwiatkowski A, Hull PR, et al. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat Genet. 2007;39:650–4.
21. Chen H, Ho JC, Sandilands A, et al. Unique and recurrent mutations in the filaggrin gene in Singaporean Chinese patients with ichthyosis vulgaris. J Invest Dermatol. 2008;128:1669–75.
22. Nomura T, Sandilands A, Akiyama M, et al. Unique mutations in the filaggrin gene in Japanese patients with ichthyosis vulgaris and atopic dermatitis. J Allergy Clin Immunol. 2007;119:434–40.
23. Palmer CNA, Irvine AD, Terron-Kwiatkowski A, et al. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 2006;38:441–6.
24. Omary MB, Coulombe PA, McLean WHI. Intermediate filament proteins and their associated diseases. N Engl J Med. 2004;351:2087–100.
25. Lane EB. Keratins. In: Royce PM, Steinmann B, editors. Connective tissue and its heritable disorders. Molecular, genetic and medical aspects. New York: Wiley-Liss; 1993. p. 237–47.
26. Fuchs E, Cleveland DW. A structural scaffolding of intermediate filaments in health and disease. Science. 1998;279:514–9.
27. Leachman SA, Kaspar RL, Fleckman P, et al. Clinical and pathological features of pachyonychia congenita. J Investig Dermatol Symp Proc. 2005;10:3–17.
28. Bowden PE, Haley JL, Kansky A, et al. Mutation of a type II keratin gene (K6a) in pachyonychia congenita. Nat Genet. 1995;10:363–5.
29. McLean WH, Rugg EL, Lunny DP, et al. Keratin 16 and keratin 17 mutations cause pachyonychia congenita. Nat Genet. 1995;9:273–8.
30. Smith FJD, Jonkman MF, van Goor H, et al. A mutation in human keratin K6b produces a phenocopy of the K17 disorder pachyonychia congenita type 2. Hum Mol Genet. 1998;7:1143–8.
31. Liao H, Sayers JM, Wilson NJ, et al. A spectrum of mutations in keratins K6a, K16 and K17 causing pachyonychia congenita. J Dermatol Sci. 2007;48:199–205.
32. Wilson NJ, Messenger AG, Leachman SA, et al. Keratin K6c mutations cause focal palmoplantar keratoderma. J Invest Dermatol. 2010;130:425–9.
33. Reis A, Hennies H-C, Langbein L, et al. Keratin 9 gene mutations in epidermolytic palmoplantar keratoderma (EPPK). Nat Genet. 1994;6:174–9.
34. Terron-Kwiatkowski A, Paller AS, Compton J, et al. Two cases of primarily palmoplantar keratoderma associated with novel mutations in keratin 1. J Invest Dermatol. 2002;119:966–71.
35. Lai-Cheong JE, Arita K, McGrath JA. Genetic diseases of junctions. J Invest Dermatol. 2007;127:2713–25.
36. Armstrong DK, McKenna KE, Purkis PE, et al. Haploinsufficiency of desmoplakin causes a striate subtype of palmoplantar keratoderma. Hum Mol Genet. 1999;8:143–8.
37. Whittock NV, Ashton GH, Dopping-Hepenstal PJ, et al. Striate palmoplantar keratoderma resulting from desmoplakin haploinsufficiency. J Invest Dermatol. 1999;113:940–6.
38. Norgett EE, Lucke TW, Bowers B, Munro CS, et al. Early death from cardiomyopathy in a family with autosomal dominant striate palmoplantar keratoderma and woolly hair associated with a novel insertion mutation in desmoplakin. J Invest Dermatol. 2006;126:1651–4.
39. Bolling MC, Jonkman MF. Skin and heart: une liaison dangereuse. Exp Dermatol. 2009;18:658–68.
40. Alcalai R, Metzger S, Rosenheck S, et al. A recessive mutation in desmoplakin causes arrhythmogenic right ventricular dysplasia, skin disorder, and woolly hair. J Am Coll Cardiol. 2003;42:319–27.
41. Norgett EE, Hatsell SJ, Carvajal-Huerta L, et al. Recessive mutation in desmoplakin disrupts desmoplakin-intermediate filament interactions and causes dilated cardiomyopathy, woolly hair and keratoderma. Hum Mol Genet. 2000;9:2761–6.
42. Tanaka A, Lai-Cheong JE, Cafe ME, et al. Novel truncating mutations in PKP1 and DSP cause similar skin phenotypes in two Brazilian families. Br J Dermatol. 2009;160:692–7.
43. Uzumcu A, Norgett EE, Dindar A, et al. Loss of desmoplakin isoform I causes early onset cardiomyopathy and heart failure in a Naxos-like syndrome. J Med Genet. 2006;43:e5.
44. Whittock NV, Wan H, Morley SM. Compound heterozygosity for non-sense and mis-sense mutations in desmoplakin underlies skin fragility/woolly hair syndrome. J Invest Dermatol. 2002;118:232–8.
45. Jonkman MF, Pasmooij AM, Pasmans SG, et al. Loss of desmoplakin tail causes lethal acantholytic epidermolysis bullosa. Am J Hum Genet. 2005;77:653–60.
46. Asimaki A, Syrris P, Ward D, et al. Unique epidermolytic bullous dermatosis with associated lethal cardiomyopathy related to novel desmoplakin mutations. J Cutan Pathol. 2009;36:553–9.
47. Fine JD, Eady RA, Bauer EA, et al. The classification of inherited epidermolysis bullosa (EB): Report of the Third International Consensus Meeting on Diagnosis and Classification of EB. J Am Acad Dermatol. 2008;58:931–50.
48. Rugg EL, McLean WHI, Lane EB, et al. A functional “knock-out” for human keratin 14. Genes Dev. 1994;8:2563–73.

49. McLean WHI, Pulkkinen L, Smith FJD, et al. Loss of plectin causes epidermolysis-bullosa with muscular-dystrophy – cDNA cloning and genomic organization. Genes Dev. 1996;10:1724–35.
50. Smith FJ, Eady RA, Leigh IM, et al. Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. Nat Genet. 1996;13:450–7.
51. Koss-Harnes D, Jahnsen FL, Wiche G, et al. Plectin abnormality in epidermolysis bullosa simplex Ogna: non-responsiveness of basal keratinocytes to some anti-rat plectin antibodies. Exp Dermatol. 1997;6:41–8.
52. Pfendner E, Uitto J. Plectin gene mutations can cause epidermolysis bullosa with pyloric atresia. J Invest Dermatol. 2005;124:111–5.
53. Varki R, Sadowski S, Uitto J, et al. Epidermolysis bullosa. II. Type VII collagen mutations and phenotype-genotype correlations in the dystrophic subtypes. J Med Genet. 2007;44:181–92.
54. Jobard F, Bouadjar B, Caux F, et al. Identification of mutations in a new gene encoding a FERM family protein with a pleckstrin homology domain in Kindler syndrome. Hum Mol Genet. 2003;12:925–35.
55. Cassidy AJ, van Steensel MAM, Steijlen PM, et al. A homozygous missense mutation in TGM5 abolishes epidermal transglutaminase 5 activity and causes acral peeling skin syndrome. Am J Hum Genet. 2005;77:909–17.
56. Priolo M. Ectodermal dysplasias: an overview and update of clinical and molecular-functional mechanisms. Am J Med Genet A. 2009;149A:2003–13.
57. Itin PH, Fistarol SK. Ectodermal dysplasias. Am J Med Genet C Semin Med Genet. 2004;131C:45–51.
58. Lamartine J. Towards a new classification of ectodermal dysplasias. Clin Exp Dermatol. 2003;28:351–5.
59. Pinheiro M, Freire-Maia N. Ectodermal dysplasias: a clinical classification and a causal review. Am J Med Genet. 1994;53:153–62.
60. Ringpfeil F, McGuigan K, Fuchsel L, et al. Pseudoxanthoma elasticum is a recessive disease characterized by compound heterozygosity. J Invest Dermatol. 2006;126:782–6.
61. Matsuzaki Y, Nakano A, Jiang QJ, et al. Tissue-specific expression of the ABCC6 gene. J Invest Dermatol. 2005;125:900–5.
62. Thomas I, Kihiczak GG, Fox MD, et al. Piebaldism: an update. Int J Dermatol. 2004;43:716–9.
63. Dessinioti C, Stratigos AJ, Rigopoulos D, et al. A review of genetic disorders of hypopigmentation: lessons learned from the biology of melanocytes. Exp Dermatol. 2009;18:741–9.
64. Tomita Y, Suzuki T. Genetics of pigmentary disorders. Am J Med Genet C Semin Med Genet. 2004;131C:75–81.
65. Gronskov K, Ek J, Brondum-Nielsen K. Oculocutaneous albinism. Orphanet J Rare Dis. 2007;2:43.
66. Govani FS, Shovlin CL. Hereditary haemorrhagic telangiectasia: a clinical and scientific review. Eur J Hum Genet. 2009;17:860–71.
67. Bideau A, Brunet G, Heyer E, et al. An abnormal concentration of cases of Rendu-Osler disease in the Valserine valley of the French Jura: a genealogical and demographic study. Ann Hum Biol. 1992;19:233–47.
68. Kjeldsen AD, Vase P, Green A. Hereditary haemorrhagic telangiectasia: a population-based study of prevalence and mortality in Danish patients. J Intern Med. 1999;245:31–9.
69. Grand'Maison A. Hereditary hemorrhagic telangiectasia. CMAJ. 2009;180:833–5.
70. Lo Muzio L. Nevoid basal cell carcinoma syndrome (Gorlin syndrome). Orphanet J Rare Dis. 2008;3:32.
71. Johnson RL, Rothman AL, Xie J, et al. Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science. 1996;272:1668–71.
72. Hahn H, Wicking C, Zaphiropoulous PG, et al. Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell. 1996;85:841–51.
73. Aszterbaum M, Rothman A, Johnson RL, et al. Identification of mutations in the human PATCHED gene in sporadic basal cell carcinomas and in patients with the basal cell nevus syndrome. J Invest Dermatol. 1998;110:885–8.
74. Knudson Jr AG. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci USA. 1971;68:820–3.
75. Yamamoto K, Yoshihashi H, Furuya N, et al. Further delineation of 9q22 deletion syndrome associated with basal cell nevus (Gorlin) syndrome: report of two cases and review of the literature. Congenit Anom (Kyoto). 2009;49:8–14.
76. Fan Z, Li J, Du J, et al. A missense mutation in PTCH2 underlies dominantly inherited NBCCS in a Chinese family. J Med Genet. 2008;45:303–8.
77. McCabe KM, Olson SB, Moses RE. DNA interstrand crosslink repair in mammalian cells. J Cell Physiol. 2009;220:569–73.
78. Hussussian CJ, Struewing JP, Goldstein AM, et al. Germline p16 mutations in familial melanoma. Nat Genet. 1994;8:15–21.
79. Kamb A, Shattuck-Eidens D, Eeles R, et al. Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. Nat Genet. 1994;8:23–6.
80. Zuo L, Weger J, Yang Q, et al. Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. Nat Genet. 1996;12:97–9.

81. Meyle KD, Guldberg P. Genetic risk factors for melanoma. Hum Genet. 2009;126:499–510.
82. Nelson AA, Tsao H. Melanoma and genetics. Clin Dermatol. 2009;27:46–52.
83. Gudbjartsson DF, Sulem P, Stacey SN, et al. ASIP and TYR pigmentation variants associate with cutaneous melanoma and basal cell carcinoma. Nat Genet. 2008;40:886–91.
84. Raimondi S, Sera F, Gandini S, et al. MC1R variants, melanoma and red hair color phenotype: a meta-analysis. Int J Cancer. 2008;122:2753–60.
85. Badminton MN, Elder GH. Molecular mechanisms of dominant expression in porphyria. J Inherit Metab Dis. 2005;28:277–86.
86. Schneider-Yin X, Harms J, Minder EI. Porphyria in Switzerland, 15 years experience. Swiss Med Wkly. 2009;139:198–206.
87. Kato M, Kimura H, Seki M, et al. Omenn syndrome–review of several phenotypes of Omenn syndrome and RAG1/RAG2 mutations in Japan. Allergol Int. 2006;55:115–9.
88. Villa A, Notarangelo LD, Roifman CM. Omenn syndrome: inflammation in leaky severe combined immunodeficiency. J Allergy Clin Immunol. 2008;122:1082–6.
89. McLean WHI. Combing the genome for the root cause of baldness. Nat Genet. 2008;40:1270–1.
90. Sprecher E. Disentangling the roots of inherited hair disorders. Nat Genet. 2008;40:265–6.
91. Esparza-Gordillo J, Weidinger S, Folster-Holst R, et al. A common variant on chromosome 11q13 is associated with atopic dermatitis. Nat Genet. 2009;41:596–601.
92. Nair RP, Duffin KC, Helms C, et al. Genome-wide scan reveals association of psoriasis with IL-23 and NF-kappaB pathways. Nat Genet. 2009;41:199–204.
93. Zhang XJ, Huang W, Yang S, et al. Psoriasis genome-wide association study identifies susceptibility variants within LCE gene cluster at 1q21. Nat Genet. 2009;41:205–10.
94. Sandilands A, Sutherland C, Irvine AD, et al. Filaggrin in the frontline: role in skin barrier function and disease. J Cell Sci. 2009;122:1285–94.
95. Fallon PG, Sasaki T, Sandilands A, et al. A homozygous frameshift mutation in the murine filaggrin gene facilitates enhanced percutaneous allergen priming. Nat Genet. 2009;41:602–8.
96. de Cid R, Riveira-Munoz E, Zeeuwen PL, et al. Deletion of the late cornified envelope LCE3B and LCE3C genes as a susceptibility factor for psoriasis. Nat Genet. 2009;41:211–5.
97. Reversade B, Escande-Beillard N, Dimopoulou A, et al. Mutations in PYCR1 cause cutis laxa with progeroid features. Nat Genet. 2009;41:1016–21.

Chapter 20
Molecular Aspects of Skin Aging

Michael J. Murphy

The past two decades have seen significant strides, not only in our understanding of the pathobiology of human skin aging, but also in the search for targeted modalities to treat or possibly prevent some of the changes that come with this process. Human skin aging can be categorized as either (a) chronologic/intrinsic aging or (b) extrinsic aging, of which photoaging is a major component [1–5].

Chronologic skin aging is a slow, progressive, cumulative, and degradative process, similar to aging at noncutaneous sites, and is influenced by several factors, including genetic predisposition, diet, lifestyle (i.e., smoking), and hormone levels [1–5]. It is characterized by a number of molecular and cellular events in the skin, such as replicative senescence, telomere shortening, decreased proliferative capacity, cell cycle and apoptosis alterations, oxidative damage, mitochondrial dysfunction, mutations in mitochondrial DNA (mtDNA), reduced DNA repair mechanisms, matrix metalloproteinase dysregulation, decreased responses to mitogenic stimuli, and changes in stem cell function [1–8]. Aged skin is less resistant to shearing forces, shows increased fragility and a limited capacity to regenerate, and is at risk of chronic wound formation (see Chap. 17) [9]. The study of "progeroid" syndromes, such as Werner syndrome, Hutchinson-Gilford progeria syndrome, and xeroderma pigmentosum, has been helpful in our understanding of the skin aging process [10–12]. These inherited premature aging disorders are characterized by defects in DNA transcription, replication, recombination, and repair [10–12]. A recent gene expression profiling study demonstrated that transcription alterations in Werner syndrome were similar to those seen in chronologic aging [11]. Up to 91% of genes displayed comparable expression changes in both groups, suggesting that Werner syndrome is characterized by an acceleration of normal aging mechanisms [11].

Extrinsic aging is primarily the result of environmentally induced changes, superimposed on the physiologic manifestations of chronologic aging. While exposure to ultraviolet (UV) radiation is regarded as the most important exogenous factor ("photoaging"), additional environmental insults include other forms of ionizing/nonionizing radiation, trauma, air pollutants, chemicals, and infectious agents [1]. UV radiation leads to skin damage through a variety of mechanisms, including the upregulation of immune responses [13]. It is known that high levels of and/or long-term UV exposure induce persistent single strand nuclear DNA breaks, pyrimidine dimer formation, UV-signature mutations in *TP53*, *CDKN2A*/p16, *RAS* and *PTCH* genes, mtDNA aberrations, and other types of DNA lesions in human skin (see Chap. 9) [14–20]. Of note, in the absence of pyrimidine

M.J. Murphy (✉)
Division of Dermatopathology, Department of Dermatology, University of Connecticut Health Center, 21 South Rd., Farmington, CT 06030, USA
e-mail: drmichaelmurphy@netscape.net

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*, Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_20,

dimer repair, mutations in the DNA sequence can occur [20]. Changes in the form of C→T and CC→TT transitions are described as UV-signature mutations [20]. In addition, epigenetic alterations in the skin (i.e., promoter hypo-/hyper-methylation, histone and chromatin modifications) may also be a response to UV exposure [20]. These changes are paralleled by altered expression patterns of genes representing a variety of functional classes, including cell cycle control, apoptosis, transcription factors, receptors, transporters, signaling intermediates, growth factors, intermediary metabolism, hormones, translation factors, tumor suppressor genes, and oncogenes [14–16, 18, 20, 21]. Of note, these alterations are detectable in chronically sun-exposed, but morphologically normal skin adjacent to a variety of skin cancers, and appear related to sunburn history in most cases [14, 16, 18, 20, 21]. It is postulated that many of the identified genes with UV-altered expression play no role in UV-induced carcinogenesis, but may turn out to be useful as markers of underlying genomic instability and photoaging [17, 18].

While the majority of human photoaging changes have long been considered to be secondary to UV-B radiation exposure, recent evidence suggests that both UV-A and infrared A (IRA) radiation play significant roles in this process [22–24]. A recent study identified early and stable markers of UV-A-induced effects which may be predictive of UV-A related damage *in vivo* [23]. Meloni et al. [23] reported that the cellular response to UV-A is mediated by a number of different pathways that function in early and delayed adaptive or repair mechanisms (fibrillin-1, involucrin, IL-10, and decorin), early defense mechanisms (collagen VII, GPX, and GSR), and tissue damage processes (elastin, MMP-1, and MMP-9). A study by Calles et al. [24] determined that the IRA-induced gene expression signature differed in some respects from that known to be due to UV (both UV-A and UV-B) exposure. Of note, changes in extracellular matrix metabolism, calcium homeostasis, MAPK signaling, IP_3 signaling, IL-6 signaling, stress signaling, and apoptosis pathways were found to represent IRA-response mechanisms [24]. Similar to UV-induced events, MMP-1 is upregulated upon IRA exposure [24]. However, UV and IRA radiation produce opposite responses for a number of other genes (IL6ST, FN1, BAX, BAD, STAT3, TNFRSF6B) [24]. Although, IRA exposure induces similar biological effects to UV radiation (i.e., decreased *de novo* synthesis of collagen and increased expression of the collagen-degrading enzyme MMP-1), it does so through different underlying molecular mechanisms, and in particular, disruption of the mitochondrial electron transport chain [25]. It remains to be determined if these specific molecular alterations can be translated into different morphologic changes *in vivo*. However, evidence does suggests that current sun-protection strategies should be reconsidered, and steps to protect against IRA-induced photoaging, and perhaps photocarcinogenesis, are necessary [24, 25].

Both chronologic aging and photoaging result in cumulative changes in skin structure, function, and appearance [1–5]. The clinical signs of chronologically aged skin include pallor, smoothness, xerosis, laxity, loss of elasticity, and fine wrinkles. In contrast, photoaged skin appears coarsely wrinkled with erythema, telangiectasias, roughness, pigmentary changes (including lentigines and guttate hypomelanosis), with an increased incidence of benign, premalignant and malignant keratinocytic lesions, such as seborrheic keratosis, actinic keratosis, basal cell carcinoma, and squamous cell carcinoma. Microscopically, young skin demonstrates a well-organized and compact dermal extracellular matrix, whereas in aged skin, this becomes disorganized and loose, with an overall decrease in dermal thickness [26]. Both chronologic and photoaged skin are characterized by reduced expression of collagen genes, with a marked decrease in collagen synthesis, and an increase in the ratio of collagen III to collagen I (largely due to loss of the latter) [26]. However, photoaging is distinguished by its variable epidermal thickness and a selective increase in the production of specific dermal matrix proteins, such as elastin [26]. Chronic sun-exposed skin can demonstrate either epidermal hypertrophy or atrophy, and shows variable superficial dermal accumulation of dystrophic, truncated, and thickened elastic fibers (termed "solar elastosis") (Fig. 20.1). Dysregulation of genes encoding the structural proteins of elastic fibers (i.e., ↑ elastin and ↓ fibrillin), secondary to

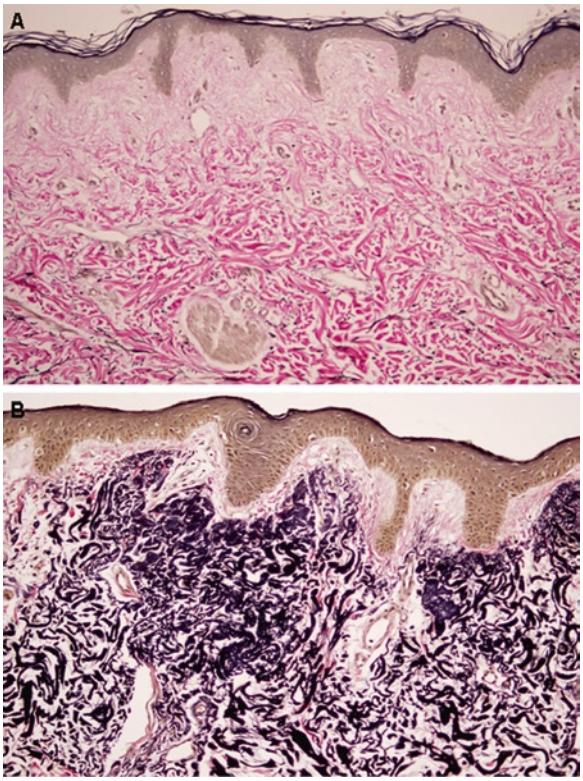

Fig. 20.1 Solar elastosis. (**a**) Normal (sun-protected) and (**b**) photodamaged (sun-exposed) skin stained with Verhoeff-Van Gieson. Solar elastosis is characterized by increased staining of short, thickened, and disorganized elastic fibers in the photo-damaged dermis

UV exposure, may be the basis of the solar elastotic features seen [27]. Although the phenotypically evident changes in chronically sun-damaged skin are largely localized to the dermis, a recent *in vivo* gene expression profiling study on full-thickness human skin samples suggested a major role for the epidermis in the pathobiology of these events [27]. In contrast with the microscopic characteristics of photoaging, chronologically aged skin shows epidermal thinning, flattening of rete ridges, and general atrophy of the dermal extracellular matrix with reduction of both collagen and elastic fibers. In addition, there is slowing of epidermal turnover with decreased keratinocyte mitotic activity. Unlike photoaging, elastin gene expression is reduced in the chronologic aging process [1]. While the clinical and histopathological features of chronologically aged and photoaged skin appear distinct, recent evidence points to these processes having some common characteristics, including partially overlapping molecular pathogenic pathways [1–5].

Numerous studies continue to be undertaken in an effort to elucidate the pathobiological mechanisms of both aging processes. Many investigations have employed human keratinocyte primary/organ cultures, dermal fibroblast cultures, and/or animal models [1, 28, 29]. However, conventional cell cultures do not accurately reproduce physiological conditions, and skin explants may show marked inter-individual variability [23]. In a number of instances, studies have been accomplished on skin biopsy specimens, taken from different cutaneous sites of individuals from different age groups [13, 26, 30–32]. However, prospective studies on long-term, and indeed single or short-term, experimental exposure to UV radiation in humans are difficult to design, due to both ethical concerns and the logistics of acquiring data over the lifespan of study subjects. Recent reports confirm the biological relevance of *in vitro* human skin equivalent cultures for use in

studies on the aging process and potential treatment/prevention strategies [23, 29, 33, 34]. With this model, the effects of environmental insults (i.e., UV, IRA, and chemicals) and potential therapeutic intervention on human skin can be investigated at both cellular and molecular levels. Human skin equivalent cultures are designed to reproduce key structural and functional features of natural skin and closely mimic *in vivo* events. In this regard, they can possess a multilayered structure, comprised of a stratified and differentiated layer of epidermis containing keratinocytes and melanocytes, alone or in combination with a subjacent basement membrane zone and fibroblast-rich dermal matrix [33]. A stratum corneum forms at the air–liquid interface allowing for the application of topical compounds [33]. Technologies used in these studies have included light and electron microscopy, flow cytometry, immunohistochemistry, immunoblotting, and enzyme-linked immunosorbent assays [28, 29, 35, 36]. In particular, the employment of nucleic acid-based methodologies, such as cDNA/oligonucleotide microarrays, reverse transcription-polymerase chain reaction (RT-PCR), and serial analysis of gene expression (SAGE), are now broadening our understanding of the molecular pathways associated with skin aging [13, 26, 29–42]. This has been facilitated by rapid advances in genomic technologies and bioinformatics. Results from these investigations are uncovering biomarkers of the skin aging process and revealing novel targets for antiaging drug development. In addition, some of these findings could be employed in preventive strategies for photoinduced cutaneous malignancies.

Comparative investigations of global gene expression in skin biopsies from young (2–28 years old) *vs.* older (60–80 years old) subjects have been accomplished, using tissue from: (a) sun-protected sites (i.e., buttock, breast, foreskin, and upper inner arm), in order to study chronologic aging; and (b) sun-exposed sites (i.e., forearm), in order to study the combination of photoaging and chronologic aging effects [13, 26, 30–32]. Holtkötter et al. [26] demonstrated several hundred differentially expressed genes as a function of chronologic skin aging, by analyzing sun-protected breast skin from young *vs.* older individuals. Aged skin showed upregulation of APP, PAP2-B, DSC1, and KIF5B; downregulation of SUN2, IGFBP4, FOS, IKBA, and HSPC254; and, as expected, reduced expression of collagen gene transcripts [26]. Many of these genes had not been previously reported to be associated with skin aging processes. Lener et al. [31] identified 43 downregulated transcripts and 62 upregulated transcripts in older (68–72 years old) foreskin samples compared with young (2–3 years old) foreskin samples. Their results suggested that the chronologic aging process in human skin is associated with the dysregulation of various cellular processes, such as DNA binding, transcriptional activation/repression, cell cycle control, cytoskeletal changes, inflammatory response, signal transduction, and metabolism [31]. Other studies have compared the transcriptome of chronologically aged and photoaged skin [13, 30, 32]. While both processes show similar gene expression profiles, aberrations are generally more exaggerated with photoaging [13]. Both aging processes show reduced expression of genes associated with stratum corneum lipid biosynthesis/metabolism and epidermal differentiation (keratins and cornified envelope components) [13, 30]. Downregulated genes include those related to epidermal cholesterol synthesis (HMGCS1, HMGCR, MVK, PMVK, MVD, IDI1, FDPS, FDFT1, SQLE, CYP51A1, SC4MOL, NSDHL, and DHCR7), major cellular cholesterol influx pathway (LDLR and SCARB1), fatty acid synthesis (PKM2, CS, ACLY, ACACA, and FASN), fatty acid uptake (SLC27A2 and ACSL1), sphingolipid biosynthesis and processing (SPTLC2, LASS, DEGS, UGCG, and COL4A3BP), and lamellar body secretion (ABHD5) [30]. Of note, the major cellular cholesterol efflux pathway (ABCA1) is found to be upregulated in aged skin [30]. The reduced capacity of aged skin to both maintain epidermal barrier function and recover from barrier damage could be due to dysregulation in these fundamental pathways [13, 30]. Both chronologic aging and photoaging demonstrate similar gene ontology themes, including immune and inflammatory response, extracellular matrix, and peptidase activity, but with photoaging showing more exaggerated changes in these common effects [13]. In addition, Lee et al. [32] have reported that S100A8 (a calcium-binding protein associated with keratinocyte differentiation, inflammation, and wound healing) is induced by acute UV

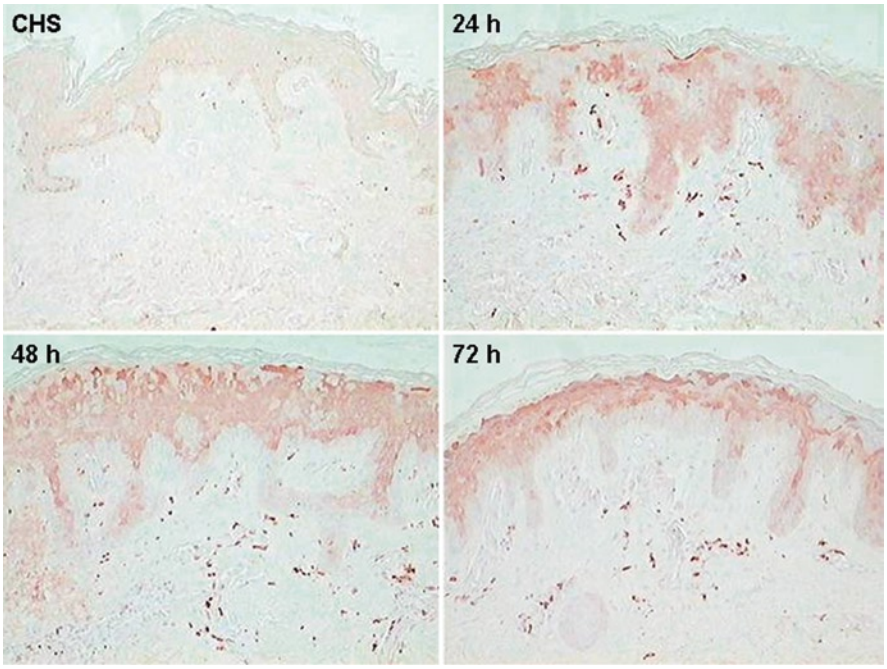

Fig. 20.2 *In vivo* pattern of S100A8 expression as a function of ultraviolet (*UV*) irradiation. The figures show S100A8-immunoreactivity in control human skin (*CHS*) and buttock skin from young subjects at 24, 48, and 72 h post-UV irradiation. UV irradiation increases the level of S100A8 expression both in the epidermis and dermis of human skin (Courtesy of Dr. Jin Ho Chung, Department of Dermatology, Seoul National University College of Medicine and Laboratory of Cutaneous Aging Research, Seoul National University Hospital, Seoul, Korea)

exposure, as well as being upregulated in chronologically aged and photoaged skin compared with young skin (Fig. 20.2). S100A8 may have important novel roles in skin aging and its functions could be potentially modulated by targeted therapeutic intervention during the aging process. Of note, transcriptome changes that are specific to one form of aging are also found [13]. For instance, increased elastin gene expression is unique to photoaged skin, consistent with the microscopically evident solar elastotic changes commonly seen at chronically sun-exposed sites [13].

In another study, Aoki et al. [37] used oligonucleotide microarray technology to investigate the transcriptome of solar lentigo, a cutaneous manifestation of chronic sun-exposure, which demonstrates basilar epidermal melanin hyperpigmentation on light microscopy. Solar lentigo showed increased expression of genes associated with inflammation, fatty acid metabolism, and melanocyte regulation, and reduced expression of cornified envelope-related genes [37]. Results were correlated with in situ hybridization and immunohistochemical studies, which demonstrated decreased proliferation and differentiation of lesional keratinocytes on a background of chronic inflammation [37].

A number of different modalities, alone or in combination, are used in the prevention and treatment of skin aging, particularly photoinduced changes [3–5, 29, 33–36, 38–43]. These include sunscreens, fillers, botulinum toxin, microdermabrasion, and photodynamic therapy/laser resurfacing, in addition to topical fluorouracil, antioxidants, retinoids, synthetic peptides, and *N*-acetyl glucosamine (NAG). Many of these therapeutic strategies have been shown to improve the signs of skin aging, including reducing the appearance of fine lines, wrinkles, and hyperpigmented spots, and increasing skin hydration and exfoliation.

Recent studies have evaluated the effects of a number of these therapeutic modalities on the transcriptomic profiles of human skin equivalent cultures, human skin explants, and human skin

samples [29, 33–36, 38–42]. These investigations were designed to identify changes in skin biomarker expression that are therapy-induced and likely linked to their antiaging benefits. Mullins et al. [29] demonstrated that human skin equivalent cultures treated with the peptide palmitoyl-lysine-threonine (pal-KT) showed increased expression of basement membrane (collagen IV, laminins I and IV), dermal matrix (collagens I, III, and VI, elastin, fibronectin, CD44, and vimentin), and epidermal differentiation (CK1 and CK10) molecules. Bissett et al. [34] found that topical NAG therapy of human skin equivalent cultures led to dose-dependent decreases in melanin production, in addition to altered expression (upregulation and downregulation) of genes associated with sugar metabolism, antioxidant enzymes, cell growth/division, epidermal differentiation, and skin pigmentation (i.e., melanin synthesis, melanosome transport). Many of these changes occurred at early time points during treatment [34]. In another study, the treatment of *in vitro* skin cultures with agents which promote skin barrier function (i.e., hexamidine and niacinamide) induced an upregulation of stratum corneum lipid pathways [33]. Finally, an *ex vivo* study, employing human skin explants, found that topical retinol application resulted in increased expression of CRABP2 and HBEGF (genes relevant for retinoid-like activity), that was associated with increased keratinocyte proliferation and epidermal thickness [38]. These studies have provided important information concerning *in vitro* genomic changes associated with drug-induced cellular effects, such as reduced melanin content and epidermal thickening. *In vivo* genomic, proteomic, and/or biochemical analyses will be required to determine if these changes in gene expression play a direct role in the phenotypic effects of these drugs in the clinical setting and, therefore, support their use as topical antiaging products.

The *in vivo* molecular effects of a number of other antiaging strategies have been investigated. Karimipour et al. [35, 36] used quantitative real-time PCR and other technologies to determine the molecular effects of different microdermabrasion procedures on both photoaged and sun-protected skin. Aluminum oxide microdermabrasion was found to activate a dermal remodeling cascade, with upregulation of cytokines (IL-1β and TNF-α), transcription factors (AP-1/c-jun and NF-κB), and matrix metalloproteinases (MMP-1, MMP-3 and MMP-9) [35]. However, there was minimal epidermal injury, and new collagen synthesis was identified in only a minority of subjects (associated with ↑ procollagen I mRNA) or did not occur at all [35]. In a second study, Karimipour et al. [36] noted that use of more aggressive, but still nonablative, coarse-grit microdermabrasion led to epidermal injury (↑ CK16) and dermal remodeling, associated with collagen biosynthetic pathway induction (↑ procollagens I and III, ↑ HSP47, ↑ prolyl 4-hydroxylase), in addition to predicted cytokine, transcription factor, and matrix metalloproteinase upregulation (Fig. 20.3). Sachs et al. [39] determined that topical fluorouracil treatment for photodamaged skin (and actinic keratoses) also increased the gene expression of the effectors of epidermal injury (CK16), inflammation (IL-1β), extracellular matrix degradation (MMP-1 and MMP-3), and procollagens I and III. Photodynamic therapy/laser therapy for photoaging has also been reported to be associated with an upregulation of procollagen I and III transcripts *in vivo*, in addition to other cellular and molecular alterations [40–42]. Therefore, these therapeutic modalities induce common changes in photodamaged skin that are consistent with a wound-healing response. Importantly, treatments which induce some degree of epidermal injury lead to molecular alterations that are associated with clinical improvement in the signs of skin aging.

In conclusion, it is envisioned that future studies, employing genomics-based technologies and other methodologies, and utilizing both *in vitro* and *in vivo* models, will continue to drive the development of treatments for skin aging. Many of the current therapeutic modalities have been used empirically. Ongoing investigations will likely uncover novel therapeutic targets, facilitating the development of more directed therapy, with the potential of personalized medicine for individual patients. Controlled clinical studies will be required to determine whether the molecular alterations that are obtained with many current and future treatments translate into improvements in the clinical appearance of the signs of skin aging.

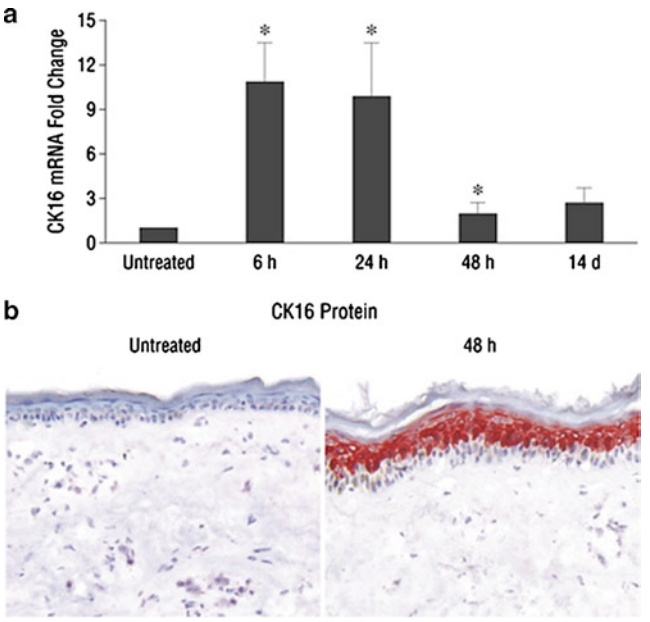

Fig. 20.3 Molecular analysis of aggressive microdermabrasion in photoaged skin. Coarse-grit microdermabrasion induces (**a**) CK16 mRNA and (**b**) CK16 protein expression in human skin *in vivo*. Abbreviations: h, hours; d, days (From Karimipour et al. [36]. Reprinted with permission from the American Medical Association, Copyright © 2009)

References

1. Makrantonaki E, Zouboulis CC, William J. Cunliffe scientific awards. Characteristics and pathomechanisms of endogenously aged skin. Dermatology. 2007;214:352–60.
2. Callaghan TM, Wilhelm KP. A review of ageing and an examination of clinical methods in the assessment of ageing skin. Part I: cellular and molecular perspectives of skin ageing. Int J Cosmet Sci. 2008;30:313–22.
3. Baumann L. Skin ageing and its treatment. J Pathol. 2007;211:241–51.
4. Herschthal J, Kaufman J. Cutaneous aging: a review of the process and topical therapies. Exp Rev Dermatol. 2007;2:753–61.
5. Yaar M, Gilchrest BA. Photoageing: mechanism, prevention and therapy. Br J Dermatol. 2007;157:874–87.
6. Sárdy M. Role of matrix metalloproteinases in skin ageing. Connect Tissue Res. 2009;50:132–8.
7. Mimeault M, Batra SK. Recent advances on skin-resident stem/progenitor cell functions in skin regeneration, aging and cancers and novel anti-aging and cancer therapies. J Cell Mol Med. 2010;14:116–34.
8. Zouboulis CC, Adjaye J, Akamatsu H, et al. Human skin stem cells and the ageing process. Exp Gerontol. 2008;43:986–97.
9. Varani J. Skin damage in the aged: it's more than cosmetic. Exp Rev Dermatol. 2009;4:549–51.
10. Capell BC, Tlougan BE, Orlow SJ. From the rarest to the most common: insights from progeroid syndromes into skin cancer and aging. J Invest Dermatol. 2009;129:2340–50.
11. Kyng KJ, May A, Kølvraa S, et al. Gene expression profiling in Werner syndrome closely resembles that of normal aging. Proc Natl Acad Sci USA. 2003;100:12259–64.
12. Kyng K, Croteau DL, Bohr VA. Werner syndrome resembles normal aging. Cell Cycle. 2009;8:2323.
13. Robinson MK, Binder RL, Griffiths CE. Genomic-driven insights into changes in aging skin. J Drugs Dermatol. 2009;8(7 Suppl):s8–11.
14. Dakubo GD, Jakupciak JP, Birch-Machin MA, et al. Clinical implications and utility of field cancerization. Cancer Cell Int. 2007;7:2.
15. Takata M, Saida T. Early cancers of the skin: clinical, histopathological, and molecular characteristics. Int J Clin Oncol. 2005;10:391–7.
16. Hubbard K, Steinberg ML, Hill H, et al. Mitochondrial DNA deletions in skin from melanoma patients. Ethn Dis. 2008;18 Suppl 2:S2-38–43.

17. Eshaghian A, Vleugels RA, Canter JA, et al. Mitochondrial DNA deletions serve as biomarkers of aging in the skin, but are typically absent in nonmelanoma skin cancers. J Invest Dermatol. 2006;126:336–44.
18. Steinberg ML, Hubbard K, Utti C, et al. Patterns of persistent DNA damage associated with sun exposure and the glutathione S-transferase M1 genotype in melanoma patients. Photochem Photobiol. 2009;85:379–86.
19. Brozyna A, Zbytek B, Granese J, et al. Mechanism of UV-related carcinogenesis and its contribution to nevi/melanoma. Exp Rev Dermatol. 2007;2:451–69.
20. Greinert R. Skin cancer: new markers for better prevention. Pathobiology. 2009;76:64–81.
21. Fabricius EM, Bezeluk A, Kruse-Boitschenko U, et al. Clinical significance of telomerase activity in basal cell carcinomas and in tumour-free surgical margins. Int J Oncol. 2003;23:1389–99.
22. Sjerobabski Masnec I, Poduje S. Photoaging. Coll Antropol. 2008;32 Suppl 2:177–80.
23. Meloni M, Farina A, de Servi B. Molecular modifications of dermal and epidermal biomarkers following UVA exposures on reconstructed full-thickness human skin. Photochem Photobiol Sci. 2010;9:439–47.
24. Calles C, Schneider M, Macaluso F, et al. Infrared A radiation influences the skin fibroblast transcriptome: mechanisms and consequences. J Invest Dermatol. 2010;130:1524–36.
25. Schroeder P, Calles C, Benesova T, et al. Photoprotection beyond ultraviolet radiation–effective sun protection has to include protection against infrared A radiation-induced skin damage. Skin Pharmacol Physiol. 2010;23:15–7.
26. Holtkötter O, Schlotmann K, Hofheinz H, et al. Unveiling the molecular basis of intrinsic skin aging(1). Int J Cosmet Sci. 2005;27:263–9.
27. Urschitz J, Iobst S, Urban Z, et al. A serial analysis of gene expression in sun-damaged human skin. J Invest Dermatol. 2002;119:3–13.
28. Soroka Y, Ma'or Z, Leshem Y, et al. Aged keratinocyte phenotyping: morphology, biochemical markers and effects of Dead Sea minerals. Exp Gerontol. 2008;43:947–57.
29. Mullins LA, Jarrold BB, Linter K, et al. In vitro skin biomarker responses to a new antiaging peptide, Pal-KT. J Am Acad Dermatol. 2009;60 Suppl 1:AB82.
30. Jarrold BB, Mullins LA, Binder RL, et al. Expression profiles of stratum corneum lipid metabolism pathways associated with intrinsic and extrinsic aging. J Am Acad Dermatol. 2009;60 Suppl 1:AB28.
31. Lener T, Moll PR, Rinnerthaler M, et al. Expression profiling of aging in the human skin. Exp Gerontol. 2006;41:387–97.
32. Lee YM, Kim YK, Eun HC, et al. Changes in S100A8 expression in UV-irradiated and aged human skin *in vivo*. Arch Dermatol Res. 2009;301:523–9.
33. Osborne R, Mullins LA, Jarrold BB. Understanding metabolic pathways for skin anti-aging. J Drugs Dermatol. 2009;8(7 Suppl):s4–7.
34. Bissett DL, Farmer T, McPhail S, et al. Genomic expression changes induced by topical N-acetyl glucosamine in skin equivalent cultures in vitro. J Cosmet Dermatol. 2007;6:232–8.
35. Karimipour DJ, Kang S, Johnson TM, et al. Microdermabrasion: a molecular analysis following a single treatment. J Am Acad Dermatol. 2005;52:215–23.
36. Karimipour DJ, Rittié L, Hammerberg C, et al. Molecular analysis of aggressive microdermabrasion in photoaged skin. Arch Dermatol. 2009;145:1114–22.
37. Aoki H, Moro O, Tagami H, et al. Gene expression profiling analysis of solar lentigo in relation to immunohistochemical characteristics. Br J Dermatol. 2007;156:1214–23.
38. Bellemère G, Stamatas GN, Bruère V, et al. Antiaging action of retinol: from molecular to clinical. Skin Pharmacol Physiol. 2009;22:200–9.
39. Sachs DL, Kang S, Hammerberg C, et al. Topical fluorouracil for actinic keratoses and photoaging: a clinical and molecular analysis. Arch Dermatol. 2009;145:659–66.
40. Orringer JS, Hammerberg C, Hamilton T, et al. Molecular effects of photodynamic therapy for photoaging. Arch Dermatol. 2008;144:1296–302.
41. Orringer JS, Kang S, Johnson TM, et al. Connective tissue remodeling induced by carbon dioxide laser resurfacing of photodamaged human skin. Arch Dermatol. 2004;140:1326–32.
42. Orringer JS, Voorhees JJ, Hamilton T, et al. Dermal matrix remodeling after nonablative laser therapy. J Am Acad Dermatol. 2005;53:775–82.
43. Tierney EP, Hanke CW. Recent advances in combination treatments for photoaging: review of the literature. Dermatol Surg. 2010;36:829–40.

Chapter 21
Pharmacogenetics and Pharmacogenomics I: Linking Diagnostic Classification to Therapeutic Decisions

Michael J. Murphy, Carlo Pincelli, Diane M. Hoss, and Riccardo G. Borroni

Innovations in medical therapy have been associated with increasing life expectancy, improved quality of life, and with a decreasing need for hospitalizations and surgery. However, these benefits are not achieved without significant drawbacks. Medications are not completely safe and effective for everyone. Spear et al. [1] analyzed the efficacy of major drugs used to treat several important diseases. The heterogeneity of therapeutic responses was evident, ranging from a low of 25% (for cancer chemotherapeutics) to a high of 80% (for COX-2 inhibitors), with response rates for most drugs falling in the range of 50–75% [1]. Adverse drug reactions (ADRs) represent another important problem, leading to ~6% of hospitalizations (>2 million per year) and annual health care costs estimated at $1.5–$4 billion in the United States [2–4]. ADRs are now the fourth leading cause of mortality in the United States, resulting in >100,000 deaths per year [2–4]. Drug efficacy and toxicity are affected by a number of factors, including patient age, sex, hepatic and renal function, drug–drug interactions, diet, lifestyle, and comorbidities. Many drugs also have narrow therapeutic indices (i.e., the therapeutic dose is close to the toxic dose). In addition, DNA sequence variations are known to play a major role in the inter-individual variability of drug response and ADRs [1]. Pharmacogenomic testing could facilitate a more targeted approach to treatment, by predicting which patients are more likely to respond to a drug, as well as those at increased risk for developing an ADR [1]. The tailoring of drug therapy to the individual patient ("personalized medicine") is an exciting possibility for clinicians.

The completion of the Human Genome Project has facilitated the study of naturally occurring sequence variations, or DNA polymorphisms. About 90% of these are single nucleotide polymorphisms (SNPs), or changes in genomic sequences (alleles) at single base pairs (bp), that exist in normal individuals at a frequency of 1% or greater. With a total of 3.12 billion nucleotides and an SNP frequency at 1 in 1,250 between two unrelated individuals, it is estimated that any two patients should differ by about three million bp – or 0.1% of the total genome. During the last 10 years, more than ten million SNPs have been identified [5]. The International HapMap project has genotyped approximately four million common SNPs in a genome-wide map of SNP-tagged haplotypes [6]. Although the majority of SNPs are present in intergenic or perigenic regions, about 30,000–100,000

R.G. Borroni (✉)
Department of Medicine, Dermatology Division,
University of Modena and Reggio Emilia, Via del Pozzo, 71, 41100, Modena, Italy
e-mail: riccardoborroni@gmail.com

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_21,

occur in the coding or regulatory regions of genes, and can change protein expression and/or function, possibly affecting drug responses. Other less common polymorphisms that can influence the therapeutic effect of drugs include: (a) haplotypes, which are a set of coinherited closely linked genetic markers (alleles or a set of SNPs) on an individual chromosome; (b) variable number tandem repeats (VNTRs) or minisatellites (repeats of sequences >5 bp in length); (c) short tandem repeat polymorphisms (STRPs) or microsatellites (repeats of sequences 1–5 bp in length); and (d) copy number variations (CNVs), representing differences in the number of copies of a particular sequence present in an individual's genome, as a result of insertions, duplications, or deletions [7, 8]. In the study of cancer chemotherapeutic agents, it is important to also consider somatic mutations within tumor cells, including loss of heterozygosity and aneuploidy [7]. In addition to roles played by polymorphisms in disease susceptibility genes and DNA repair enzymes, sequence variations in genes encoding drug-metabolizing enzymes, drug transporters, drug receptors, drug target molecules, and downstream signaling pathway molecules can affect drug pharmacokinetic and pharmacodynamic parameters, thereby influencing the efficacy and toxicity of a number of medications [8–10].

The disciplines of pharmacogenetics (single-gene focus) and pharmacogenomics (whole-genome focus) seek to understand differences in drug response as a function of gene variability. Pharmacogenetics, a term coined more than 50 years ago, describes the study of genetic factors that influence the response to drugs, including efficacy and toxicity [7, 10]. Typically, pharmacogenetic studies have focused on one single gene (or a few genes) at a time. With the completion of the Human Genome Project, and advances in high-throughput DNA sequencing, SNP mapping, and bioinformatics, we have seen a transition from pharmacogenetic to pharmacogenomic investigations [7]. Pharmacogenomic strategies employ genomic and proteomic technologies to study the influence of genetic variations on drug response parameters at a whole-genome level. The goal is to achieve a correlation between gene expression patterns and drug efficacy and toxicity. The terms pharmacogenetics and pharmacogenomics are often used interchangeably. However, according to the International Conference on Harmonization (ICH) – Guidance for Industry: E15 Definitions for Genomic Biomarkers, Pharmacogenomics, Pharmacogenetics, Genomic Data, and Sample Coding Categories [11]: pharmacogenomics is defined as "*the study of variations of DNA and RNA characteristics as related to drug response*," while pharmacogenetics is a subset of pharmacogenomics and is defined as "*the study of variations in DNA sequence as related to drug response.*"

Many current treatment modalities, including those used in dermatology, have been developed empirically and show relatively nonspecific and narrow therapeutic indices. The goal of pharmacogenomics is the personalization of therapy based on an individual's genotype, including the selection of appropriate medication(s) for the patient's genetic make-up, the optimization of drug efficacy, and the minimization of the risk for ADRs. In addition, pharmacogenomic investigations can facilitate drug discovery and development by identifying novel therapeutic targets. A broader goal of this discipline is the reduction of healthcare costs. A recent review by Huang and Ratain [7] proposed five stages of pharmacogenomics research, which included: (a) determining the role of genetics in drug response; (b) screening and identifying genetic markers; (c) validating genetic markers; (d) clinical utility assessment; and (e) pharmacoeconomic impact. In general, two strategies have been used to evaluate how genetic changes are associated with variations in drug response and toxicity: candidate gene and genome-wide [7]. Candidate gene approaches are generally hypothesis-driven, and focus on one or more candidate genes or pathways, chosen based on evidence that the gene product is involved in variations in pharmacokinetics or pharmacodynamics. In contrast, a genome-wide strategy is hypothesis-generating and employed when little information is available concerning gene-drug effects [7].

As is evident from the other chapters in this book, dermatology is rapidly integrating "new" information from recent molecular discoveries with the "classical" clinical and histopathological features of a large number of skin conditions. However, direct patient benefit has yet to be realized

in many instances. A pharmacogenomic approach to the investigation of skin diseases allows for the translation of findings from bench-to-bedside and aids in the therapeutic decision-making process for each individual patient [12]. In addition, pharmacogenomic awareness among dermatologists will facilitate the development of safer and more effective drugs for patients with skin disorders.

This chapter summarizes current pharmacogenomic data relevant to a number of major skin diseases, including psoriasis, melanoma, and non-melanoma skin cancers. Germline genetic effects on disease phenotype and drug sensitivity, and the identification of biomarkers of drug response from gene expression profiling studies, are discussed. In addition, potential areas for dermatologic translational research are explored. It is important to consider that much of the pharmacogenomic data pertaining to medications used in dermatology have not been derived from their study in patients with skin diseases. For example, both corticosteroids and 5-fluorouracil (5-FU) have long been used as therapeutic agents for a number of skin diseases, but their specific molecular effects in this setting are largely unknown [7, 9, 13–15]. In many instances, pharmacogenomic data have been obtained from the study of these and other drugs in patients with systemic disease states or from *in vitro* models [7, 9, 10, 13–15].

Psoriasis

Psoriasis is a chronic inflammatory skin disease that affects up to 2.5% of Caucasian populations [16–18]. The clinical presentation is heterogeneous, and includes psoriasis vulgaris (chronic plaque psoriasis), guttate psoriasis, pustular psoriasis (both generalized and palmoplantar variants), and erythrodermic psoriasis. In addition to skin disease, nail changes and arthropathy are commonly present [16–18]. Psoriasis is a multifactorial disease with a complex pathogenesis, involving polygenic predisposition and environmental triggering factors, such as infection, trauma, and drugs [16–18]. A genetic basis for this disease is supported by the fact that a concordance rate of 63–73% is seen in monozygotic twins, as compared to 17–20% for dizygotic twins. The HLA-Cw*0602 allele of HLA-Cw6 is the main genetic determinant of psoriasis. This gene, present on chromosome 6p21, has been designated "psoriasis susceptibility 1" (PSORS1), and accounts for ~35% of psoriasis genetic risk [16–18]. However, due to high linkage disequilibrium in this region, other candidate genes at PSORS1 may also be involved. From the results of genome-wide linkage scans, several additional disease susceptibility loci, PSORS2-PSORS9, have also been proposed [16–18], but the strength of their association with psoriasis susceptibility is variable. A recent study by Elder et al. [17] reported an association between psoriasis and seven genetic loci: HLA-C, IL-12B, IL-23R, IL-23A, IL-4/IL-13, TNFAIP3, and TNIP1. Most candidate genes at susceptibility loci still await confirmation in large, independent association studies on homogeneous populations. Their biological functions also have to be validated in order to be considered relevant to the pathogenesis of this disease.

The presence of the HLA-Cw*0602 allele typically correlates with early onset (<40 years old) and more severe psoriasis, in addition to a positive family history [10]. PSORS1 appears to be associated with psoriasis vulgaris and even more strongly with the guttate variant, but not with palmoplantar pustulosis, suggesting similarities between psoriasis vulgaris and guttate psoriasis, and important differences between these variants and the pustular subtype [19]. The different carriage rates of TNF -238*A and TNF -308*A between psoriasis vulgaris and palmoplantar pustulosis also support the genetic distinction between these disease variants [20]. Table 21.1 provides a list of reported polymorphisms that are associated with distinct clinical features in patients with psoriasis.

A wide variety of therapeutic modalities are available for the treatment of psoriasis (Tables 21.2–21.4). These include: (a) topical agents, such as corticosteroids, vitamin D analogs (i.e., calcipotriol, tacalcitol),

Table 21.1 Reported polymorphisms that are associated with distinct clinical features in patients with psoriasis

Allele	Phenotype	Reference
HLA-Cw*0602	Early onset of disease	[111]
	More frequent guttate and eruptive psoriasis	
	More frequent exacerbations with throat infections	
	Higher incidence of the Koebner's phenomenon	
	More extensive disease	
	Less frequent dystrophic nail changes	
	More frequent remissions during pregnancy	
TNFA -238*A(G→A)	Early onset of disease	[20, 112]
	Associated with psoriasis vulgaris variant	
TNFA -308*A(G→A)	? Protective effect	[20]
	Associated with less severe skin disease	
	? Marker of more severe joint involvement	
ACC haplotype (-1082/-819/-592) of IL-10 promoter region	Lower PASI score and limited extent of disease	[113]
ATA haplotype (-1082/-819/-592) of IL-10 promoter region	Persistent eruptions	[112]
IL-10.G13 microsatellite allele	? Associated with familial early onset psoriasis	[114]
IL-10.G9 microsatellite allele	Protective effect for familial psoriasis	[113]
-1082 heterozygous (G/A) genotype of IL-10 promoter region	Late onset in Caucasians	[115]
IL-1B-511*1 (-511*C) homozygous genotype	Late onset in Caucasians	[111]
-2763A allele of IL-10 promoter region and AAGC haplotype (-3575/-2763/-1082/-592) of IL-10 promoter region	Late onset in a Thai population	[116]
VEGF +405CC genotype and C allele	Severe psoriasis (PASI ≥ 12)	[117]
	Early onset of disease	
VEGF –460TT (C>T) genotype	Early onset of disease	[118]
IL-23R, IL-23A, IL-12B, IL-13, IL-15	Some variants associated with disease development	[119–124]
	Other variants confer protection	

PASI Psoriasis Area and Severity Index

Table 21.2 Response to treatment modalities in psoriasis. Percentage of patients achieving PASI 75 reduction at approximately 12 weeks with current therapeutic approaches (Adapted from Leon et al. [22])

Drug	Responding patients (%)
Goeckerman and RePUVA	100
Calcipotriene plus PUVA	87
Cyclosporine	78.2–80.3
Infliximab	80
Adalimumab 40 mg every other week	53
Adalimumab 40 mg/week	80
PUVA	63
Methotrexate	60
NB-UVB	55
Acitretin	52
Etanercept 50 mg twice weekly	49
Etanercept 25 mg twice weekly	34
Efalizumab	31.4
Alefacept	21

PASI 75 75% or greater reduction in Psoriasis Area and Severity Index score from baseline, *PUVA* psoralen plus ultraviolet A, *RePUVA* retinoid plus PUVA, *NB-UVB* narrow band-ultraviolet B

Table 21.3 Associations between genetic polymorphisms and drug response in patients with psoriasis (Adapted from data by Ryan et al. [10], except where indicated)

Drug	Polymorphism	Association	Reference
Methotrexate	SNP rs35592 (intron 9, ABCC1) and SNP rs6532049 (intron 1, ABCG2)	Drug efficacy	[10]
	Five SNPs in ABCC1	Drug toxicity	[10]
	SNP in SLC19A1	Drug toxicity	[10]
	TYMS alleles (2R *vs.* 3R), and G>C SNPs	Unclear; possible variable drug sensitivity	[10]
Cyclosporine[a]	SNPs in CYP3A4, CYP3A5, and ABCB1; Presence of HLA-DR1 allele	Conflicting reports regarding drug pharmacokinetics, toxicity, and side effects	[10]
Acitretin	VEGF -460TT (C>T) genotype	Drug response	[10, 118]
	APOE e4 allele (+3937C/+4075C)	No association with drug response	[10]
TNF-α inhibitors[a]	Promoter region of TNF at -238, -308, and -857	Conflicting reports; no polymorphism definitively associated with drug response	[10]
	Promoter region of TNF -1031T>C	Response to adalimumab (subjectively determined)	[10]
	HLA-Cw*0602	Conflicting or nonsignificant data with regard to drug response	[10]
	*0404 and *0101 haplotype of HLA-DRB1 (shared epitope)	Drug response	[125]
	IL-10 1087 GG haplotype	Drug response	[126]
	IL-1RN (two repeats of A-allele in intron 2) and TGFB1 (rare C-allele in codon 25) haplotype	Drug response	[126]
	IL-10 promoter microsatellites R3, G13, R2-G13, and R3-G9	Drug response	[127]
	FcGR IIIa -158 VV	Drug response	[128]
	TNFR-1 +36 G-allele	Drug response	[129]
	LTα 1–1-1–1 haplotype	Drug response	[130]
	IBD 5 (5q31) TT	Drug response	[131]
Topical vitamin D analogs	A, F, and T alleles of -1012A>G, Fok1, and Taq1VDR, respectively	Conflicting results with regard to drug response	[10]
	Possible polymorphisms of VDR	Clinical response correlated with increased VDR transcripts in skin lesions; nonresponders showed no increase in VDR levels	[10]
Coal tar	GSTM1-null allele	Possible risk of higher mutagen exposure	[10]
IL-12/23p40 therapies	IL-23R, IL-23A, and IL-12B	Effects on drug response are currently unknown	[10]

[a] Most data pertaining to the associations between genetic polymorphisms and drug response come from other disease states and conditions (i.e., rheumatoid arthritis, Crohn's disease, and organ transplant recipients)

retinoids (tazarotene), coal tar, and dithranol; (b) phototherapies [i.e., PUVA (psoralen plus ultraviolet A) and NB-UVB (narrow band-ultraviolet B)]; and (c) different classes of systemic agents, including methotrexate, cyclosporine and rhIL-11 (inhibitors of NF-kB and calcineurin/p38 pathways), pimecrolimus (selective inflammatory cytokine release inhibitor), acitretin (vitamin A derivative/retinoid), IL-10, alefacept (CD2 signaling inhibitor), and TNF-α inhibitors (i.e., infliximab, etanercept, adalimumab) [10]. While many of the older therapeutic modalities were developed empirically, biologics are specifically designed to target key molecules and mechanisms that are involved in the

Table 21.4 Effects of different therapeutic modalities on gene expression in patients with psoriasis

Treatment	Template	General findings	Notable changes	Reference
IL-10	PBMC	934 genes upregulated and 837 genes downregulated *in vivo*, including chemokines, cytokines, surface markers, phagocytosis-related genes	Phagocytosis-related genes generally upregulated; early regulation of chemokines and signal transduction mediators; HO-1 upregulated	[132]
Alefacept	Skin samples	Only histological responders showed marked reductions in the tissue expression of inflammatory genes of the type 1 pathway	Modified genes included IFN-γ, STAT1, MIG, iNOS, IL-8, and IL-23 subunits	[133]
Alefacept	PBMC Skin samples	"Unexpected" early increased expression of proinflammatory genes, including STAT1, IL-8, and MIG in PBMC, and iNOS and IFN-γ in skin; downregulation of CD2, CD69, RANTES, STAT4, and GZMB	Mixed agonist and antagonist effects; decreased CD8+ T-cells; gene expression at baseline and at 6hr treatment were different for nonresponders (↑ CD69, ↑ CD3D) and responders (↑ Foxp3, ↑ CD2, ↑ MYD88)	[23]
Alefacept	PBMC	Upregulation of TOAG1 and downregulation of RHAMM in responding patients	Changes preceded clinical improvement; TOAG1/RHAMM ratio enhanced distinction of responders and nonresponders; Foxp3 and CD69 were nondiscriminatory	[24]
Alefacept	PBMC	A disease response classifier using 23 genes was created to accurately predict response to drug (Fig. 21.1)	Notable genes included CREM, MAFF, WEE1, PLCB1, CLIC1/NCC27, NLRP1, and CCL5/RANTES	[134]
Cyclosporine rhIL-11	Skin samples	159 genes were differentially expressed in the disease state; 41 transcripts rapidly modulated by these drugs	Many of the changes preceded clinical improvement; cyclosporine specifically regulated CCNF, MMP-12, and HBP17; rhIL-11 specifically increased GATA3, CRIP1, and TNXA levels	[25]
Cyclosporine	PBMC Skin samples	220 early response genes in skin downregulated by drug; downregulation of proinflammatory type 1 (p40, IFN-γ, STAT1), Th17 (IL-23, IL-17, IL-22, CCL20, DEFB4, S100A12), and dendritic cell (TNF, iNOS) genes; no changes in PBMC transcripts	Early gene expression changes in response to drug are localized in the skin rather than the blood. Therefore, therapeutic activity of the drug is in the skin, further supporting the assumption that psoriasis is a disease of cutaneous immune modulation rather than circulating lymphocytes. Gene expression correlated with clinical score (i.e., epidermal thickness, PASI)	[135]

Pimecrolimus	PBMC	Downregulation of genes associated with the macrolactam target pathway, cellular activation and proliferation, cellular metabolism, signal transduction, transcription factors, inflammatory mediators, lymphocyte recruitment, chemotaxis, and cellular migration; kallistatin, an inflammation regulating protein, was upregulated; no significant changes in T-cell markers	Absence of a systemic T-cell suppressive effect of the drug; genes that showed changes in expression were not related to side effects or toxicity	[136]
Dithranol	PBMC	18 genes showed differential expression in diseased stage compared with remission stage	17 genes, including IL-8, ANXA3, COX2, G0S2, TNFAIP6, and PBEF, were upregulated in diseased stage; 1 gene, CDKN1C, was upregulated in remission stage	[137]
Phototherapy	Skin samples	315 genes modulated by phototherapy; 248 genes (142 up; 106 down) were changed by Dead Sea treatment, 116 (71 up; 45 down) by NB-UVB, and 49 (37 up; 12 down) were modulated regardless of treatment	Differentially regulated genes included S100 calcium-binding proteins, dendritic cell markers, TNF-α target genes, matrix metalloproteinases, and NF-κB target genes; IGFBP7 is underexpressed in psoriatic epidermis, but is inducible by UVB	[138]

PBMC peripheral blood mononuclear cells, *NB-UVB* narrow band-ultraviolet B, *PASI* Psoriasis Area and Severity Index

pathogenesis of psoriasis. In addition, the recent identification of the IL-23/Th17 axis as a major player in the pathobiology of psoriasis has provided a basis for novel therapeutic strategies in this disease. Anti-IL-12/23p40 monoclonal antibodies (i.e., ustekinumab and ABT-874), which target the p40 common subunit of both IL-23 and IL-12, are now proving to be beneficial in the treatment of patients with psoriasis [21]. Despite advances in our understanding of disease pathogenesis and the availability of newer biologic agents, a significant proportion of patients with psoriasis still do not respond to treatment (Table 21.2). Many drugs are also associated with significant costs and/or toxicities, including side effects related to immunosuppression [10, 22]. Pharmacogenetic and pharmacogenomic markers of treatment response could be employed to individualize care for patients with psoriasis. In this regard, the evaluation of SNPs in drug-metabolizing enzymes, drug transporters, cytokines, and disease susceptibility genes can help determine patient response and risk of toxicity associated with psoriasis treatments (Table 21.3) [10]. Pharmacogenomic approaches, employing whole-genome applications such as cDNA microarray technology, supplemented by quantitative real-time polymerase chain reaction (PCR) in some instances, have been used to investigate global transcript expression associated with responses to currently available treatments, and to distinguish those patients who are likely to respond to a particular therapeutic modality from those who may not (Table 21.4 and Fig. 21.1). In addition, an individual patient's response to a specific treatment

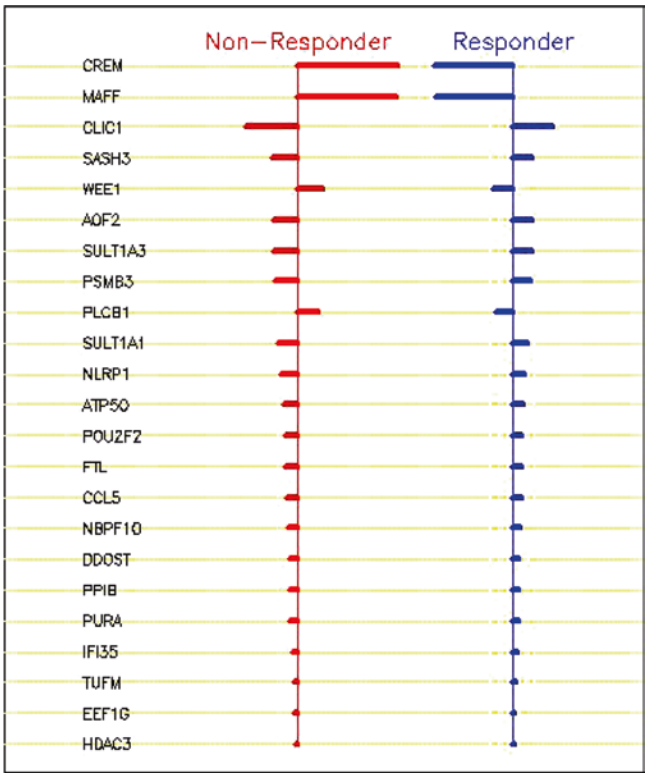

Fig. 21.1 Centroid expression of 23 psoriasis-related genes in a genomic classifier for nonresponders (red) and responders (blue) to Alefacept. *Lines* to the *left* indicate relative increased levels of expression; *lines* to the *right* indicate relative decreased levels of expression. For example, CREM and MAFF are completely opposite in their expression in these two groups (down in nonresponders, up in responders) (Reprinted from Suárez-Fariñas et al. [134], Open Access article)

can change over time, and pharmacogenomic strategies may identify biomarkers of intra-individual drug response variability [10]. Unfortunately, pharmacogenetic/pharmacogenomic data for a number of drugs used in psoriasis patients are currently unavailable (i.e., cyclosporine, methotrexate, acitretin, TNF-α inhibitors, and anti-IL-12/23p40 therapies). Much of the data pertaining to the associations between genetic polymorphisms and drug efficacy and toxicity come from other disease states and conditions (i.e., rheumatoid arthritis, Crohn's disease, and organ transplant recipients) [10]. In some instances, conflicting results have been reported (i.e., significance of baseline CD69 levels in alefacept responsive and nonresponsive patients with psoriasis) [23, 24]. However, some very interesting data have also been published. For instance, drug-induced differences in transcript expression may be restricted to the first hours of therapy and normalize thereafter. Therefore, there may be a short window of opportunity in which to analyze particular genes in order to discriminate between responders and nonresponders to certain drugs (i.e., Foxp3 in patients treated with alefacept) [23]. In such cases, baseline gene expression could be used to identify *a priori* those patients who are likely to benefit from a particular treatment. In other instances, transcript levels could be used to distinguish between responders and nonresponders throughout the course of therapy (i.e., TOAG-1 and RHAMM in patients treated with alefacept) [24]. In addition, changes in gene expression that precede clinical therapeutic response can also be uncovered [24, 25]. These findings may help identify genes that are intimately involved in disease progression, as opposed to those whose expression may simply mirror clinical improvement [25]. The ability to "genomically" identify potential responders would allow for the relatively confident selection and institution of specific therapies. As a corollary, probable nonresponders could be spared ineffective, expensive, and/or potentially toxic medications. Personalized medicine for patients with psoriasis represents the ability to select for appropriate and cost-effective therapy, and adjust dosage in order to maximize benefit and minimize toxicity [10]. With the increased use of molecular technologies, additional genetic factors associated with disease pathogenesis, phenotype, and/or progression will be determined, and novel therapeutic targets will be uncovered. Potential targets include CCR6, IL-22, MAPK, PPAR-γ, selectin, and the JAK-STAT signaling pathway [16, 26]. Some of these concepts are discussed in Chap. 14.

Melanoma

The worldwide incidence of melanoma is increasing faster than that of any other cancer; although, recent evidence suggests that this rise may have peaked [27]. In the United States, invasive melanoma is the sixth most common cancer in men and the seventh in women; the lifetime probability of developing this tumor is 1 in 37 for males and 1 in 56 for females [28]. An estimated 68,130 new cases of cutaneous melanoma were diagnosed in 2010, with 8,700 estimated deaths from this disease [28]. Approximately 84% of cutaneous melanomas are locally confined; 8% of patients are diagnosed after the tumor has spread regionally; and 4% are diagnosed with distant metastasis [28]. For the remaining 4% of patients, the staging information is unknown [28]. The 5-year survival rates for localized, regional, and distant disease are 98%, 62%, and 15%, respectively [28].

The alkylating agent dacarbazine (DTIC) is the only FDA-approved chemotherapeutic agent for treatment of metastatic melanoma; although, responses are infrequently seen (5–10% of patients) and are generally short-lived [29]. Other chemotherapeutic agents, such as carmustine (BCNU), temozolomide, taxanes, and platinum-analogs, have equally poor efficacy in this setting [29]. In addition, there are two FDA-approved biological response modifiers for metastatic melanoma, interleukin-2 (IL-2) and interferon-α2b (IFN-α2b) [29]. Studies report that high-dose IL-2

results in durable responses in only 10–20% of stage IV melanoma patients, and is associated with severe, albeit short-lived, toxicities. IFN-α2b is an approved adjuvant immunotherapy for stage III melanoma, and while demonstrating a 10–20% improvement in relapse-free survival, no clear effect on melanoma-related mortality is seen [29–30]. A large set of genes are found to be differentially regulated in IFN-sensitive and IFN-resistant melanoma cell lines, identifying both sensitivity- and resistance-associated genomic signatures [31–35].

A recent study explored the impact of cytokine gene polymorphisms on clinical outcome for stage IV melanoma patients treated with biochemotherapy (cisplatin, vinblastine, and DTIC; combined with IL-2 and IFN-α) [36]. The IFN-γ +874 SNP was found to be significantly associated with treatment response, progression-free survival, and overall survival [36]. When three gene polymorphisms (IFN-γ +874, IL-10-1082G>A, and ERCC1 codon 118) were combined, four distinct groups of patients with significantly different outcomes were identified [36]. Another study reported that a 32 bp deletion polymorphism in the chemokine receptor 5 gene (CCR5Delta32) was significantly associated with decreased survival in stage IV melanoma patients receiving immunotherapy (IFN, IL-2, or vaccination) [37]. Because current therapy for advanced melanoma utilizes cytotoxic agents and biological response modifiers that mediate tumor regression by different mechanisms, combined testing for multiple polymorphisms could certainly generate more accurate pharmacogenomic information than single SNP analysis.

Significant progress has been made in our understanding of the cellular, molecular, and genetic basis for melanoma. The traditional classification of melanoma into four subtypes (i.e., acral lentiginous, superficial spreading, nodular, and lentigo maligna), which is based on clinical–histopathological features [38], is now being challenged by the results of molecular studies [39–43]. This integration of clinical, morphologic, and genomic data may help to characterize each individual melanoma and guide the selection of novel target-oriented drugs in clinical trials (Fig. 5.1 and Table 21.5).

The mitogen-activated protein kinase (MAPK) signaling pathway (RAS/RAF/MEK/ERK) has been found to be constitutively activated in up to 80–90% of melanomas [44, 45]. The two most common mechanisms for this activation are gain-of-function mutations in either NRAS (15–30% of melanomas) or BRAF (50–70% of melanomas) (Fig. 5.1). Therefore, drugs that target this pathway are of considerable interest (Table 21.5). Since the discovery of BRAF mutations in this tumor, several targeted multikinase inhibitors which decrease BRAF activity have been developed [30, 46–51]. For example, the broad-spectrum multikinase inhibitor sorafenib (BAY 43-9006) targets BRAF, VEGF receptor (VEGFR)-2, VEGFR-3, platelet-derived growth factor receptor (PDGFR)-β, FMS-like tyrosine kinase 3, and KIT. Unfortunately, early clinical studies using sorafenib in melanoma patients, as a single agent or in combination with chemotherapy, demonstrated little benefit beyond disease stabilization [46–51]. Clinical trials are now ongoing with second-generation selective and nonselective RAF inhibitors, such as PLX4032, SB-590885/GSK2118436, XL-281, and RAF-265 ([45–60]; www.clinicaltrials.gov). In recent Phase I and Phase II trials, XL-281 (ARAF, BRAF, and CRAF inhibitor) and PLX4032 ($BRAF^{V600E}$ inhibitor) were shown to have single-agent antitumor activity in patients with melanoma, with the achievement of objective responses (Table 21.5). These studies indicate the potential therapeutic value of single-agent therapy against a mutated oncogene in melanoma. However, not all patients respond to this treatment; and dose-limiting toxicities, primary and secondary drug resistance, and the development of therapy-related cutaneous squamous cell carcinomas/keratoacanthomas (in up to 30% of patients) represent issues that must be addressed. While PLX4032 selectively inhibits downstream MEK/ERK signaling and cellular activation in $BRAF^{V600E}$ mutant cells, it paradoxically activates this signaling pathway in cells with wild-type BRAF. It therefore has the potential to induce carcinogenesis in cells lacking the $BRAF^{V600E}$ mutation. These findings emphasize the requirement for current and future clinical studies of BRAF inhibitors to select for patients who have BRAF-mutant melanomas.

Table 21.5 Published clinical studies in melanoma patients for targeted drugs either alone or in combination with immunotherapy/chemotherapy

Drug	Mechanism of action	Target molecule(s)	Study phase	Reference
BAY 43-9006 (sorafenib)	Nonselective multikinase inhibitor	RAF kinases, VEGFR-2, VEGFR-3, PDGFR, p38MAPK, FLT3, KIT, FMS, RET	II	[139]
			I/II (with carboplatin/ paclitaxel)	[140]
			III (with carboplatin/ paclitaxel)	[141]
			I (with dacarbazine)	[142]
			II (with dacarbazine)	[143, 144]
			II (with temozolomide)	[145]
			I (with IFN-α2a)	[146]
			II (with IFN-α2b)	[147, 148]
Oblimersen	Antisense oligonucleotide	Bcl-2	III (with dacarbazine)	[149]
CCI-779 (temsirolimus)	mTOR inhibitor	mTOR, PTEN/PI3K-AKT pathway	II	[150]
PS-341 (bortezomib)	Proteasome inhibitor	NF-κB	II	[151]
SU5416 (semaxanib)	Kinase inhibitor	VEGFR, FLK-1/KDR	II	[152, 153]
			II (with thalidomide)	[154]
Imatinib mesylate	Tyrosine kinase inhibitor	KIT, PDGFR, BCR-ABL	II	[65–67]
PI-88	Enzyme inhibitor	Heparanase	II	[155]
AZD6244	Kinase inhibitor	MEK	I	[156]
PD-0325901	Kinase inhibitor	MEK	I/II	[157]
MDX-010 (ipilimumab)	Monoclonal antibody	CTLA-4	II	[75]
			II (with dacarbazine)	[75]
			I/II (with IL-2)	[76]
			II (with vaccine)	[77]
			II (with vaccine)	[78]
			III (+/− vaccine)	[79]
CP-675,206 (tremelimumab, formerly ticilimumab)	Monoclonal antibody	CTLA-4	I	[80, 81]
			I/II	[82, 83]
Bevacizumab	Monoclonal antibody	VEGF-A	II (with IFN-α2b)	[158]
17-AAG	HSP90 inhibitor	HSP90	I	[159]
CALGB 500104	Farnesylation transferase inhibition	RAS	I	[160]
XL-281	Kinase inhibitor	RAF kinases (ARAF, BRAF, CRAF)	I	[161]
PLX4032	Kinase inhibitor	$BRAF^{V600E}$	I/II	[162]

A Phase II trial of PLX4032 in $BRAF^{V600E}$ melanoma patients is currently open in the United States and Australia, and a worldwide Phase III randomized trial comparing PLX4032 with DTIC chemotherapy in $BRAF^{V600E}$ melanoma patients is also enrolling. In addition, MEK, which is directly activated by BRAF, is another potential drug target in patients with melanoma (Table 21.5) [46–51].

Imatinib mesylate, a tyrosine kinase inhibitor of BCR-ABL, KIT, and PDGFR, is an FDA-approved treatment for both chronic myelogenous leukemias (which harbor the BCR-ABL fusion

protein) and gastrointestinal stromal tumors (GISTs; which harbor oncogenic KIT and/or PDGFRA mutations) [52]. There is a strong association between specific activating mutations of KIT with clinical responses to imatinib in GISTs and mastocytosis [53–55]. In a study of 102 primary melanomas, KIT mutations were identified in 17% of chronic sun-damaged cutaneous, 11% of acral, and 21% of mucosal melanomas, but not in any melanomas on skin without chronic sun damage; supporting a role for KIT as an oncogene in a subset of tumors [51, 56]. In addition, KIT gene amplification has been found to be present in 6% of chronic sun-damaged, 7% of acral, and 8% of mucosal melanomas [51, 56]. Similar rates of KIT alterations in acral and mucosal melanomas, but lower rates (~2%) in chronic sun-damaged cutaneous tumors are reported by other studies [51]. Point mutations in KIT result in constitutive activation of the c-KIT protein in melanoma cells, and the activation of downstream proliferative and prosurvival signaling pathways [51]. At the protein level, immunohistochemical studies have shown c-KIT expression in 81% of mucosal and acral melanomas [57]. Interestingly, cases with activating mutations are commonly positive for c-KIT protein expression; although, this is not uniformly the case. Furthermore, many tumors that do not have detectable gene mutation or amplification show high expression levels of c-KIT protein [57–59]. Inhibition of KIT signaling has been shown *in vitro* to inhibit proliferation of cultured melanoma cells [60, 61]. In addition, several anecdotal case reports have noted remarkable responses to small molecule KIT inhibitors (imatinib, sorefenib, and dasatinib) in patients with widely metastatic melanoma [51, 62–64]. However, recent Phase II trials of imatinib reported that, among 63 patients with melanoma, only one clinical response was seen (in a patient with an acral tumor) [51, 65–67]. Importantly, these patients' melanomas were not tested for the presence of a KIT (or PDGFRA) mutation, with only c-KIT (and PDGFRA) immunohistochemistry being performed. C-KIT receptor protein expression, in the absence of downstream signaling activity, has not been shown to be highly predictive of clinical response to imatinib [51, 57, 68, 69]. More specifically, KIT mutations, and not gene amplifications, appear to be associated with drug response in melanoma patients [51]. These findings clearly illustrate the importance of proper patient selection prior to imatinib treatment, including KIT and PDGFRA gene mutational analysis. In this regard, a number of multicenter Phase II clinical trials, using imatinib, in addition to sunitinib, nilotinib, and dasatanib, for the treatment of metastatic melanomas with KIT genomic aberrations (i.e., from acral, mucosal, and chronically sun-damaged sites) have been initiated.

The molecular chaperone, heat-shock protein 90 (HSP90), regulates the folding and function of newly synthesized proteins, including the kinases – BRAF, CRAF, CDK4, and CDK6 [70]. Inhibition of HSP90 by 17-allylamino, 17-demethoxygeldanamycin (17-AAG) results in the degradation of its client proteins through the ubiquitin-dependent proteasomal pathway, thereby facilitating the targeting of several pathways simultaneously. Melanoma patients have been found to respond to 17-AAG without significant toxicity (Table 21.5) [71, 72]. Interestingly, NRAS and BRAF mutations seem to influence the response to 17-AAG [72].

Many treatment-responsive patients ultimately relapse as a result of acquired resistance to selective kinase-targeted therapies. This may be due to a number of factors, including alternative activation of MAPK signaling (CRAF bypass signaling), other BRAF mutations or amplifications, mutations in RAS genes (HRAS, KRAS, or NRAS), mutations in MEK1, activation of alternative pathways that may drive proliferation and resistance to apoptosis (PI3K-AKT), or upregulation of escape pathways (CMET, KIT, FGFR, and EGFR) [29, 46–51, 73]. As a result of the intrinsic redundancy in the multiple genetic pathways that are activated in melanoma, it is likely that the use of synergistic combinations of mutation-targeted agents will be required to achieve optimal outcomes and overcome potential drug resistance in patients with metastatic disease [29, 46–51, 73]. In addition to MAPK-related mechanisms, other possible therapeutic targets in melanoma include GNAQ, CDK4, ERBB4, and ETV1, as well as PI3K-AKT, apoptosis, DNA repair, angiogenesis, ubiquitin-proteosome and epigenetic pathways

[29, 46–51]. Clinical trials evaluating novel drugs directed against some of these targets are currently underway [29, 46–51].

Melanoma tumors can demonstrate spontaneous immune-mediated regression [29, 74–83]. In addition, tumor-specific cytotoxic T-cells and antibodies may be found in the peripheral blood of melanoma patients [29, 74–83]. Therefore, immunotherapy could be an effective treatment strategy for individuals with this disease [29, 74–83]. One approach is the enhancement of antimelanoma immune responses through the optimization of T-cell activation. The latter involves interactions between the T-cell receptor (TCR), the costimulatory receptor CD28, and the ligands CD80 and CD86 [29, 74–83]. T-cell inhibition is mediated by the inhibitory receptor, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a molecule that shares 30% structural homology with CD28, and is expressed by activated T-cells and T-regulatory cells (Tregs) [29]. CTLA-4 binds CD80/CD86 with greater affinity than CD28 does, thereby inhibiting CD28-mediated T-cell activation and IL-2 production [29]. CTLA-4 is critical in maintaining immune tolerance to self-antigens, but may also limit host responses to tumor antigens and the efficacy of vaccine therapy. CTLA-4 blockade, either alone or in combination with melanoma-specific vaccines, has been explored as a potential strategy to treat advanced-stage melanoma (Table 21.5) [74–83]. A recent Phase III clinical trial found that patients with advanced, previously treated melanoma who received ipilimumab (MDX-010, a monoclonal antibody targeting CTLA-4), with or without a gp100 peptide vaccine, showed improved overall survival compared with those who received gp100 alone [79]. Importantly, this clinical trial was the first randomized study to show an improvement in overall survival in advanced melanoma, where few treatment options exist [79]. However, not all patients have responded well to CTLA-4 blockade, and some have developed severe autoimmune reactions. Of note, the presence of serum antibodies against the cancer-associated antigen, NY-ESO-1, has been found to be associated with efficacy of anti-CTLA-4 therapy. In addition, metastatic tumors at different sites in an individual patient can demonstrate distinct immunological signatures and local microenvironmental changes, possibly explaining the variable responses to immunotherapy seen in some patients. Variations in the CTLA-4 gene could also influence the response to its inhibition in patients with metastatic melanoma. In a recent study, three SNPs in this gene were found to be associated with responses to CTLA-4 blockade: proximal promoter SNPs, rs4553808 and rs11571327, and the nonsynonymous SNP rs231775 [74]. A haplotype analysis, that included seven SNPs, suggested that the common haplotype TACCGGG is associated with no response, whereas the haplotype TGCCAGG does predict treatment response. Unfortunately, no specific haplotype or SNP predicts which patients will develop the severe autoimmune reactions triggered by CTLA-4 blockade therapy [74]. Other potential immunological approaches in melanoma patients include the use of Toll-like receptor antagonists (i.e., imiquimod) and a HLA-B7/β2-microglobulin gene transfer product [29].

In the future, molecular technologies could be used to determine pathway activation and indicate which combinations of drugs would be most effective in an individual melanoma patient. In this regard, the employment of laser capture-microdissection to isolate both melanoma cells and “normal-appearing” surrounding tissue, followed by nucleic acid extraction and amplification, would facilitate gene expression profiling and genotyping for both germline aberrations and somatic mutations (i.e., those acquired by melanoma cells) in routine surgical specimens. Disease outcome may depend on a combination of both the tumor genome and the inherited germline genome. Determination of germline DNA alterations could be used to assess the host pharmacogenomic profile. This strategy could have important consequences for clinical trial design, with the incorporation of pharmacogenomics into inclusion (and exclusion) criteria. Previous studies of targeted drugs may have failed in part because of inadequate melanoma characterization, resulting in the inclusion of few to no potentially treatment-responsive patients.

Non-Melanoma Skin Cancers

Actinic Keratosis

Actinic keratoses (AKs), cutaneous precancerous lesions commonly found on the skin of middle-aged and elderly individuals at chronically sun-exposed sites, represent the second most common reason for visits to a dermatologist [84–87]. AKs show overlapping phenotypic (histopathological) and genotypic (tumor suppressor gene and proto-oncogene) alterations with squamous cell carcinoma (SCC), and the risk of transformation to SCC, albeit small, correlates with the number of AKs present on an individual per year [84–87]. In a patient with multiple AKs, the risk of progression to SCC increases substantially over the patient's lifetime; although, up to a quarter of AKs may spontaneously regress [87]. A variety of treatment modalities for AKs are available, including topical 5-FU, imiquimod, photodynamic therapy, cryosurgery, curettage, and excision [84–88]. Emerging treatments include ingenol mebutate (PEP005) and COX-1/2 inhibitors [87, 88]. The annual cost of AK management in the United States is estimated to be ~$1.2 billion [87].

5-FU is an antimetabolite chemotherapeutic agent which inhibits the synthesis of thymine, a building block of DNA [84]. It has been used topically for the treatment of AKs for more than 40 years. However, its specific molecular effects in this setting are largely unknown, and much of the pharmacogenomic data for this drug have been obtained from studies of its systemic use in patients with non-cutaneous malignancies [7, 9]. Germline polymorphisms in a number of genes (CYP2A6, DPYD, TYMS, and MTHFR) have been found to influence the response to 5-FU [7, 9]. A recent study by Sachs et al. [84] used real-time reverse transcription (RT)-PCR and enzyme-linked immunosorbent assay to determine the molecular changes induced by topical 5-FU application in AKs and photodamaged skin. Treatment resulted in increased expression of genes associated with epidermal injury (KRT16), inflammation (IL-1β), extracellular matrix degradation (MMP-1 and MMP-3), and procollagens I and III, which were accompanied by improvements in the clinical signs of AKs and photoaging [84]. This study suggested that remodeling of the dermal matrix, which followed epidermal injury and an inflammatory phase of wound healing, is the mechanism for the improved clinical appearance of AKs and photodamaged skin seen with this drug [84].

Imiquimod, an immune response modifier with agonist effects against the Toll-like receptor-7, is approved for the treatment of AKs, in addition to superficial basal cell carcinoma (BCC), and warts (verrucae) [85, 86]. Recently, Torres et al. [86] investigated the mechanism of action of imiquimod, using oligonucleotide microarrays and real-time RT-PCR to determine differential gene expression in AKs before and after topical 5% imiquimod application. Treatment resulted in the upregulation of a wide variety of transcripts involved in both innate and adaptive immune responses, including chemokine, type 1 IFN-inducible and pattern-recognition receptor genes (Fig. 21.2) [86]. The effect of topical imiquimod therapy on gene expression in warts and BCC has also been investigated [89–91].

Basal Cell Carcinoma (BCC)

BCC is the most common malignant neoplasm of the skin, accounting for up to 80% of non-melanoma skin cancers [92, 93]. Ultraviolet radiation exposure is the major risk determinant, but other factors include skin phototype, immunosuppression, and genetic susceptibility (i.e., BCC is increased in patients with xeroderma pigmentosum, albinism, and basal cell nevus syndrome [BCNS; also known as Gorlin syndrome]) [92, 93].

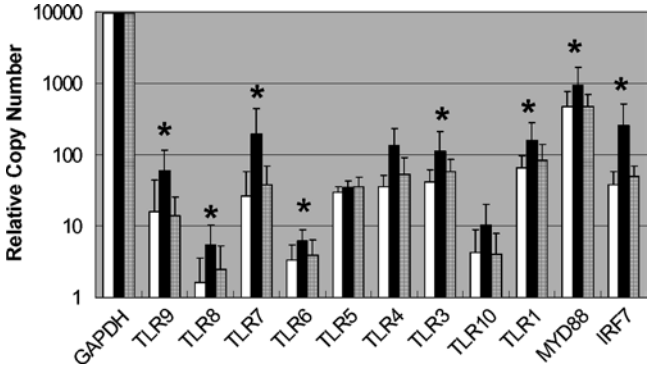

Fig. 21.2 Imiquimod treatment of actinic keratosis (*AK*) increases expression of pattern-recognition receptors of the innate immune system, including *TLR1*, *TLR3*, *TLR6*, *TLR7*, *TLR8*, *TLR9*, *MyD88*, and *IRF7*. Figure illustrates basal TLR, IRF7, and MyD88 gene expression in skin biopsies as determined by real-time RT-PCR. *White bars* represent pre-treatment AK, *black bars* represent during imiquimod treatment (maximum response value from week 1, week 2, and week 4 treatment times), and *hatched bars* represent 4-weeks post-treatment. *Asterisks* indicate those genes that had p-values <0.05, comparing expression in pre-treatment AK samples to the maximum response expression in samples from subjects ($n = 13$) during imiquimod treatment (Reprinted from Torres et al. [86], Open Access article)

Germline and most sporadic BCCs harbor mutations in components of the hedgehog (HH) signaling pathway (Fig. 7.1) [92, 93]. HH, a key regulator of cell growth and differentiation during development, controls epithelial and mesenchymal interactions in many tissues during embryogenesis. PTCH1 is inactivated (loss-of-function mutations) in ~90% of tumors [92, 93]. Without functional PTCH1, a downstream regulator SMO cannot be repressed, and the HH pathway is continuously activated. Up to 10% of BCCs carry gain-of-function mutations in the SMO gene [92, 93]. Constitutive HH pathway signaling is known to promote BCC carcinogenesis [92, 93]. Other genomic aberrations in BCC have been identified in genes which regulate skin color (i.e., MC1R), members of the PI3K-AKT and Wnt pathways, DNA damage repair genes, FoxM1, and TP53 [93].

A wide variety of therapeutic modalites are available for the management of BCCs [12, 90–91]. Fortunately, BCCs are typically characterized by slow, local growth, and surgical excision is curative in most cases. Other invasive procedures include electro-desiccation and curettage, cryosurgery, and Mohs micrographic surgery. A number of topically applied agents, such as imiquimod and 5-FU, are commonly used to treat the superficial subtype of this tumor. A few patients show progression to locally advanced and unresectable [94, 95] or, even more rarely, metastatic disease [96]. Radiotherapy and chemotherapy, alone or in combination, are generally administered to patients in these instances. A greater understanding of the molecular pathogenesis of BCC is necessary in order to identify novel therapeutic and preventive strategies in patients with this tumor.

Urosevic et al. [90] recently identified 1,305 genes that were differentially regulated in BCC as a function of topical imiquimod application. Treatment resulted in the upregulation of opioid growth factor receptor, cytokines, and IFN-α and IFN-γ inducible genes, and downregulation of bcl-2 and transcripts involved in the HH signaling pathway [90]. Wuest et al. [91] used real-time PCR and immunohistochemistry to demonstrate selective transcriptional and/or protein upregulation of Notch pathway members (Notch1, Jagged1, and Delta1) in tumor cells of superficial BCC following treatment with imiquimod.

5-FU is widely used in the treatment of superficial BCC. In addition to its effect as a pyrimidine analog, recent evidence suggests that 5-FU also downregulates target molecules of the HH signaling pathway [93]. Pharmacogenomic studies with this drug have not been undertaken in BCC patients;

although, data are available from the use of 5-FU in patients with systemic malignancies [7, 9]. The outcome of 5-FU-based chemotherapy is influenced by a number of germline polymorphisms involving catabolic (CYP2A6 and DPYD) and target (TYMS and MTHFR) genes.

The potential for chemopreventive and therapeutic approaches in BCC using other currently available and investigational drugs has been recently reviewed [92]. These include topical retinoids, nonsteroidal anti-inflammatory drugs (NSAIDs), DNA repair enhancers, and α-Difluoromethylornithine (DMFO). As for other tumors, many of the treatment strategies for BCC have been developed empirically. However, increasing knowledge of the molecular pathways involved in the pathogenesis of BCC has facilitated the development of a number of potentially effective mechanism-based therapies, such as HH pathway antagonists (anti-SMO), vitamin D3 (VDR transcriptional signaling, anti-SMO), itraconazole (anti-SMO), non-SMO/HH pathway inhibitors (anti-GLI), and FoxM1 inhibitors [92]. A number of Phase I and Phase II clinical trials with some of these medications have been completed or are ongoing in patients with advanced BCC or BCNS [92]. While many of these drugs have been found to be effective in the prevention and/or treatment of BCC, pharmacogenetic/pharmacogenomic data are relatively sparse. For example, COX-2 gene polymorphisms are believed to be associated with BCC risk [92]; however, the effect of these polymorphisms on the response of BCC tumors to COX-2 inhibitors (i.e., NSAIDs) is unknown. An interesting development in BCC-targeted therapy is the emergence of HH pathway-directed drugs. Small molecule inhibitors of HH signaling were first noted to suppress proliferation and induce apoptosis of tumor cells in BCC mouse models [97]. Topical cyclopamine (a naturally occurring SMO inhibitor) was reported to induce rapid regression of four BCCs in a single patient [98]. In a recent Phase I clinical trial, systemic GDC-0449 (a synthetic SMO antagonist) was found to have significant antitumor activity in patients with locally advanced or metastatic BCC [99]. The BCC tumors in this study were found to have elevated mRNA levels of GLI1 (HH target), as well as PTCH1 and SMO gene mutations [99]. GDC-0449 has now entered Phase II testing in patients with advanced BCC and BCNS. However, resistance to GDC-0449 single-agent therapy can occur, secondary to acquired mutations in the SMO gene [100, 101]. The simultaneous use of multiple pathway antagonists (i.e., HH and non-HH) in individuals with BCC may be required for optimal benefit. In addition, regressed BCCs may contain a small population of residual viable cells, some of which could lead to the regrowth of tumors secondary to HH pathway reactivation on drug withdrawal [102]. Therefore, HH signaling should be evaluated in BCCs before and during drug treatment, microscopic review of all clinically regressed tumors should be undertaken, and all patients must be carefully followed on cessation of drug therapy.

Current Issues and Future Perspectives

While pharmacogenomics is important for drug development, its application in drug approval and release is currently under discussion. Indeed, regulatory authorities in Europe (EMEA), the United States (FDA), and Japan (MHLW) have long recognized the significance of pharmacogenomics in drug design, and have already issued guidelines to address the genetic heterogeneity of target patient populations when developing a new chemical entity [103, 104]. To become an effective clinical tool, pharmacogenomics will require the genotyping of large clinical populations in order to better identify those patients, before the initiation of drug treatment, who are likely to either be nonresponders or suffer ADRs. Homogeneity of subpopulations studied is crucial in order to obtain consistent results, as genetic polymorphisms can be population-specific. Sponsoring pharmaceutical companies are now including a genetic extension in many clinical trial protocols, enabling them to collect and store patient samples for genetic analysis.

A potential problem is that some pharmacogenomic tests for drug response determination may also contain important secondary information, such as an individual's predisposition to another disease [105, 106]. The way in which any "discovered" information could be disseminated and used is an obvious issue. Unlike public genetic databases, those of pharmaceutical companies may not be subject to the same governance that ensures transparency and adequate oversight. The ethical, legal, and social implications ("ELSI") of control, ownership, and profit-sharing of banked DNA require responsible debate [107, 108].

An important policy issue is whether existing practices that allow "off-label use" of medications will carry over to pharmacogenomics-based drugs, and whether health insurance companies and drug benefit plans will cover such approaches [109]. One concern is that pharmacogenomics would refocus management to an "easier to treat" subset of patients, and exclude other patients with unfavorable genetic backgrounds, even though a proportion of the latter might still benefit from a particular drug. Moreover, healthcare policy-makers rely on evidence-based medicine and adopt economizing practices, such as restricted formularies, while at the same time expecting the best cost-to-benefit ratio. Although drugs specifically developed for each "genetic" subset of patients may be more expensive, the associated reduction of ineffective and/or harmful prescriptions could help reduce overall healthcare costs. The ultimate goals of pharmacogenomics are to increase therapeutic effectiveness and to prevent ADRs. Individual pharmacogenomic profiles that could identify potential treatment responders would improve recruitment efficiency and allow clinical trials to be smaller, faster, and less expensive [110]. This approach could also rescue drugs that are abandoned at later developmental stages or withdrawn from the market because of severe ADRs in a small number of patients.

Pharmacogenomic testing in the field of dermatology is still in its infancy. As we move forward, the goal of this discipline is the individualization of therapy on the basis of a patient's genotype ("personalized medicine"). This will include the selection of appropriate medication(s), optimization of drug efficacy, and minimization of the risk for ADRs. In addition, pharmacogenomic investigations will facilitate drug development by identifying novel therapeutic targets in patients with skin diseases.

References

1. Spear BB, Heath-Chiozzi M, Huff J. Clinical application of pharmacogenetics. Trends Mol Med. 2001;7:201–4.
2. Griffin JP. The cost of adverse drug reactions. Adverse Drug React Toxicol Rev. 1997;16:75–8.
3. Pirmohamed M, Breckenridge AM, Kitteringham NR, et al. Fortnightly review: adverse drug reactions. BMJ. 1998;316:1295–8.
4. Lazarou J, Pomerantz BH, Corey PN. Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. JAMA. 1998;279:1200–5.
5. Sherry ST, Ward MH, Kholodov M, et al. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res. 2001;29:308–11.
6. The International HapMap Consortium. A haplotype map of the human genome. Nature. 2005;437:1299–320.
7. Huang RS, Ratain MJ. Pharmacogenetics and pharmacogenomics of anticancer agents. CA Cancer J Clin. 2009;59:42–55.
8. Nakamura Y. DNA variations in human and medical genetics: 25 years of my experience. J Hum Genet. 2009;54:1–8.
9. Yamayoshi Y, Iida E, Tanigawara Y. Cancer pharmacogenomics: international trends. Int J Clin Oncol. 2005;10:5–13.
10. Ryan C, Menter A, Warren RB. The latest advances in pharmacogenetics and pharmacogenomics in the treatment of psoriasis. Mol Diagn Ther. 2010;14:81–93.
11. International Conference on Harmonization (ICH) – Guidance for Industry: E15 definitions for genomic biomarkers, pharmacogenomics, pharmacogenetics, genomic data and sample coding categories. http://www.fda.gov/RegulatoryInformation/Guidances/ucm129286.htm. Accessed 15 Aug 2010.

12. Grealy R, Griffiths LR. Current status of pharmacogenomics testing for anti-tumor drug therapies: approaches to non-melanoma skin cancer. Mol Diagn Ther. 2009;13:65–72.
13. Stojadinovic O, Lee B, Vouthounis C, et al. Novel genomic effects of glucocorticoids in epidermal keratinocytes: inhibition of apoptosis, interferon-gamma pathway, and wound healing along with promotion of terminal differentiation. J Biol Chem. 2007;282:4021–34.
14. Nguyen VT, Arredondo J, Chernyavsky AI, et al. Pemphigus vulgaris IgG and methylprednisolone exhibit reciprocal effects on keratinocytes. J Biol Chem. 2004;279:2135–46.
15. Franchimont D, Galon J, Vacchio MS, et al. Positive effects of glucocorticoids on T cell function by upregulation of IL-7 receptor alpha. J Immunol. 2002;168:2212–8.
16. Rácz E, Prens EP. Molecular pathophysiology of psoriasis and molecular targets of antipsoriatic therapy. Expert Rev Mol Med. 2009;11:e38.
17. Elder JT, Bruce AT, Gudjonsson JE, et al. Molecular dissection of psoriasis: integrating genetics and biology. J Invest Dermatol. 2010;130:1213–26.
18. Das RP, Jain AK, Ramesh V. Current concepts in the pathogenesis of psoriasis. Indian J Dermatol. 2009;54:7–12.
19. Asumalahti K, Ameen M, Suomela S, et al. Genetic analysis of PSORS1 distinguishes guttate psoriasis and palmoplantar pustulosis. J Invest Dermatol. 2003;120:627–32.
20. Mossner R, Kingo K, Kleensang A, et al. Association of TNF -238 and -308 promoter polymorphisms with psoriasis vulgaris and psoriatic arthritis but not with pustulosis palmoplantaris. J Invest Dermatol. 2005;124:282–4.
21. Di Cesare A, Di Meglio P, Nestle FO. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. J Invest Dermatol. 2009;129:1339–50.
22. Leon A, Nguyen A, Letsinger J, et al. An attempt to formulate an evidence-based strategy in the management of moderate-to-severe psoriasis: a review of the efficacy and safety of biologics and prebiologic options. Expert Opin Pharmacother. 2007;8:617–32.
23. Haider AS, Lowes MA, Gardner H, et al. Novel insight into the agonistic mechanism of alefacept in vivo: differentially expressed genes may serve as biomarkers of response in psoriasis patients. J Immunol. 2007;178:7442–9.
24. Keeren K, Friedrich M, Gebuhr I, et al. Expression of tolerance associated gene-1, a mitochondrial protein inhibiting T cell activation, can be used to predict response to immune modulating therapies. J Immunol. 2009;183:4077–87.
25. Oestreicher JL, Walters IB, Kikuchi T, et al. Molecular classification of psoriasis disease-associated genes through pharmacogenomic expression profiling. Pharmacogenomics J. 2001;1:272–87.
26. Hedrick MN, Lonsdorf AS, Hwang ST, et al. CCR6 as a possible therapeutic target in psoriasis. Expert Opin Ther Targets. 2010;14:911–22.
27. Lens MB, Dawes M. Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. Br J Dermatol. 2004;150:179–85.
28. Jemal A, Siegel R, Xu J, et al. Cancer statistics, 2010. CA Cancer J Clin. 2010;60:277–300.
29. Ji Z, Flaherty KT, Tsao H. Molecular therapeutic approaches to melanoma. Mol Aspects Med. 2010;31:194–204.
30. Tarhini AA, Agarwala SS. Cutaneaus melanoma: available therapy for metastatic disease, Dermatol. Ther. 2006;19:19–25.
31. Certa U, Wilhelm-Seiler M, Foser S, et al. Expression modes of interferon-alpha inducible genes in sensitive and resistant human melanoma cells stimulated with regular and pegylated interferon-alpha. Gene. 2003;315:79–86.
32. Craven RA, Stanley AJ, Hanrahan S, et al. Identification of proteins regulated by interferon-alpha in resistant and sensitive malignant melanoma cell lines. Proteomics. 2004;4:3998–4009.
33. Certa U, Seiler M, Padovan E, et al. High density oligonucleotide array analysis of interferon-alpha2a sensitivity and transcriptional response in melanoma cells. Br J Cancer. 2001;85:107–14.
34. Certa U, Seiler M, Padovan E, et al. Interferon-a sensitivity in melanoma cells: detection of potential response marker genes. Recent Results Cancer Res. 2002;160:85–91.
35. Krepler C, Certa U, Wacheck V, et al. Pegylated and conventional interferon-alpha induce comparable transcriptional responses and inhibition of tumor growth in a human melanoma SCID mouse xenotransplantation model. J Invest Dermatol. 2004;123:664–9.
36. Liu D, O'Day SJ, Yang D, et al. Impact of gene polymorphisms on clinical outcome for stage IV melanoma patients treated with biochemotherapy: an exploratory study. Clin Cancer Res. 2005;11:1237–46.
37. Ugurel S, Schrama D, Keller G, et al. Impact of the CCR5 gene polymorphism on the survival of metastatic melanoma patients receiving immunotherapy. Cancer Immunol Immunother. 2008;57:685–91.
38. Clark Jr WH, From L, Bernardino EA, et al. The histogenesis and biologic behavior of primary human malignant melanomas of the skin. Cancer Res. 1969;29:705–27.

39. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005;353:2135–47.
40. Bastian BC, LeBoit PE, Hamm H, et al. Chromosomal gains and losses in primary cutaneous melanomas detected by comparative genomic hybridization. Cancer Res. 1998;58:2170–5.
41. Ren S, Liu S, Howell Jr P, et al. The impact of genomics in understanding human melanoma progression and metastasis. Cancer Control. 2008;15:202–15.
42. Gerami P, Mafee M, Lurtsbarapa T, et al. Sensitivity of fluorescence in situ hybridization for melanoma diagnosis using RREB1, MYB, Cep6, and 11q13 probes in melanoma subtypes. Arch Dermatol. 2010;146:273–8.
43. Viros A, Fridlyand J, Bauer J, et al. Improving melanoma classification by integrating genetic and morphologic features. PLoS Med. 2008;5:e120.
44. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature. 2002;417:949–54.
45. Cohen C, Zavala-Pompa A, Sequeira JH, et al. Mitogen-actived protein kinase activation is an early event in melanoma progression. Clin Cancer Res. 2002;8:3728–33.
46. Wellbrock C, Hurlstone A. BRAF as therapeutic target in melanoma. Biochem Pharmacol. 2010;80:561–7.
47. Shepherd C, Puzanov I, Sosman JA. B-RAF inhibitors: an evolving role in the therapy of malignant melanoma. Curr Oncol Rep. 2010;12:146–52.
48. Flaherty KT, Hodi FS, Bastian BC. Mutation-driven drug development in melanoma. Curr Opin Oncol. 2010;22:178–83.
49. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. Nature. 2007;445:851–7.
50. Nathanson KL. Using genetics and genomics strategies to personalize therapy for cancer: focus on melanoma. Biochem Pharmacol. 2010;80:755–61.
51. Davies MA, Samuels Y. Analysis of the genome to personalize therapy for melanoma. Oncogene. 2010;29:5545–55.
52. Lasota J, Miettinen M. Clinical significance of oncogenic KIT and PDGFRA mutations in gastrointestinal stromal tumours. Histopathology. 2008;53:245–66.
53. Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol. 2003;21:4342–9.
54. Frost MJ, Ferrao PT, Hughes TP, et al. Juxtamembrane mutant V560GKit is more sensitive to imatinib (STI571) compared with wild-type c-kit whereas the kinase domain mutant D816VKit is resistant. Mol Cancer Ther. 2002;1:1115–24.
55. Ma YS, Zeng S, Metcalfe DD, et al. The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors; kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations. Blood. 2002;99:1741–4.
56. Curtin JA, Busam K, Pinkel D, Bastian BC. Somatic activation of KIT in distinct subtypes of melanoma. J Clin Oncol. 2006;24:4340–6.
57. Torres-Cabala CA, Wang WL, Trent J, et al. Correlation between KIT expression and KIT mutation in melanoma: a study of 173 cases with emphasis on the acral-lentiginous/mucosal type. Mod Pathol. 2009;22:1446–56.
58. Ashida A, Takata M, Murata H, et al. Pathological activation of KIT in metastatic tumors of acral and mucosal melanomas. Int J Cancer. 2009;124:862–8.
59. Rivera RS, Nagatsuka H, Gunduz M, et al. C-kit protein expression correlated with activating mutations in KIT gene in oral mucosal melanoma. Virchows Arch. 2008;452:27–32.
60. Fisher DE. Microphthalmia: a signal responsive transcriptional regulator in development. Pigment Cell Res. 2000;13 Suppl 8:145–9.
61. Jiang X, Zhou J, Yuen NK, et al. Imatinib targeting of KIT-mutant oncoprotein in melanoma. Clin Cancer Res. 2008;14:7726–32.
62. Hodi FS, Friedlander P, Corless CL, et al. Major response to imatinib mesylate in KIT-mutated melanoma. J Clin Oncol. 2008;26:2046–51.
63. Lutzky J, Bauer J, Bastian BC. Dose-dependent, complete response to imatinib of a metastatic mucosal melanoma with a K642E KIT mutation. Pigment Cell Melanoma Res. 2008;21:492–3.
64. Quintás-Cardama A, Lazar AJ, Woodman SE, et al. Complete response of stage IV anal mucosal melanoma expressing KIT Val560Asp to the multikinase inhibitor sorafenib. Nat Clin Pract Oncol. 2008;5:737–40.
65. Wyman K, Atkins MB, Prieto V, et al. Multicenter Phase II trial of high-dose imatinib mesylate in metastatic melanoma: significant toxicity with no clinical efficacy. Cancer. 2006;106:2005–11.
66. Kim KB, Eton O, Davis DW, et al. Phase II trial of imatinib mesylate in patients with metastatic melanoma. Br J Cancer. 2008;99:734–40.
67. Ugurel S, Hildenbrand R, Zimpfer A, et al. Lack of clinical efficacy of imatinib in metastatic melanoma. Br J Cancer. 2005;92:1398–405.

68. Hofmann UB, Kauczok-Vetter CS, Houben R, et al. Overexpression of the KIT/SCF in uveal melanoma does not translate into clinical efficacy of imatinib mesylate. Clin Cancer Res. 2009;15:324–9.
69. Ivan D, Niveiro M, Diwan AH, et al. Analysis of protein tyrosine kinases expression in the melanoma metastases of patients treated with Imatinib Mesylate (STI571, Gleevec). J Cutan Pathol. 2006;33:280–5.
70. Banerji U. Heat shock protein 90 as a drug target: some like it hot. Clin Cancer Res. 2009;15:9–14.
71. Sharp S, Workman P. Inhibitors of the HSP90 molecular chaperone: current status. Adv Cancer Res. 2006; 95:323–48.
72. Banerji U, Affolter A, Judson I, et al. BRAF and NRAS mutations in melanoma: potential relationships to clinical response to HSP90 inhibitors. Mol Cancer Ther. 2008;7:737–9.
73. Smalley KSM, Haass NK, Brafford P, et al. Multiple signaling pathways must be targeted to overcome therapeutic resistance in cell lines derived from melanoma metastases. Mol Cancer Ther. 2006;5: 1136–44.
74. Breunis WB, Tarazona-Santos E, Chen R, et al. Influence of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) common polymorphisms on outcome in treatment of melanoma patients with CTLA-4 blockade. J Immunother. 2008;31:586–90.
75. Fischkoff S, Hersch E, Weber J, et al. Durable responses and long-term progression-free survival observed in a phase II study of mdx-010 alone or in combination with dacarbazine (DTIC) in metastatic melanoma. 2005 ASCO Annual Meeting Proceedings. J Clin Oncol. 2005;23 Suppl 1:7525.
76. Maker AV, Phan GQ, Attia P, et al. Tumor regression and autoimmunity in patients treated with cytotoxic T lymphocyte-associated antigen 4 blockade and interleukin 2: a phase I/II study. Ann Surg Oncol. 2005;12:1005–16.
77. Attia P, Phan GQ, Maker AV, et al. Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. J Clin Oncol. 2005;23:6043–53.
78. Weber JS, Targan S, Scotland R, et al. Phase II trial of extended dose CTLA-4 antibody ipilimumab (formerly MDX-010) with a multi-peptide vaccine for resected stages IIIC and IV melanoma. 2006 ASCO Annual Meeting Proceedings Part I. J Clin Oncol. 2006;24(Suppl):2510.
79. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363:711–23.
80. Ribas A, Camacho LH, Lopez-Berestein G, et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675, 206. J Clin Oncol. 2005;23:8968–77.
81. Bulanhagui CA, Ribas A, Pavlov D, et al. Phase I clinical trials of ticilimumab: Tumor responses are sufficient but not necessary for prolonged survival. J Clin Oncol. 2006;24:461s.
82. Reuben M, Lee BN, Li C, et al. Biologic and immunomodulatory events after CTLA-4 blockade with ticilimumab in patients with advanced malignant melanoma. Cancer. 2006;106:2437–44.
83. Gomez-Navarro J, Sharma A, Bozon VA, et al. Dose and schedule selection for the anti-CTLA4 monoclonal antibody ticilimumab in patients (pts) with metastatic melanoma. J Clin Oncol. 2006;24:460s.
84. Sachs DL, Kang S, Hammerberg C, et al. Topical fluorouracil for actinic keratoses and photoaging: a clinical and molecular analysis. Arch Dermatol. 2009;145:659–66.
85. Torres A, Storey L, Anders M, et al. Microarray analysis of aberrant gene expression in actinic keratosis: effect of the Toll-like receptor-7 agonist imiquimod. Br J Dermatol. 2007;157:1132–47.
86. Torres A, Storey L, Anders M, et al. Immune-mediated changes in actinic keratosis following topical treatment with imiquimod 5% cream. J Transl Med. 2007;5:7.
87. Fenske NA, Spencer J, Adam F. Actinic keratoses: past, present and future. J Drugs Dermatol. 2010; 9(5 Suppl):s45–9.
88. Ulrich M, Drecoll U, Stockfleth E. Emerging drugs for actinic keratosis. Expert Opin Emerg Drugs. 2010;15:545–55.
89. Jacobs S, Grussendorf-Conen EI, Rösener I, et al. Molecular analysis of the effect of topical imiquimod treatment of HPV 2/27/57-induced common warts. Skin Pharmacol Physiol. 2004;17:258–66.
90. Urosevic M, Oberholzer PA, Maier T, et al. Imiquimod treatment induces expression of opioid growth factor receptor: a novel tumor antigen induced by interferon-alpha? Clin Cancer Res. 2004;10:4959–70.
91. Wuest M, Dummer R, Urosevic M. Induction of the members of Notch pathway in superficial basal cell carcinomas treated with imiquimod. Arch Dermatol Res. 2007;299:493–8.
92. So PL, Tang JY, Epstein EH. Novel investigational drugs for basal cell carcinoma. Expert Opin Investig Drugs. 2010;19:1099–112.
93. Dummer R, Karpova MB, Barysch MJ. Basal cell carcinomas: molecular abnormalities and molecularly targeted therapies. Expert Rev Dermatol. 2009;4:355–69.
94. Walling HW, Fosko SW, Geraminejad PA, et al. Aggressive basal cell carcinoma: presentation, pathogenesis, and management. Cancer Metastasis Rev. 2004;23:389–402.
95. Kovarik CL, Stewart D, Barnard JJ. Lethal basal cell carcinoma secondary to cerebral invasion. J Am Acad Dermatol. 2005;52:149–51.

96. Wadhera A, Fazio M, Bricca G, et al. Metastatic basal cell carcinoma: a case report and literature review. How accurate is our incidence data? Dermatol Online J. 2006;12:7.
97. Williams JA, Guicherit OM, Zaharian BI, et al. Identification of a small molecule inhibitor of the hedgehog signaling pathway: effects on basal cell carcinoma-like lesions. Proc Natl Acad Sci USA. 2003;100:4616–21.
98. Tabs S, Avci O. Induction of the differentiation and apoptosis of tumor cells *in vivo* with efficiency and selectivity. Eur J Dermatol. 2004;14:96–102.
99. Von Hoff DD, LoRusso PM, Rudin CM, et al. Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. N Engl J Med. 2009;361:1164–72.
100. Rudin CM, Hann CL, Laterra J, et al. Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449. N Engl J Med. 2009;361:1173–8.
101. Yauch RL, Dijkgraaf GJ, Alicke B, et al. Smoothened mutation confers resistance to a Hedgehog pathway inhibitor in medulloblastoma. Science. 2009;326:572–4.
102. Dlugosz AA, Talpaz M. Following the hedgehog to new cancer therapies. N Engl J Med. 2009;361:1202–5.
103. US Food and Drug Administration. Drug development and drug interactions: regulatory guidance and manual for policies and procedures. http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093606.html. Accessed 11 Feb 2010.
104. Ishiguro A, Toyoshima S, Uyama Y. Current Japanese regulatory situations of pharmacogenomics in drug administration. Expert Rev Clin Pharmacol. 2008;1:505–14.
105. Netzer C, Biller-Andorno N. Pharmacogenetic testing, informed consent and the problem of secondary information. Bioethics. 2004;18:344–60.
106. Roses AD. Pharmacogenetics and future drug development and delivery. Lancet. 2000;355:1358–61.
107. Nuffield Council on Bioethics. Pharmacogenetis: ethical issues. London: Nuffield Council on Bioethics; 2003. http://www.nuffieldbioethics.org/go/ourwork/pharmacogenetics/publication_314.html. Accessed 11 Feb 2010.
108. Marks AD, Steinberg KK. The ethics of access to online genetic databases: private or public? Am J Pharmacogenomics. 2002;2:207–12.
109. Roses AD. Pharmacogenetics and the practice of medicine. Nature. 2002;405:857–65.
110. Fijal BA, Hall JM, Witte JS. Clinical trials in the genomic era: effects of protective genotypes on sample size and duration of trial 2000. Control Clin Trials. 2000;21:7–20.
111. Gudjonsson JE, Karason A, Runarsdottir EH, et al. Distinct clinical differences between HLA-Cw*0602 positive and negative psoriasis patients – an analysis of 1019 HLA-C- and HLA-B-typed patients. J Invest Dermatol. 2006;126:740–5.
112. Reich K, Mossner R, Konig IR, et al. Promoter polymorphisms of the genes encoding tumor necrosis factor-alpha and interleukin-1beta are associated with different subtypes of psoriasis characterized by early and late disease onset. J Invest Dermatol. 2002;118:155–63.
113. Kingo K, Kõks S, Silm H, et al. IL-10 promoter polymorphisms influence disease severity and course in psoriasis. Genes Immun. 2003;4:455–7.
114. Hensen P, Asadullah K, Windemuth C, et al. Interleukin-10 promoter polymorphism IL10.G and familial early onset psoriasis. Br J Dermatol. 2003;149:381–5.
115. Craven NM, Jackson CW, Kirby B, et al. Cytokine gene polymorphisms in psoriasis. Br J Dermatol. 2001;144:849–53.
116. Wongpiyabovorn J, Hirankarn N, Ruchusatsawat K, et al. Association of the interleukin-10 distal promoter (-2763A/C) polymorphism with late-onset psoriasis. Clin Exp Dermatol. 2008;33:186–9.
117. Young HS, Summers AM, Bhushan M, et al. Single-nucleotide polymorphisms of vascular endothelial growth factor in psoriasis of early onset. J Invest Dermatol. 2004;122:209–15.
118. Young HS, Summers AM, Read IR, et al. Interaction between genetic control of vascular endothelial growth factor production and retinoid responsiveness in psoriasis. J Invest Dermatol. 2006;126:453–9.
119. Nair RP, Duffin KC, Helms C, et al. Collaborative Association Study of Psoriasis. Genome-wide scan reveals association of psoriasis with IL-23 and NF-kappaB pathways. Nat Genet. 2009;41:199–204.
120. Capon F, Di Meglio P, Szaub J, et al. Sequence variants in the genes for the interleukin-23 receptor (IL23R) and its ligand (IL12B) confer protection against psoriasis. Hum Genet. 2007;122:201–6.
121. Cargill M, Schrodi SJ, Chang M, et al. A large-scale genetic association study confirms IL12B and leads to the identification of IL23R as psoriasis-risk genes. Am J Hum Genet. 2007;80:273–90.
122. Liu Y, Helms C, Liao W, et al. A genome-wide association study of psoriasis and psoriatic arthritis identifies new disease loci. PLoS Genet. 2008;4:e1000041.
123. Smith RL, Warren RB, Eyre S, et al. Polymorphisms in the IL-12beta and IL-23R genes are associated with psoriasis of early onset in a UK cohort. J Invest Dermatol. 2008;128:1325–7.
124. Duffin KC, Krueger GG. Genetic variations in cytokines and cytokine receptors associated with psoriasis found by genome-wide association. J Invest Dermatol. 2009;129:827–33.
125. Criswell LA, Lum RF, Turner KN, et al. The influence of genetic variation in the HLA-DRB1 and LTA-TNF regions on the response to treatment of early rheumatoid arthritis with methotrexate or etanercept. Arthritis Rheum. 2004;50:2750–6.

126. Padyukov L, Lampa J, Heimburger M, et al. Genetic markers for the efficacy of tumour necrosis factor blocking therapy in rheumatoid arthritis. Ann Rheum Dis. 2003;62:526–9.
127. Schotte H, Schluter B, Drynda S, et al. Interleukin 10 promoter microsatellite polymorphisms are associated with response to long term treatment with etanercept in patients with rheumatoid arthritis. Ann Rheum Dis. 2005;64:575–81.
128. Louis E, El Ghoul Z, Vermeire S, et al. Association between polymorphism in IgG Fc receptor IIIa coding gene and biological response to infliximab in Crohn's disease. Aliment Pharmacol Ther. 2004;19:511–9.
129. Pierik M, Vermeire S, Steen KV, et al. Tumour necrosis factor-alpha receptor 1 and 2 polymorphisms in inflammatory bowel disease and their association with response to infliximab. Aliment Pharmacol Ther. 2004;20:303–10.
130. Taylor KD, Plevy SE, Yang H, et al. ANCA pattern and LTA haplotype relationship to clinical responses to anti-TNF antibody treatment in Crohn's disease. Gastroenterology. 2001;120:1347–55.
131. Urcelay E, Mendoza JL, Martinez A, et al. IBD5 polymorphisms in inflammatory bowel disease: association with response to infliximab. World J Gastroenterol. 2005;11:1187–92.
132. Jung M, Sabat R, Krätzschmar J, et al. Expression profiling of IL-10-regulated genes in human monocytes and peripheral blood mononuclear cells from psoriatic patients during IL-10 therapy. Eur J Immunol. 2004;34:481–93.
133. Chamian F, Lowes MA, Lin SL, et al. Alefacept reduces infiltrating T cells, activated dendritic cells, and inflammatory genes in psoriasis vulgaris. Proc Natl Acad Sci USA. 2005;102:2075–80.
134. Suárez-Fariñas M, Shah KR, Haider AS, et al. Personalized medicine in psoriasis: developing a genomic classifier to predict histological response to Alefacept. BMC Dermatol. 2010;10:1.
135. Haider AS, Lowes MA, Suárez-Fariñas M, et al. Identification of cellular pathways of "type 1," Th17 T cells, and TNF- and inducible nitric oxide synthase-producing dendritic cells in autoimmune inflammation through pharmacogenomic study of cyclosporine A in psoriasis. J Immunol. 2008;180:1913–20.
136. Rappersberger K, Komar M, Ebelin ME, et al. Pimecrolimus identifies a common genomic anti-inflammatory profile, is clinically highly effective in psoriasis and is well tolerated. J Invest Dermatol. 2002;119:876–87.
137. Koczan D, Guthke R, Thiesen HJ, et al. Gene expression profiling of peripheral blood mononuclear leukocytes from psoriasis patients identifies new immune regulatory molecules. Eur J Dermatol. 2005;15:251–7.
138. Hochberg M, Zeligson S, Amariglio N, et al. Genomic-scale analysis of psoriatic skin reveals differentially expressed insulin-like growth factor-binding protein-7 after phototherapy. Br J Dermatol. 2007;156:289–300.
139. Eisen T, Ahmad T, Flaherty KT, et al. Sorafenib in advanced melanoma: a Phase II randomised discontinuation trial analysis. Br J Cancer. 2006;95:581–6.
140. Flaherty KT, Brose M, Schuchter LM, et al. Sorafenib combined with carboplatin and paclitaxel for metastatic melanoma: progression-free survival and response versus B-raf status. Proc 4th International Conference on Targeted Anti-Cancer Therapy (TAT) 2006. Ann Oncol. 2006;17 Suppl 3:iii33.
141. Agarwala SS, Keilholz U, Hogg D, et al. Randomized phase III study of paclitaxel plus carboplatin with or without sorafenib as second-line treatment in patients with advanced melanoma. 2007 ASCO Annual Meeting Proceedings Part I. J Clin Oncol. 2007;25 Suppl 18:8510.
142. Eisen T, Ahmad T, Marais R, et al. Phase I trial of sorafenib (BAY-43-9006) combined with dacarbazine (DTIC) in patients with metastatic melanoma. Eur J Cancer Suppl. 2005;3:349.
143. McDermott DF, Sosman JA, Gonzalez R, et al. Double-blind randomized phase II study of the combination of sorafenib and dacarbazine in patients with advanced melanoma: a report from the 11715 Study Group. J Clin Oncol. 2008;26:2178–85.
144. Lorigan P, Corrie P, Chao D, et al. Phase II trial of sorafenib combined with dacarbazine in metastatic melanoma patients [abstract]. J Clin Oncol. 2006;24:8012.
145. Amaravadi R, Schuchter LM, McDermott DF, et al. Updated results of a randomized phase II study comparing two schedules of temozolomide in combination with sorafenib in patients with advanced melanoma. J Clin Oncol (ASCO Annu Meet Proc). 2007;25:8527.
146. Robert C, Lassau N, Angevin E, et al. Phase I trial of sorafenib (BAY 43-9006) in combination with interferon alpha 2a in patients with unresectable and/or metastatic renal cell carcinoma and malignant melanoma. Eur J Cancer Suppl. 2005;3:254.
147. Gollob JA, Rathmell WK, Richmond TM, et al. Phase II trial of sorafenib plus interferon alfa-2b as first- or second-line therapy in patients with metastatic renal cell cancer. J Clin Oncol. 2007;25:3288–95.
148. Ryan CW, Goldman BH, Lara Jr PN, et al. Sorafenib with interferon alfa-2b as first-line treatment of advanced renal carcinoma: a phase II study of the Southwest Oncology Group. J Clin Oncol. 2007;25:3296–301.
149. Bedikian AY, Millward M, Pehamberger H, et al. Oblimersen Melanoma Study Group. Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. J Clin Oncol. 2006;24:4738–45.
150. Margolin K, Longmate J, Baratta T, et al. CCI-779 in metastatic melanoma: a phase II trial of the California Cancer Consortium. Cancer. 2005;104:1045–8.

151. Markovic SN, Geyer SM, Dawkins F, et al. A phase II study of bortezomib in the treatment of metastatic malignant melanoma. Cancer. 2005;103:2584–9.
152. Kuenen BC, Tabernero J, Baselga J, et al. Efficacy and toxicity of the angiogenesis inhibitor SU5416 as a single agent in patients with advanced renal cell carcinoma, melanoma, and soft tissue sarcoma. Clin Cancer Res. 2003;9:1648–55.
153. Peterson AC, Swiger S, Stadler WM, et al. Phase II study of the Flk-1 tyrosine kinase inhibitor SU5416 in advanced melanoma. Clin Cancer Res. 2004;10:4048–54.
154. Mita MM, Rowinsky EK, Forero L, et al. A phase II, pharmacokinetic, and biologic study of semaxanib and thalidomide in patients with metastatic melanoma. Cancer Chemother Pharmacol. 2007;59:165–74.
155. Lewis KD, Robinson WA, Millward MJ, et al. A phase II study of the heparanase inhibitor PI-88 in patients with advanced melanoma. Invest New Drugs. 2008;26:89–94.
156. Adjei AA, Cohen RB, Franklin W, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral, small-molecule mitogen-activated protein kinase kinase 1/2 inhibitor AZD6244 (ARRY-142886) in patients with advanced cancers. Clin Oncol. 2008;26:2139–46.
157. Lorusso P, Krishnamurthi S, Rinehart JR, et al. A phase 1-2 clinical study of a second generation oral MEK inhibitor, PD 0325901 in patients with advanced cancer [abstract 3011]. J Clin Oncol. 2005;23:16s.
158. Varker KA, Biber JE, Kefauver C, et al. A randomized phase 2 trial of bevacizumab with or without daily low-dose interferon alfa-2b in metastatic malignant melanoma. Ann Surg Oncol. 2007;14:2367–76.
159. Banerji U, O'Donnell A, Scurr M, et al. Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J Clin Oncol. 2005;23:4152–61.
160. Gajewski TK, Johnson J, Linette G, et al. Phase II study of the farnesyltransferase inhibitor R115777 in advanced melanoma: CALGB 500104. J Clin Oncol. 2006;24:18S.
161. Schwartz GK, Robertson S, Shen A, et al. A phase I study of XL281, a selective oral RAF kinase inhibitor, in patients (Pts) with advanced solid tumors [abstract 3513]. J Clin Oncol. 2009;27(Suppl):15.
162. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med. 2010;363:809–19.

Chapter 22
Pharmacogenetics and Pharmacogenomics II: Genetic Determinants of Drug Responses and Adverse Drug Reactions

Stephane Dalle, Sandra Knowles, and Neil H. Shear

The ability to predict efficacy and safety is crucial for drug discovery and development. To date, there are only a few genetic biomarkers whose clinical validity in predicting drug response has been clearly established; for example, HER-2/neu-positivity in breast cancer as a predictor of response to trastuzumab (Herceptin) [1]. Patient variability in response to medications can range from failure to demonstrate an expected therapeutic effect to development of an adverse reaction, resulting in significant patient morbidity and mortality, as well as increasing healthcare costs. In order to optimize treatment response and decrease adverse drug reactions, pharmacogenetics and pharmacogenomics are being increasingly utilized. Pharmacogenetics is the study of genetic variation and its effects on the response to drugs, vaccines, or other pharmaceutical agents [2]. Pharmacogenomics more broadly involves genome-wide analysis of the genetic determinants of drug efficacy and toxicity. Primary candidate genes of interest include those encoding for drug receptors, metabolizing enzymes, and transporters. However, selection of optimal drug therapy may also involve disease susceptibility genes indirectly affecting drug responses [3].

Despite increasing investment in drug discovery and development, only one in ten new pharmaceuticals which progresses to clinical testing will subsequently reach the commercial market. Approximately half of all drug failures are attributed to problems with efficacy and toxicity, not anticipated from preclinical studies [4]. Pharmacogenetics is now routinely used and requested during the drug development process, at both pre-approval and post-approval stages [5]. The goal of both pharmacogenetics and pharmacogenomics is to personalize therapy based on an individual's genotype and to better tailor each treatment for a given patient (both from an efficacy and safety viewpoint).

Dermatologists are at the clinical frontline to recognize, treat, and provide their expertise when cutaneous adverse reactions occur. In addition, various potentially toxic medications are used in the treatment of serious dermatologic disorders. This chapter provides an overview of pharmacogenetics and pharmacogenomics for the practicing physician vis-à-vis genetic determinants of drug response and adverse drug reactions.

S. Dalle (✉)
Service de Dermatologie, Centre Hospitalier Lyon-Sud, Université Claude Bernard Lyon I,
165 chemin du grand Revoyet, 69495 Pierre-Bénite, France
e-mail: stephane.dalle@chu-lyon.fr

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_22,

Drug Used in the Treatment of Dermatologic Diseases

Dapsone

Hematologic toxicities, especially drug-related hemolytic anemia and methemoglobinemia, are occasionally associated with dapsone use, primarily in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency. G6PD deficiency is the most commonly inherited human enzyme defect. Long-term administration of dapsone at standard doses in patients with intact G6PD can result in the development of methemoglobinemia in 15% of cases, which is generally not clinically significant. The hematotoxicity of dapsone is not caused by the drug itself, but by its hydroxylamine metabolites. These are formed through N-hydroxylation in the liver via the actions of various cytochrome P450 enzymes, including CYP3A4, CYP2E1, and CYP2C9. Dapsone hydroxylamine depletes glutathione within G6PD-deficient cells. The nitroso derivative then causes peroxidation reactions, leading to rapid hemolysis. Studies have investigated the utility of co-administering cimetidine (a CYP3A4 inhibitor) with dapsone. This could potentially reduce the risk of methemoglobinemia by decreasing production of the intermediate N-hydroxylated metabolites. Coleman et al. demonstrated that cimetidine decreased methemoglobin levels by 27% in patients concurrently on dapsone for the treatment of dermatitis herpetiformis [6–9]. It has been suggested that cimetidine be used in combination with dapsone, particularly when doses of the latter exceed 200 mg daily [8].

Azathioprine

Azathioprine is actively used in dermatology for immunobullous diseases, severe atopic dermatitis, chronic actinic dermatitis, and various other skin conditions. However, azathioprine has a capacity to cause profound myelosuppression. Azathioprine is a prodrug that is metabolized to its active metabolite, 6-thioguanine, through a series of enzymatic steps. Thiopurine methyl transferase (TPMT) is one of the two principal metabolic pathways that convert azathioprine metabolites to inactive compounds. Low or absent TPMT activity correlates with an increased risk of severe profound neutropenia, as a result of excessive accumulation of intracellular 6-thioguanine [10]. TPMT gene polymorphisms can affect its enzymatic activity and the ability to detoxify azathioprine. The TPMT gene is thought to follow an autosomal co-dominant inheritance pattern and its activity appears to be trimodal: less that 1% (1 in 300 individuals) are homozygous for very low TPMT activity ($TPMT^{L}/TPMT^{L}$); roughly 10% are heterozygous with intermediate activity ($TPMT^{H}/TPMT^{L}$); and approximately 90% are homozygous, demonstrating the high methylator phenotype ($TPMT^{H}/TPMT^{H}$) [11]. TPMT activity can be determined in peripheral red blood cell lysates and testing for these polymorphisms can be useful in preventing azathioprine toxicities. Due to the significantly increased risk of developing myelosuppression, clinicians have advocated the assessment of TPMT status prior to prescribing azathioprine. The British Association of Dermatologists recommends that "pre-treatment TPMT measurements should be performed in all patients prescribed azathioprine for treatment of dermatological conditions" [12]. TPMT testing is routine in many jurisdictions, but may not be available or may be too expensive in others. Prudent practice dictates the use of genetic testing and the cautious introduction of azathioprine.

Methotrexate

Methotrexate is a first-line systemic therapy for psoriasis, although in approximately 20% of patients the response is moderate to poor. In addition, up to 30% of patients are unable to continue

Table 22.1 Pharmacogenetics impact on drug efficacy

Drug	Mechanism of action	Implicated gene	Polymorphism	Impact
Adalimumab	Anti-TNF-alpha mAb	TNF-alpha promoter gene	238GG, 308GG, 857CC	Increased response
Etanercept	Anti-TNF-alpha fusion protein	TNF-alpha promoter gene	308 GG	Increased response
			308 AG	Decreased response
		Interleukin-10 gene	1087 GG	Increased response
			1087 AG	Decreased response
Infliximab	Anti-TNF-alpha mAb	TNF-alpha promoter gene	308 GG	Increased response
			308 AG	Decreased response
			308 AA	Decreased response
Methotrexate	Antimetabolite Antifolate	TS	TS 5′-UTR 3R allele	Decreased response

mAb monoclonal antibody
TNF tumor necrosis factor
TS thymidylate synthase

therapy as a result of hepatotoxicity and gastrointestinal toxicity [13]. Studies in patients with psoriasis and rheumatoid arthritis suggest that functional single nucleotide polymorphisms (SNPs) in genes relevant to methotrexate metabolism may influence both efficacy and toxicity of this drug (Table 22.1). Recent studies have demonstrated that identification of specific polymorphisms of enzymes involved in folate, pyrimidine, and purine metabolism could be useful in predicting clinical response and toxicity to methotrexate in patients with psoriasis [14–16]. The thymidylate synthase (TS) 5′-untranslated region (UTR) 3R/3R homozygous genotype is strongly associated with adverse events in psoriasis patients who take methotrexate without folic acid. In addition, the 3′-UTR 6 bp deletion is significantly associated with adverse events, regardless of folic acid supplementation. Furthermore, variations in pharmacodynamic factors can also influence methotrexate treatment outcomes. It has been shown that response and toxicity to methotrexate are significantly correlated with SNPs in the ABCC1 (ATP-binding cassette, subfamily C, member 1) and ABCG2 (ATP-binding cassette, subfamily G, member 2) efflux transporter genes [15]. It must be noted that most of these studies have been undertaken in patients with rheumatologic diseases. TS polymorphisms need to be tested in large groups of dermatology patients in order to verify their ability to predict adverse drug reactions in this cohort [17].

TNF-Alpha Blockers

Tumor necrosis factor (TNF)-alpha-neutralizing strategies represent a major breakthrough in the treatment of patients with psoriasis. Systemic therapy with TNF-alpha blockers, methotrexate, or both agents, is now considered standard care for patients with moderate to severe psoriatic arthropathy, that is extensive or aggressive in nature or which significantly impacts quality of life [18, 19]. However, there is significant heterogeneity in patient response to the TNF-alpha blockers. Since therapy with these agents is expensive and bears potential risks, predictors of treatment response would be clinically useful in order to select the best therapeutic approach for each patient. The impact of genetic variation on response to TNF-alpha blockers is currently under evaluation (Table 22.1). TNF-alpha gene polymorphisms have been shown to predict therapeutic response to TNF-alpha blockers in rheumatoid arthritis, spondyloarthritis, and psoriatic arthritis patients. Most pharmacogenomic studies have focused on TNF-alpha [20, 21] and TNF-alpha receptor [22] genes and promoter polymorphisms. The prevalence of these TNF polymorphisms varies widely according to the patient's ethnic origin [23].

Several SNPs of interest have been identified in the human TNF-alpha gene [24]. The most well-documented of these SNPs are at position −308 of the TNF-alpha gene promoter and involve the substitution of guanine (G) for adenine (A), with the creation of two alleles (TNF*1 (G) and TNF*2 (A)) and three genotypes (GG, GA, and AA) [25]. For patients with rheumatoid arthritis, some studies have found that the 308 TNF-alpha promoter GG genotype correlates with better response rates, while others have found no association [26, 27]. A recent meta-analysis found an increased frequency of the A allele in patients who did not respond to anti-TNF therapy [27]. A significant association between the TNF-alpha promoter 308 AG polymorphism and responsiveness to anti-TNF therapy was noted, suggesting that individuals who carry the A allele have a poorer response to anti-TNF therapy than those with the G allele [27]. Other studies have also shown this response variability for infliximab [28] and adalimumab [29]. In addition, a combination of diplotypes 308 GG in the TNF-alpha promoter and 1087 GG in the interleukin-10 gene is associated with better responses to etanercept (anti-TNF fusion recombinant protein) [30]. Furthermore, patients with the GG genotype for the exon 6 TNF receptor-II polymorphism show a poorer response to anti-TNF-alpha therapy [31]. Additionally, differences in pharmacogenetics between monoclonal antibodies (i.e., infliximab) and the soluble receptor (i.e., etanercept) may exist.

Response variation could also be linked to the level of functional circulating TNF-alpha. Its concentration is partially genetically determined (308 TNF-alpha gene polymorphism), and is predictive of the clinical response [32]. Other recent investigations of the TNF receptor superfamily 1b (TNFSF1b) gene failed to correlate the 676 T > G polymorphism with either response to therapy (adalimumab, infliximab) or disease severity [26, 33]. Additionally, Fc-gamma receptor type IIIA polymorphisms have been shown to influence therapeutic response in patients with either rheumatoid or psoriatic arthritis treated with TNF-blocking agents [34, 35].

These studies illustrate the highly relevant role of pharmacogenomics and pharmacogenetics in determining a patient's response to TNF-blockers. Much of this data has been obtained in patients with rheumatoid arthritis, psoriatic arthritis, and Crohn's disease. For these patients, pharmacogenetics has the potential to optimize therapy and improve clinical outcome. However, large-scale investigations are required before a pharmacogenetic approach is applicable to routine clinical practice [36, 37]. Furthermore, studies are needed in patients with dermatologic conditions, such as psoriasis, before data can be extrapolated to this group of individuals.

Cutaneous Adverse Drug Events

Genetic factors have long been postulated to be important in drug hypersensitivity, but only recently have some specific mechanisms/pathways been elucidated. Given the pivotal role of the major histocompatibility complex (MHC) in the immune response, many of the early studies on drug hypersensitivity reactions focused on human leukocyte antigen (HLA) phenotyping (Table 22.2). The genetic associations may be: (1) drug specific (i.e., HLA-B*5701 is associated with abacavir hypersensitivity reaction); (2) ethnic and drug specific [i.e., carbamazepine-induced Stevens–Johnson syndrome-toxic epidermal necrolysis spectrum (SJS/TEN) is associated with HLA-B*1502 in Asians, but not in Caucasians]; or (3) phenotype, ethnic, and drug specific (HLA-B*1502 is associated with carbamazepine-induced SJS/TEN, but not exanthematous rashes or drug-induced hypersensitivity reactions).

Although the pathogenesis of SJS/TEN has not been fully elucidated, the extreme rarity of these drug reactions has pointed to individual susceptibility. Fas ligand [38] and Toll-like receptor 3 [39] gene polymorphisms have been recently studied in Japanese populations. Some of these polymorphisms have been found in association with SJS/TEN in this ethnic group. Confirmatory studies are needed, as well as screening of other non-japanese cohorts.

Table 22.2 Susceptibility genes associated with cutaneous drug eruptions

Drug	Clinical presentation	Genetics	Population
Abacavir	Hypersensitivity syndrome	HLA-B*5701 allele	White, Spanish, Thai
Allopurinol	Hypersensitivity syndrome, SJS	HLA-B*5801 allele	Han Chinese, Europeans, Japanese
Carbamazepine	SJS/TEN	HLA-B*1502 allele	Han Chinese, European
Cetuximab	Skin rash	Fc-gamma-RIIA Fc-gamma-RIIIA	Not determined
Erlotinib	Skin rash	ABCG2 promoter EGFR intron 1	Not determined
Nevirapine	Skin rash	HLA-DRB101 allele HLA-B*3505 allele	Australian, European Whites, Thai

SJS/TEN Stevens-Johnson syndrome-toxic epidermal necrolysis

Abacavir

Cutaneous hypersensitivity to abacavir has been linked with HLA phenotype. Abacavir is a nucleoside reverse-transcriptase inhibitor with activity against the human immunodeficiency virus (HIV). It is available for once daily use in combination with other antiretroviral agents. The most important adverse effect of abacavir is a hypersensitivity reaction, affecting 2–9% of patients during the first weeks of treatment [40, 41]. Symptoms and signs of this hypersensitivity reaction to abacavir include combinations of fever, constitutional symptoms, gastrointestinal and respiratory tract symptoms, and skin rash that become more severe with continued dosing. This reaction to abacavir is strongly associated with the presence of the HLA-B*5701 allele. At least two levels of specificity single out HLA-B*5701 as a genetic determinant of this drug hypersensitivity. The first point of specificity occurs during drug targeting of endogenous proteins or peptides to create a unique ligand. This step considerably narrows the pool of potential ligands available for host MHC-I molecules. The second layer of specificity occurs through selective binding and presentation of one or more of these ligands by particular MHC-I molecules, as a result of the polymorphic nature of the antigen-binding cleft [42].

HLA-B*5701 screening, prior to initiation of abacavir, with the subsequent exclusion from treatment of any positive patients, may help to reduce the risk of hypersensitivity reactions to this drug [43]. The US Department of Health and Human Services "Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents" now recommends screening for HLA-B*5701 before initiating treatment with an abacavir-containing regimen. HLA-B*5701-positive patients should not be prescribed abacavir [44].

In addition, patch testing has been used both as a research tool in pharmacogenetic studies and as an adjunctive test to identify patients with abacavir hypersensitivity reactions – demonstrating 100% sensitivity for HLA-B*5701 in these settings. Patients generally have positive patch tests for at least 6 years after experiencing an abacavir hypersensitivity reaction [45].

Nevirapine

Nevirapine is a potent non-nucleoside reverse-transcriptase inhibitor that is frequently used as one of the basic components for highly active antiretroviral therapy. However, nevirapine induces cutaneous adverse drug reactions in 15–20% of cases [46]. The skin reactions range from a mild, localized maculopapular rash to a diffuse maculopapular eruption and/or generalized bullous

lesions. Severe fatal reactions, including SJS/TEN, have been observed in 0.3% of patients [47]. Some patients may also develop fever and internal organ involvement. Nevirapine-associated hypersensitivity commonly occurs within 14–21 days of treatment initiation, and is more rapid and severe with nevirapine rechallenge. In an Australian population, the presence of a HLA-DRB101 allele was associated with an increased risk of hypersensitivity syndrome [48]. These results were subsequently confirmed in a French population [49]. However, susceptibility HLA-alleles may vary according to the population analyzed. For example, a recent study undertaken in Thai patients identified HLA-B*3505 as a marker of nevirapine-induced rash [50].

Allopurinol

Allopurinol, which is used in the treatment of gout and hyperuricemia, is a frequent cause of severe drug hypersensitivity reactions. Allopurinol-induced severe cutaneous eruptions are associated with a strong genetic predisposition. Genetic polymorphisms in the MHC region, particularly the HLA-B*5801 allele, are highly associated with individuals at risk for allopurinol-induced hypersensitivity reactions, SJS/TEN or TEN [51]. These findings were first demonstrated in a Han Chinese population, and subsequently confirmed in European and Japanese cohorts [52, 53].

Carbamazepine

Carbamazepine is a widely prescribed anticonvulsant often associated with drug-induced hypersensitivity syndrome (DIHS), also called DRESS (drug rash with eosinophilia and systemic symptoms). DIHS/DRESS is defined as a cutaneous drug eruption associated with visceral involvement and an eosinophilia greater than 1.5×10^9/L or atypical lymphocytosis, occurring 2–3 weeks after initiation of the drug. The detection of drug-specific lymphocytes in patients with a history of carbamazepine – induced hypersensitivity reaction suggests that this response has an immune-mediated component [54].

Several recent studies have shown that the HLA-B*1502 allele is strongly associated with carbamazepine-induced SJS and TEN in subjects of Chinese/Asian ethnicity, but not with carbamazepine-induced exanthematous eruptions or DHIS/DRESS [55, 56]. One of the first reports showed that HLA-B*1502 was present in 100% of carbamazepine-induced SJS patients, but in only 3% of patients tolerating carbamazepine and in 9% of the general population [55]. These data suggest that Asians, especially Han Chinese, who have the HLA-B*1502 allele are at an increased risk of developing SJS/TEN when exposed to carbamazepine. Assuming a 0.25% incidence of induced SJS/TEN in newly prescribed carbamazepine patients in Taiwan and a 3% false-positive rate for HLA-B*1502, the number of patients required to be screened is calculated to be 407, in order to prevent one case of SJS/TEN [57]. HLA-B*1502 has a strikingly variable occurrence among different ethnic groups. It is present in 10–15% of individuals from southern China, Thailand, Malaysia, Indonesia, the Philippines, and Taiwan, and has a prevalence rate of at least 2–4% in other southern Asian groups. It is uncommon in Japanese and Korean populations (<1%) and in European Caucasians (0–0.1%). Interestingly, one study of 12 French and German patients with carbamazepine-induced SJS/TEN found that all 4 HLA-B*1502-positive individuals were of Asian ancestry [58]. The Federal Drug Administration (FDA) and other regulatory bodies have advised physicians to test Asian patients for the HLA-B*1502 allele prior to prescribing carbamazepine, and to consider the potential risk of skin reactions when instituting other antiepileptic drugs, such as phenytoin or fosphenytoin [59].

In addition, since carbamazepine is metabolized by the cytochrome P450 enzyme CYP3A4, genetic variants of this enzyme may play a role in hypersensitivity reactions to this drug [60].

EGF Receptor Inhibitors

Acneiform (pseudoacne) eruptions, paronychia, and alopecia are among the many adverse dermatologic effects reported with the use of epidermal growth factor receptor inhibitors (EGFR-Is), which are employed in the treatment of advanced stage cancer. Acneiform eruptions occur in more than 50% of patients treated with these agents. The acneiform lesions usually present as pruritic, erythematous follicular papules that may evolve into pustules. Comedones are generally absent. The typical distribution is similar to acne vulgaris, involving the scalp, face, neck, shoulder, and upper trunk. The eruption usually occurs about a week after starting treatment. Importantly, the presence and intensity of acneiform eruptions due to EGFR-Is have a positive correlation with patient survival. Some authors have suggested that treatment response with cetuximab may be improved by increasing the dose until an acneiform eruption appears [61]. Fc-gamma receptor polymorphisms were first determined to be correlated with this response in patients with metastatic colorectal cancer [62]. More recently, both ABCG2 promoter and EGFR intron 1 polymorphisms have been linked with gastrointestinal and skin toxicities to erlotinib. In multivariate analyses, polymorphisms of EGFR intron 1 correlated with plasma levels of erlotinib and were the strongest predictors for the development of an acneiform rash [63]. These findings may be useful when considering dose or schedule changes in patients experiencing toxicities to these drugs.

Minocycline

Minocycline has been associated with the development of DIHS/DRESS, usually occurring 2–4 weeks after therapy is initiated [64]. In these patients, DHIS may have a prolonged course, lasting up to several months. This unusual protracted course, even after withdrawal of the drug, in individuals with heavily pigmented skin raises the possibility of a pharmacological susceptibility based on ethnicity. Glutathione S-transferases (GST) and UDP-glucuronosyltransferases (UGT) are potentially involved in the metabolism of minocycline. GST polymorphisms include homozygous mutations (i.e., GSTT1 and GSTM1) that are particularly frequent in African and West Indian populations, and responsible for a decrease in enzymatic detoxification activity. Although the impact of these genetic variants on DHIS incidence and characteristics has not been fully elucidated, these genes remain interesting candidates [65].

Current and Future Perspectives

The goals of pharmacogenomics are the reduction of adverse drug events and the enhancement of therapeutic effectiveness. Pharmacogenomics as it applies to the field of dermatology is still in its formative years; although G6PD testing is routine practice where dapsone is considered the drug of choice [8]. Recent studies have provided insight into the strong association of the HLA-B*5701 allele with abacavir-induced hypersensitivity reactions, as well as the importance of TPMT measurements prior to prescribing azathioprine. With the increasing use of expensive biological response modifiers, pharmacogenomics may help to better identify patient responders and/or reduce

adverse drug reactions. Tests for measuring individual genotypes are available in many centers, but often only as research tools. Such assays must become cost-efficient and more accessible to a larger market. In addition, ethical, legal, and regulatory issues need to be addressed. A vision for the future utility of genetic testing involves the employment of genomic data to rank therapeutic choices, in an effort to exclude the use of drugs associated with a high risk of toxicity in a specific individual.

References

1. Katsanis SH, Javitt G, Hudson K. Public health. A case study of personalized medicine. Science. 2008;320:53–4.
2. Roses AD. Pharmacogenetics in drug discovery and development: a translational perspective. Nat Rev Drug Discov. 2008;7:807–17.
3. Ameen M, Smith CH, Barker JN. Pharmacogenetics in clinical dermatology. Br J Dermatol. 2002;146:2–6.
4. Kramer JA, Sagartz JE, Morris DL. The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. Nat Rev Drug Discov. 2007;6:636–49.
5. International Conference on Harmonisation; Guidance on E15 Pharmacogenomics Definitions and Sample Coding; Availability. Notice. Fed Regist. 2008;73:19074–19076.
6. Coleman MD. Dapsone: modes of action, toxicity and possible strategies for increasing patient tolerance. Br J Dermatol. 1993;129:507–13.
7. Coleman MD. Dapsone toxicity: some current perspectives. Gen Pharmacol. 1995;26:1461–7.
8. Coleman MD. Improvement of patient tolerance to dapsone: current and future developments. Dermatol Online J. 2007;13:18.
9. Coleman MD, Coleman NA. Drug-induced methaemoglobinaemia. Treatment issues. Drug Saf. 1996;14:394–405.
10. Teml A, Schaeffeler E, Herrlinger KR, et al. Thiopurine treatment in inflammatory bowel disease: clinical pharmacology and implication of pharmacogenetically guided dosing. Clin Pharmacokinet. 2007;46:187–208.
11. Patel AA, Swerlick RA, McCall CO. Azathioprine in dermatology: the past, the present, and the future. J Am Acad Dermatol. 2006;55:369–89.
12. Anstey AV, Wakelin S, Reynolds NJ. Guidelines for prescribing azathioprine in dermatology. Br J Dermatol. 2004;151:1123–32.
13. Van Dooren-Greebe RJ, Kuijpers AL, Mulder J, et al. Methotrexate revisited: effects of long-term treatment in psoriasis. Br J Dermatol. 1994;130:204–10.
14. Campalani E, Arenas M, Marinaki AM, et al. Polymorphisms in folate, pyrimidine, and purine metabolism are associated with efficacy and toxicity of methotrexate in psoriasis. J Invest Dermatol. 2007;127:1860–7.
15. Warren RB, Smith RL, Campalani E, et al. Genetic variation in efflux transporters influences outcome to methotrexate therapy in patients with psoriasis. J Invest Dermatol. 2008;128:1925–9.
16. Warren RB, Smith RL, Campalani E, et al. Outcomes of methotrexate therapy for psoriasis and relationship to genetic polymorphisms. Br J Dermatol. 2009;160:438–41.
17. Pincelli C, Pignatti M, Borroni RG. Pharmacogenomics in dermatology: from susceptibility genes to personalized therapy. Exp Dermatol. 2009;18:337–49.
18. Gottlieb A, Korman NJ, Gordon KB, et al. Guidelines of care for the management of psoriasis and psoriatic arthritis: Section 2. Psoriatic arthritis: overview and guidelines of care for treatment with an emphasis on the biologics. J Am Acad Dermatol. 2008;58:851–64.
19. Menter A, Gottlieb A, Feldman SR, et al. Guidelines of care for the management of psoriasis and psoriatic arthritis: Section 1. Overview of psoriasis and guidelines of care for the treatment of psoriasis with biologics. J Am Acad Dermatol. 2008;58:826–50.
20. Miceli-Richard C, Comets E, Verstuyft C, et al. A single tumour necrosis factor haplotype influences the response to adalimumab in rheumatoid arthritis. Ann Rheum Dis. 2008;67:478–84.
21. Kang CP, Lee KW, Yoo DH, et al. The influence of a polymorphism at position -857 of the tumour necrosis factor alpha gene on clinical response to etanercept therapy in rheumatoid arthritis. Rheumatology (Oxford). 2005;44:547–52.
22. Toonen EJ, Coenen MJ, Kievit W, et al. The TNF Receptor Superfamily member 1b 676T>G polymorphism in relation to response to infliximab and adalimumab treatment and disease severity in rheumatoid arthritis. Ann Rheum Dis. 2008;67:1174–7.
23. Delaney NL, Esquenazi V, Lucas DP, et al. TNF-alpha, TGF-beta, IL-10, IL-6, and INF-gamma alleles among African Americans and Cuban Americans. Report of the ASHI Minority Workshops: Part IV. Hum Immunol. 2004;65:1413–9.

24. Abraham LJ, Kroeger KM. Impact of the -308 TNF promoter polymorphism on the transcriptional regulation of the TNF gene: relevance to disease. J Leukoc Biol. 1999;66:562–6.
25. Wilson AG, de Vries N, Pociot F, et al. An allelic polymorphism within the human tumor necrosis factor alpha promoter region is strongly associated with HLA A1, B8, and DR3 alleles. J Exp Med. 1993;177:557–60.
26. Maxwell JR, Potter C, Hyrich KL, et al. Association of the tumour necrosis factor-308 variant with differential response to anti-TNF agents in the treatment of rheumatoid arthritis. Hum Mol Genet. 2008;17:3532–8.
27. Lee YH, Rho YH, Choi SJ, et al. Association of TNF-alpha -308 G/A polymorphism with responsiveness to TNF-alpha-blockers in rheumatoid arthritis: a meta-analysis. Rheumatol Int. 2006;27:157–61.
28. Mugnier B, Balandraud N, Darque A, et al. Polymorphism at position -308 of the tumor necrosis factor alpha gene influences outcome of infliximab therapy in rheumatoid arthritis. Arthritis Rheum. 2003;48:1849–52.
29. Cuchacovich M, Soto L, Edwardes M, et al. Tumour necrosis factor (TNF)alpha -308 G/G promoter polymorphism and TNFalpha levels correlate with a better response to adalimumab in patients with rheumatoid arthritis. Scand J Rheumatol. 2006;35:435–40.
30. Padyukov L, Lampa J, Heimburger M, et al. Genetic markers for the efficacy of tumour necrosis factor blocking therapy in rheumatoid arthritis. Ann Rheum Dis. 2003;62:526–9.
31. Fabris M, Tolusso B, Di Poi E, et al. Tumor necrosis factor-alpha receptor II polymorphism in patients from southern Europe with mild-moderate and severe rheumatoid arthritis. J Rheumatol. 2002;29:1847–50.
32. Marotte H, Arnaud B, Diasparra J, et al. Association between the level of circulating bioactive tumor necrosis factor alpha and the tumor necrosis factor alpha gene polymorphism at -308 in patients with rheumatoid arthritis treated with a tumor necrosis factor alpha inhibitor. Arthritis Rheum. 2008;58:1258–63.
33. Ongaro A, De Mattei M, Pellati A, et al. Can tumor necrosis factor receptor II gene 676T>G polymorphism predict the response grading to anti-TNFalpha therapy in rheumatoid arthritis? Rheumatol Int. 2008;28: 901–8.
34. Canete JD, Suarez B, Hernandez MV, et al. Influence of variants of Fc{gamma}receptors IIA and IIIA on the ACR and EULAR responses to anti-TNF{alpha} therapy in rheumatoid arthritis. Ann Rheum Dis. 2008;68:1547–52.
35. Tutuncu Z, Kavanaugh A, Zvaifler N, et al. Fcgamma receptor type IIIA polymorphisms influence treatment outcomes in patients with inflammatory arthritis treated with tumor necrosis factor alpha-blocking agents. Arthritis Rheum. 2005;52:2693–6.
36. Kooloos WM, de Jong DJ, Huizinga TW, et al. Potential role of pharmacogenetics in anti-TNF treatment of rheumatoid arthritis and Crohn's disease. Drug Discov Today. 2007;12:125–31.
37. Kooloos WM, Guchelaar HJ, Huizinga TW, et al. Comment on: investigation of candidate polymorphisms and disease activity in rheumatoid arthritis patients on methotrexate. Rheumatology (Oxford). 2009;48:1176–7. author reply 1177.
38. Ueta M, Sotozono C, Inatomi T, et al. Association of Fas Ligand gene polymorphism with Stevens-Johnson syndrome. Br J Ophthalmol. 2008;92:989–91.
39. Ueta M, Sotozono C, Inatomi T, et al. Toll-like receptor 3 gene polymorphisms in Japanese patients with Stevens-Johnson syndrome. Br J Ophthalmol. 2007;91:962–5.
40. Hughes AR, Brothers CH, Mosteller M, et al. Genetic association studies to detect adverse drug reactions: abacavir hypersensitivity as an example. Pharmacogenomics. 2009;10:225–33.
41. Hughes AR, Spreen WR, Mosteller M, et al. Pharmacogenetics of hypersensitivity to abacavir: from PGx hypothesis to confirmation to clinical utility. Pharmacogenomics J. 2008;8:365–74.
42. Chessman D, Kostenko L, Lethborg T, et al. Human leukocyte antigen class I-restricted activation of CD8+ T cells provides the immunogenetic basis of a systemic drug hypersensitivity. Immunity. 2008;28:822–32.
43. Mallal S, Phillips E, Carosi G, et al. HLA-B*5701 screening for hypersensitivity to abacavir. N Engl J Med. 2008;358:568–79.
44. Panel on Antiretroviral Guidelines for Adult and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. January 10, 2011;1–166. Available at http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf. Accessed January 29, 2011.
45. Shear NH, Milpied B, Bruynzeel DP, et al. A review of drug patch testing and implications for HIV clinicians. AIDS. 2008;22:999–1007.
46. Carr A, Cooper DA. Adverse effects of antiretroviral therapy. Lancet. 2000;356:1423–30.
47. Metry DW, Lahart CJ, Farmer KL, et al. Stevens-Johnson syndrome caused by the antiretroviral drug nevirapine. J Am Acad Dermatol. 2001;44:354–7.
48. Martin AM, Nolan D, James I, et al. Predisposition to nevirapine hypersensitivity associated with HLA-DRB1*0101 and abrogated by low CD4 T-cell counts. AIDS. 2005;19:97–9.
49. Vitezica ZG, Milpied B, Lonjou C, et al. HLA-DRB1*01 associated with cutaneous hypersensitivity induced by nevirapine and efavirenz. AIDS. 2008;22:540–1.
50. Chantarangsu S, Mushiroda T, Mahasirimongkol S, et al. HLA-B*3505 allele is a strong predictor for nevirapine-induced skin adverse drug reactions in HIV-infected Thai patients. Pharmacogenet Genomics. 2009;19:139–46.

51. Hung SI, Chung WH, Liou LB, et al. HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol. Proc Natl Acad Sci USA. 2005;102:4134–9.
52. Kaniwa N, Saito Y, Aihara M, et al. HLA-B locus in Japanese patients with anti-epileptics and allopurinol-related Stevens-Johnson syndrome and toxic epidermal necrolysis. Pharmacogenomics. 2008;9:1617–22.
53. Lonjou C, Borot N, Sekula P, et al. A European study of HLA-B in Stevens-Johnson syndrome and toxic epidermal necrolysis related to five high-risk drugs. Pharmacogenet Genomics. 2008;18:99–107.
54. Mauri-Hellweg D, Bettens F, Mauri D, et al. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. J Immunol. 1995;155:462–72.
55. Chung WH, Hung SI, Hong HS, et al. Medical genetics: a marker for Stevens-Johnson syndrome. Nature. 2004;428:486.
56. Hung SI, Chung WH, Jee SH, et al. Genetic susceptibility to carbamazepine-induced cutaneous adverse drug reactions. Pharmacogenet Genomics. 2006;16:297–306.
57. Ferrell Jr PB, McLeod HL. Carbamazepine, HLA-B*1502 and risk of Stevens-Johnson syndrome and toxic epidermal necrolysis: US FDA recommendations. Pharmacogenomics. 2008;9:1543–6.
58. Miller JW. Of race, ethnicity, and rash: the genetics of antiepileptic drug-induced skin reactions. Epilepsy Curr. 2008;8:120–1.
59. Kuehn BM. FDA: epilepsy drugs may carry skin risks for Asians. JAMA. 2008;300:2845.
60. Gueant JL, Gueant-Rodriguez RM, Gastin IA, et al. Pharmacogenetic determinants of immediate and delayed reactions of drug hypersensitivity. Curr Pharm Des. 2008;14:2770–7.
61. Ciardiello F, Tortora G. EGFR antagonists in cancer treatment. N Engl J Med. 2008;358:1160–74.
62. Zhang W, Gordon M, Schultheis AM, et al. FCGR2A and FCGR3A polymorphisms associated with clinical outcome of epidermal growth factor receptor expressing metastatic colorectal cancer patients treated with single-agent cetuximab. J Clin Oncol. 2007;25:3712–8.
63. Rudin CM, Liu W, Desai A, et al. Pharmacogenomic and pharmacokinetic determinants of erlotinib toxicity. J Clin Oncol. 2008;26:1119–27.
64. Knowles SR, Shapiro L, Shear NH. Serious adverse reactions induced by minocycline. Report of 13 patients and review of the literature. Arch Dermatol. 1996;132:934–9.
65. Maubec E, Wolkenstein P, Loriot MA, et al. Minocycline-induced DRESS: evidence for accumulation of the culprit drug. Dermatology. 2008;216:200–4.

Chapter 23
Regulatory, Legal, Coding, Billing, Reimbursement, and Ethical Considerations for Molecular Diagnostic Testing in Dermatology and Dermatopathology

Deborah Ann Payne

Molecular diagnostics is one of the most rapidly growing areas in laboratory medicine. Current applications in dermatology and dermatopathology cover a wide range of conditions, from infectious and inflammatory diseases, to cancer, and inherited skin disorders (genodermatoses). Early molecular tests were developed for diagnosis of systemic hematologic conditions, largely due to the relative ease in accessing specimens (i.e., venapuncture), possibility of abundant tissue for analysis (i.e., whole lymph nodes), and the capabilities of molecular methodologies available at that time (i.e., Southern blot analysis). Technical advances have now permitted the extraction and analysis of template (i.e., DNA and RNA) from both smaller and routinely processed (i.e., formalin-fixed paraffin-embedded [FFPE]) specimens (i.e., skin biopsies), enabling the application of molecular diagnostic testing to other fields, including dermatology. For example, diagnostic and follow-up evaluation of cutaneous diseases with sparse cellular infiltrates (such as many cutaneous lymphoproliferative disorders) is now possible. With the advent of methods for both target and signal amplification of nucleic acids, infectious disease testing has now become the dominant area in molecular diagnostics. Many infections can present with dermatologic manifestations, including the skin-localization of microorganisms and other nonspecific sequelae, such as those related to chronic hepatitis C virus (HCV) infection (i.e., pruritus, lichen planus) or human herpesvirus-6 (HHV-6) infection (i.e., exanthem subitum in childhood, and skin eruptions following solid-organ/bone marrow transplantation) [1, 2]. In addition, advances facilitate the design of studies which evaluate the linkage between skin disease phenotypes and genomic abnormalities. The latter is particularly important with respect to genodermatoses. As translational research strives to identify increasing numbers of genes associated with dermatologic disorders, it is likely that the number of molecular assays applicable to skin diseases will continue to expand. Due to the broad spectrum and complexity of molecular analyses, there are a number of regulatory, legal, coding, billing, reimbursement, and ethical concerns which can create barriers to the adoption of this form of testing in clinical medicine. The purpose of this chapter is to discuss these issues and identify laboratory processes and resources that can facilitate broader implementation of molecular-based tests for skin diseases.

D.A. Payne (✉)
American Pathology Partners-UniPath LLC, 6116 East Warren Ave. Denver, CO 80222, USA
e-mail: dpayne@unipathllc.com

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4_23,

Regulatory and Legal Considerations

Certifications

In order to assure quality molecular testing, certifications for the Medical Director, technical personnel, and the laboratory itself are required.

Diagnostic laboratories with United States of America (USA)-based operations must comply with the requirements of the Clinical Laboratory Improvement Act (CLIA) and/or the College of American Pathologists (CAP) [3–5]. The CAP provides a checklist for molecular pathology testing that can assist in the organization and implementation of all aspects of the molecular laboratory. Links to these organizations are listed in Table 23.1. It should be noted that some states in the USA have additional requirements for laboratory certification (i.e., New York State Clinical Laboratory Evaluation Program [CLEP]). A number of documents are available from the International Organization for Standardization (ISO) for those laboratories wishing to follow the standards of the international community [6].

Molecular diagnostics is considered "high complexity testing" by CLIA. Accordingly, CLIA requires that the Medical Director have the professional qualification, certification, and/or experience outlined in Table 23.2. Some states (i.e., New York) may also require that the Medical Director be licensed (i.e., possess a certificate of qualification [CQ]) in order to perform testing on patient samples from that state. For laboratories performing genetic testing for inherited diseases, the Clinical Laboratory Improvement Advisory Committee (CLIAC) recommends additional levels of experience and/or certification for technical supervisors which include "*4 years of training or experience (or both) in genetics, two of which are in the area of molecular genetic testing for heritable diseases"* or *"current certification in molecular genetic testing by a board approved by the*

Table 23.1 Links to Web sites describing various guidances for clinical laboratory testing

Organization	Web site	Type of guidance
American College of Medical Genetics (ACMG)	http://www.acmg.net/Pages/ACMG_Activities/stds-2002/URD.htm	Ultra-rare disorders with prevalences less than 2,000 persons in the USA (i.e., X-linked ocular albinism, epidermolytic hyperkeratosis)
College of American Pathologists (CAP)	http://www.cap.org/apps/cap.portal?_nfpb=true&_pageLabel=accreditation *CAP accreditation and inspection information* Click on "About Accreditation Checklists" link	General Laboratory and Molecular Checklist (Checklist 12)
Clinical Laboratory Improvement Act (CLIA)	http://www.cms.hhs.gov/CLIA/	Waived, Moderate and High Complexity testing
Clinical Laboratory Standards Institute (CLSI)	http://www.clsi.org/	General Laboratory Practice and Molecular Pathology
Food and Drug Administration (FDA)	http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm077862.htm	Genetic Tests for Heritable Diseases
International Organization for Standardization (ISO)	http://www.iso.org/iso/search.htm?qt=medical+laboratory+testing&searchSubmit=Search&sort=rel&type=simple&published=true	General Laboratory and Reference Materials

Table 23.2 CLIA requirements for medical directors who manage and direct performance of high complexity tests

Professional degree	Laboratory board certification	Experience
Doctor of Medicine or Doctor of Osteopathy[a]	Anatomic or Clinical Pathology, or both, by the American Board of Pathology or the American Osteopathic Board of Pathology, or possess qualifications that are equivalent to those required for such certification	–
Doctor of Medicine, Doctor of Osteopathy, or Doctor of Podiatric Medicine[a]	–	• Have at least 1 year of laboratory training during medical residency (i.e., physicians certified in either hematology or hematology/medical oncology by the American Board of Internal Medicine) • Have at least 2 years of experience directing or supervising high complexity testing
Hold an earned doctoral degree in a chemical, physical, biological, or clinical laboratory science from an accredited institution[a]	Health and Human Services–approved boards: • ABB – American Board of Bioanalysis • ABB (Public Health Microbiology Certification) • ABCC – American Board of Clinical Chemistry • ABCC [24-month Commission on Accreditation in Clinical Chemistry (COMACC) accredited program] • ABFT – American Board of Forensic Toxicology • ABHI – American Board of Histocompatibility and Immunogenetics • ABMG – American Board of Medical Genetics • ABMLI – American Board of Medical Laboratory Immunology • ABMM – American Board of Medical Microbiology • NRCC – National Registry of Certified Chemists	• Two years of laboratory training or experience, or both; and • Two years of laboratory experience directing or supervising high complexity testing

[a]A current license as a laboratory director issued by the State in which the laboratory is located, if such licensing is required

Department of Health and Human Services (HHS). It should be noted that these recommendations are not regulatory in nature [7, 8]."

Requirements for appropriate training, proficiency, and competency are necessary for persons performing molecular assays (i.e., technologists). These requirements are described in the CAP checklist for Molecular Pathology, in CLIAC recommendations, and on the CLIA Web site [3, 5, 8]. However, additional certifications and licenses may be required for personnel working in certain states in the USA.

Assay Development, Performance Characteristics, and Reporting

There are two basic reagent options for molecular diagnostic testing. A laboratory can purchase commercially available (1) Food and Drug Administration (FDA)-approved/cleared kits or (2) analyte-specific reagents (ASRs), design the assay, and perform its own internal validation.

Unlike most other areas in laboratory medicine, relatively few molecular tests, in particular, those applicable to skin diseases have been cleared or approved as *in vitro* diagnostic medical devices (IVD) by the FDA. Generally limited to high-volume assays, IVD approval through the FDA utilizes the more costly Pre-Market Approval (PMA) process, whereby the performance of the IVD is typically linked to patient outcome studies. Approved tests, such as human papillomavirus (HPV) high-risk assays, link the performance of the test with a patient's risk for developing cancer. In contrast, FDA clearance uses the 510K process and focuses on the analytical performance of the assay. Manufacturers of these IVDs are permitted to train technical staff on the implementation and internal verification of these assays. While the performance characteristics of an assay have previously been validated during the approval or clearance process (see Table 23.3), the laboratory is still required to undertake additional verification procedures [7].

Most molecular tests are developed in-house (i.e., laboratory-developed tests [LDTs]), also known as "home brew" assays. These tests utilize commercially available ASRs; examples of which include polymerase chain reaction (PCR) primers, buffers, and enzymes. Such components are then integrated in order to develop a functional test. Unlike FDA-cleared or FDA-approved tests, all aspects of the assay, including its performance characteristics must be validated by the laboratory [8, 9]. Validation variables include: (1) *analytical validity*: how precise and reliable the test performs in detecting the product of interest; and (2) *clinical validity*: how well the test predicts the disease of interest. Other important factors to consider include: (1) *clinical utility*: an analysis of the risks and benefits of introducing a new test into clinical practice; and (2) more broad *ethical, legal, and social implications*. A laboratory that performs an assay which utilizes ASRs must include a disclaimer on the test report. An example of such a disclaimer is "*This test was developed and its performance characteristics determined by the Molecular Genetics Laboratory. It has not been cleared or approved by the United States Food and Drug Administration (FDA). However, such approval is not required for clinical implementation, and the test results have been shown to be clinically useful. This laboratory is CAP-accredited and CLIA-certified to perform high complexity testing* [10]."

In order to maintain compliance with regulatory entities, documentation of the assay's validation and performance characteristics (Table 23.3), in addition to records of the training, proficiency, and competency of technical staff (i.e., test operators), must be maintained. In addition, laboratories must provide documentation on various preanalytic, analytic and postanalytic aspects of a test. Several publications and online resources are available that summarize both the validation processes and reporting requirements for molecular laboratory testing (Table 23.1) [8, 9]. These publications, especially the Clinical Laboratory Standards Institute (CLSI) guidelines, address specific issues for molecular methods (MM). For example, MM1-A2, MM14-A, and MM5-A cover such aspects as mutation nomenclature, safety, sample intake information, specimen identification and accessioning, controlling for false positives, mutation detection, detection formats, nonmicroarray test validation/characterization, quality control (QC)/quality assurance (QA), proficiency testing, and reporting of results. Other CLSI guidelines focus on specific platforms, such as fluorescence in situ hybridization (FISH) (i.e., MM-7A) or different aspects of microarray analysis, including methods, preanalytic issues, QC/QA, specimen preparation, and test validation/characterization (i.e., MM12-A). The CLSI guidelines are cited in the CAP Molecular Pathology checklist. It should be noted that these are not static documents and are updated periodically as new technologies begin to replace older methodologies. Additionally, new guidelines are developed as the field of laboratory medicine expands into new areas.

Table 23.3 Performance characteristics of a molecular test or reference material (Adapted from Jennings et al. [9])

Characteristic	Example
Accuracy	Total error of the test (systemic error and random error). For qualitative assays, accuracy is equivalent to "sensitivity and specificity."
Trueness	Systemic error (bias) expressed as percent deviation from the true value. Applies to quantitative tests.
Precision	Random error expressed as coefficient of variation or standard deviation. Applies to quantitative tests.
Reproducibility	Equivalent to "precision" for qualitative or semiquantitative assays. Within-run reproducibility indicated by repeatability.
Robustness	Test precision given changes in preanalytic conditions, such as specimen storage or different formalin fixation conditions for paraffin-embedded tissues.
Linearity	The ability of a test to produce values directly proportional to analyte concentration. Applies to quantitative tests.
Reportable range	The range of results for all tests (qualitative and quantitative) that has been validated for the assay.
Reference range	Range of test results for a specific patient population.
Interfering substances	A substance that at a given concentration causes systemic error.
Analytic sensitivity	The ability of a qualitative test to provide a positive agreement as compared to a positive reference material.
Analytic specificity	The ability of a qualitative test to provide a negative agreement as compared to a negative reference material.
Limit of detection	The lowest concentration of an analyte that can be distinguished from background or a negative control.
Limit of quantification	The lowest and highest values detected by a quantitative assay that has an adequately precise and accurate linear range.
Clinical sensitivity	The proportion of patients with a specific disorder whose test result is positive.
Clinical specificity	The proportion of patients without a specific disorder whose test result is negative.

Special clinical circumstances do exist in molecular pathology which can hamper a laboratory's ability to strictly follow the CLSI guidelines. For instance, no specific numerical requirement of positive and negative cases, in order to validate an assay, is stated for all tests. With respect to dermatopathology samples, a paucity of such "informative" cases may be due to the rarity of the disorder and/or scant amounts of tissue available for testing. In these situations, the CLSI guidelines do not state which statistical values need be amended or prioritized in order to introduce a test. For instance, CLSI guideline EP12-A suggests at least 50 positive and 50 negative specimens over a time period of 10–20 days. Likewise, EP09-A2 recommends testing of at least 40 patient samples over several days in order to compare and establish bias between methods. These recommendations were likely prepared for high-volume, low-cost tests, and may not reflect the current need for molecular diagnostic testing of skin-related disorders. For the purposes of molecular dermatologic testing, obtaining such large numbers of clinical samples and performing this type of validation may be prohibitive (i.e., due to specimen availability and test cost). Consequently, some guideline requirements from the CLSI may not completely harmonize with other professional guidelines (i.e., American College of Medical Genetics [ACMG] guidelines for validating tests for Ultra-Rare Diseases [URD], which are particularly relevant to rare genodermatoses) [11]. One important issue to consider, when proposing the introduction of a molecular assay in a situation where the "ideal" number of specimens cannot be analyzed, is whether the new test is equivalent or better (i.e., decreased turn-around time, reduced cost, less invasive specimen collection, information not otherwise available, etc.) than current tests or procedures for patient management. Ultimately, it is the responsibility of the Medical Director to determine when sufficient test validation has been completed [9].

Good laboratory practice dictates that reporting of test results must include the proper nomenclature and the following information: "*patient name and identification number, name and address of the laboratory, specimen source (when appropriate), test report date, test performed, and test results* [8, 10, 12]." Additional information should include: "*patient date of birth, indication for testing, date and (if applicable) time of (specimen) collection and arrival in the laboratory, and name of the referring physician* [8]." For molecular testing of skin conditions, knowledge of the specimen source is helpful in interpreting results. For example, specimens for cutaneous lymphoma workup may include skin biopsies, whole blood, and/or bone marrow specimens. In addition, serial specimen collections are often required; for example, sequential samples from a posttransplantation patient with a skin rash are used to monitor changes in HHV-6 load. Preanalytic factors, such as delays in receiving the specimen after collection, can result in nucleic acid degradation: and potentially produce false-negative results. Therefore, the inclusion of the specimen collection time is critical for appropriate interpretation. One important aspect of result reporting is the requirement to use the Human Genome Organization (HUGO) standard nomenclature [12, 13]. The inclusion of both scientific and colloquial names of diseases may help physicians better understand any molecular findings. Stating the exact gene that is being tested (i.e., EVER1 or EVER2 in Epidermodysplasia Verruciformis [EDV]) on the report is important, because other genes may be identified in the future that are associated with the disease. Another benefit is that duplicate testing can be avoided. Likewise, inclusion of the test methodology can allow the referring physician (i.e., dermatologist) to evaluate the results of tests from different laboratories. For instance, a laboratory performing a DNA sequence-based test for an inherited disorder may analyze only a portion of a gene, while another laboratory may analyze the entire gene. Understanding both the method(s) and target(s) of the molecular test may permit a physician to determine the possible cause of any discordant results. Any report should include the results of testing (i.e., positive or negative), and also an interpretation that provides clinical and analytical information, relating the molecular findings to the patient's condition.

Legal Issues

The legal aspects of molecular diagnostics vis-à-vis gene patenting are also important to consider. With regard to molecular assays, patent claims in the USA involve: (1) composition of matter (i.e., novel gene mutation, deletion, single nucleotide polymorphism); (2) method-of-use; and (3) to a lesser extent, method of manufacture. Laboratories which utilize LDTs need to be aware of genes or methods that are the intellectual property (i.e., through patents or licenses) of others. For example, *InVivo*Scribe Technologies, LLC (San Diego, CA) holds worldwide exclusive rights to the patents for the identification and monitoring of leukemias, lymphomas, and other lymphoproliferative diseases through any amplification-based technology, including PCR (i.e., B- and T-cell clonality assays). Therefore, laboratories wishing to implement molecular tests for cutaneous lymphomas, such as mycosis fungoides, must first obtain a sublicense agreement from this company. Several databases exist to search for gene patents [14]. It should be noted that not all gene patents use standard nomenclature. Therefore, colloquial names for genes or diseases should be included in database searches. Failure to obtain a sublicense from any patent holder could result in monetary fines or a requirement that the laboratory cease performance of the test.

Coding, Billing, and Reimbursement Considerations

As is the case with all laboratory testing, reimbursement for molecular assays necessitates the proper use of International Classification of Diseases (ICD)-9 and Current Procedural Terminology (CPT) codes, in addition to the ability to bill for the service.

The submission of an appropriate ICD-9 code is required in order to identify a clinical diagnosis that justifies the performance of a molecular assay. One challenge for physicians is that multiple ICD-9 codes may be applicable to a given condition. Case in point, EDV could potentially be coded under 216.x (benign lesion), 078.x (verruca), or 757.x (genodermatosis). Some diagnosis codes may not support "medical necessity" for a particular (molecular test) CPT code, and payment may be denied for the submission of an "invalid" ICD-9 code.

CPT codes are determined by the American Medical Association and cover a wide range of medical procedures [15]. In addition, Medicare uses HCPCS (Health Care Procedure Coding System) codes. The coding of molecular tests can be quite complex, partly compounded by the fact that new molecular assays are continually being developed, and support systems must adapt in order to keep up. In 1993, there were only six codes associated with molecular testing. By 2008, this number had jumped to 21. Codes are assigned by procedure (i.e., nucleic acid isolation, digestion procedures, gel electrophoresis) rather than by analyte or analysis. CPT codes used for molecular testing of skin diseases include those for molecular microbiology and FISH, as well as those generated for newer technologies (i.e., cDNA microarrays: 88384, 88385, 88386). Modifiers (i.e., technical component [TC], professional component [PC]) and edits (rules concerning which codes can be billed with others) also exist. Molecular microbiology codes are analyte-specific and range from 87470 (*Bartonella henselae* and *Bartonella quintana*, direct probe technique) to 87904 (infectious agent phenotype analysis by nucleic acid [DNA or RNA], each additional drug tested). Within this range are codes for numerous bacteria and viruses associated with either skin infections or nonspecific skin disorders (i.e., *Borrelia burgdorferi*, mycobacteria, HHV-6, herpes simplex virus [HSV], and HPV). Typically, specific infectious entities have a separate code for each methodology (i.e., *Borrelia burgdorferi* – 87475: direct probe; 87476: amplified probe; 87477: quantification). Other codes (i.e., 87149: identification by nucleic acid probe) are also available for infectious agent detection. Molecular cytogenetic codes for FISH begin with 88271, with additional codes available for testing of increasing numbers of interphase cells for derivatives/markers (88272) and microdeletions (88273). CPT codes for FISH can also be found under the surgical pathology section (i.e., 88365–88368). The various codes for surgical pathology FISH applications reflect the quantitative/semiquantitative and manual *vs.* automated nature of morphometric analysis. Other technical CPT codes (i.e., 83890–83914) are more procedural in nature and attempt to address all technical steps required to generate a report for a molecular test (Table 23.4). According to the 2010 edition of the CPT manual, "*genetic testing modifiers should be used in conjunction with CPT and HCPCS codes to provide diagnostic granularity of service, to enable providers to submit complete and precise genetic testing information without altering test descriptors* [15]." For example, when performing clonality tests for cutaneous lymphomas, the addition of the modifier 2M or 2N designates that the test analyzed the T-cell receptor beta or the T-cell receptor gamma genes, respectively. These modifiers enable the laboratory to better articulate that multiple genes were tested; otherwise, the payer may consider the use of both of these tests, without the inclusion of modifiers, as a duplicate order.

Superficially, it would appear that molecular testing is more costly than nonmolecular-based methods. Indeed, the actual performance of a molecular assay may be more expensive than that of a conventional test. However, molecular diagnostic approaches not only demonstrate the advantage of fast, reproducible, accurate, and objective results, but also the potential of providing significant overall cost benefits in the management of individual patients and to the health care system in general (i.e., a value-based approach). Importantly, this cost effectiveness may be more broadly related to a reduction in the number of diseases, unnecessary treatments, adverse drug reactions, drug resistance, disabilities, hospital stays, time away from work, and mortality [16–19]. Some of the costs associated with molecular testing for cutaneous T-cell lymphoproliferative disorders (Chap. 10) and dermatophytoses (Chap. 15) are discussed in their respective chapters. Determination of the reimbursement rate for a molecular test utilizing multiple procedural codes is complex. Accordingly, the development of an in-house spreadsheet, linking reimbursement with each procedural code, may be

Table 23.4 Spreadsheet for calculating reimbursement for molecular assays with select technical components. Example is for TCR-γ gene rearrangement analysis for mycosis fungoides (i.e., two PCRs with capillary electrophoresis)

CPT code	CPT description	Reimbursement ($)	Number	Subtotal ($)
83890	Molecular diagnostics; molecular isolation or extraction	5.85		
83891	Isolation/extraction of highly purified nucleic acid	5.85	1	5.85
83892	Enzymatic digestion	5.85		
83894	Separation by gel electrophoresis	5.85		
83896	Nucleic acid probe, each	5.85		
83897	Nucleic acid transfer (i.e., Southern blot)	5.85		
83898	Amplification, target, each nucleic acid sequence	24.47		
83900	Amplification, multiplex, first two nucleic acid sequences	48.95	1	48.95
83901	Amplification, multiplex, each additional primer set	24.47	1	24.47
83902	Reverse transcription	20.72		
83903	Mutation scanning, by physical properties (i.e., SSCP)	24.47		
83904	Mutation identification by sequencing	24.47		
83905	Mutation identification by allele-specific transcription	24.47		
83907	Lysis of cells prior to nucleic acid extraction	19.50	1	19.50
83908	Amplification, signal, each nucleic acid sequence	24.47		
83909	Separation and identification by high-resolution technique (i.e., capillary electrophoresis)	24.47	2	48.94
83912	Interpretation and Report	5.85	1	5.85
Sum of Billed CPT codes				153.56

Table does not contain all technical codes for molecular testing. Dollar amounts are based on 2008 Medicare reimbursements and may not reflect current reimbursement rates

helpful in this respect. Table 23.4 is an example of such a spreadsheet that lists some commonly used molecular CPT codes. Of course, not all CPT codes related to molecular testing are listed in this table, and reimbursement rates for CPT codes can both vary and change among payers. Reimbursement rates for the different CPT code components associated with the performance of a molecular test range from ~$5 to ~$46 (Medicare). Of note, quantification codes (i.e., expression levels) typically reimburse at a higher rate than qualitative codes (i.e., positive *vs.* negative result), due to the added complexity of the test. It should be noted that royalties and license fees are not reimbursed. Royalties for performing tests may add a significant percentage to the assay costs. In some cases, 10% or more of the billed test may be required to be paid to the license holder.

Rates and methods of reimbursement vary significantly among payers (i.e., Medicare, insurance, self-pay). Different fee schedules exist (i.e., Clinical Laboratory Fee Schedule and MPFS under Medicare), and reimbursements are influenced by such factors as geographic location, the use of diagnosis-related groups (DRGs), test performance by an independent laboratory *vs.* in a hospital

setting (i.e., ambulatory payment classifications [APCs]), relative value systems, ratio of cost-to-charge, and/or medically unlikely edits (MUEs). In addition, there is poor comprehension by the payer community of the complex coding associated with molecular assays, and reimbursement policies are often inconsistent and out-of-date. Common problems associated with poor reimbursement are listed in Table 23.5. In some cases, issues may result from failures to transfer/communicate the relevant data. For instance, the absence of appropriate ICD-9 codes or modifiers and/or a signed Advanced Beneficiary Notification (ABN) are common causes of poor reimbursement. Delays in test reporting and/or billing can also result in rejection of billed services. Many of these issues can

Table 23.5 Frequent causes of poor reimbursement

Cause	Comment
No ICD-9 code listed or Incorrect ICD-9 code listed	Occasionally no ICD-9 code is listed on the requisition. One way of addressing this problem is to implement a process such as Electronic Physician Order Entry (POE) that prevents the order from being placed without a diagnosis code. Education of the clinic staff is another.
Payment rejected for some CPT codes	Occasionally payers will reject certain CPT codes. This can occur with molecular technical codes, because the payer may have difficulty linking the code with a specific test result (i.e., nucleic acid isolation does not detail for what test the DNA/RNA extraction was required). In other cases, the payer may deny payment due to internal policies that consider some tests experimental or not medically necessary. Education and negotiation with the third party payers may be useful in this situation.
Delays in charging for bills	Whether the delay is caused by a technical, electronic, or administrative problem or combination of all three, delays in generating invoices for tests can lead to bill rejection. Some payers will not honor bills that are greater than 90 days old. Molecular tests that are ordered reflexively (such as in the case of mycosis fungoides) can have delayed results, if (1) the test is ordered after the initial workup, (2) the specimen is sent to an outside lab, or (3) there are technical difficulties causing the test to be repeated.
Duplicate testing (multiple uses of the same CPT code)	Because molecular technical codes are used for multiple assays (i.e., many of the same codes are used for both cutaneous T- and B-cell lymphoma testing), the payer may view multiple uses of CPT codes as duplications rather than separate technical components (i.e., as required to generate a T-cell clonality result and a B-cell clonality result). The use of different modifiers (2M and 2K) can be useful in this instance.
Duplicate testing (different levels of specificity or resolution)	For molecular microbiology codes, the CPT code may not differentiate between the initial detection of the infectious agent and subsequent higher resolution testing. For example, reflexive testing from a high-risk human papillomavirus (HPV) screening test to a HPV genotyping test for HPV types 16, 18, and 45 could result in the use the same CPT code (87621).
Lack of signed Advanced Beneficiary Notification (ABN)	In cases where Medicare does not pay for the test, a signed ABN is mandatory. Some clinics may not have requisitions with an ABN attached to them, or may not have policies that require the patient to sign an ABN if the sample is collected by a patient service center rather than the physician's office. Software systems are available that recognize a test with a noncovered diagnosis and notify the physician that an ABN is required.

be mitigated through the use of software or procedural changes that alert a physician to situations which can adversely impact reimbursement. In addition, due to the use of emerging technologies, payers may not reimburse as they do not know what the test is for. In these cases, communication directly with the payer or through professional organizations (i.e., publications, position papers, etc.) may be helpful. Of note, a future trend in reimbursement may be a value-based approach, where payments reflect overall savings to the health care system.

Ethical Issues

A number of ethical concerns can arise from molecular diagnostic testing of skin disorders (Table 23.6) [20–22]. These issues can be grouped into three general categories: (1) the value of the test; (2) infrastructure for patient support and education; and (3) informed consent, confidentiality, and unanticipated consequences.

Value of the Test

The value and limitations of the test include clinical specificity and sensitivity, as well as the analytical performance of the assay (Table 23.3). Validation and verification processes should provide information

Table 23.6 Ethical issues associated with molecular testing

Considerations	Comments
What is the purpose of the test?	Purposes for molecular testing include diagnosis, prognosis, risk assessment, and research. The purpose of the test will influence various steps that can be taken after receiving the results ranging from no action to aggressive treatment.
What value does the test information offer?	If treatment options for a genetic disease exist, then there may be a clear benefit to performing a test. In cases where no treatment is available, the value of the test can be assessed by the patient. For instance, in circumstances where the patient is a child, the decision for genetic testing may be left to that individual once they have reached adulthood.
Is the test appropriate for the patient who is being tested?	Some genetic conditions are primarily associated with persons of a specific biogeographical ancestry (i.e., ethnicity).
Does the clinic have the ability to provide adequate informed consent?	Clinical staff and physicians need to be able to articulate the various advantages and risks associated with genetic testing.
Who interprets the test result?	The physician should have adequate resources with access to laboratories and experts to facilitate interpretation of test results. With a positive test for human papillomavirus (HPV), some patients may erroneously conclude that their sexual partner has been sexually active outside their relationship. In this case, the ability of the physician to explain the natural history of HPV may be helpful.
How will the test result be communicated to the patient?	The clinic should have access to a genetic counselor to help patients understand the results, and identify support groups that can assist the patient if so desired.
What confidentiality issues exist with testing? What is the risk for discrimination or stigmatization? What other unintended consequences can result from testing?	Confidentiality issues may include disclosure of results to employers or insurance companies, with the potential for job discrimination and problems of acquiring and/or maintaining health insurance coverage. In addition, interpersonal and social issues may occur.

on the technical performance of the test (i.e., nonvalidated tests may yield erroneous results). Other questions that need to be addressed include whether the assay is to be used for screening, prediction, diagnosis, prognostication, or monitoring. Likewise, is the test appropriate for the patient (i.e., age, biogeographical ancestry/ethnicity)? How will the information be used to manage or care for the patient? In other words, "is the test medically necessary?" Some of these molecular tests are extremely expensive and consideration must be made as to how the information will be used.

Infrastructure for Patient Support and Education

Does the clinic or laboratory provide the appropriate infrastructure to acquire informed consent, interpret the test, and (if necessary) provide or refer for genetic counseling? Test interpretation for inherited diseases can be difficult and may be beyond the scope of most practicing physicians. The ability to provide adequate educational support for these patients is critical. Many of these issues can be addressed by providing referral to a genetic counselor.

Informed Consent, Confidentiality, and Unanticipated Consequences

Informed consent should be obtained prior to any form of genetic testing. This process should include a discussion concerning the purpose of the test, possible results, implications for the individual and family members, availability of genetic counseling services, and potential impact on insurance coverage. Confidentiality protects the patient from discrimination and stigmatization. Inappropriate release of molecular test results can create interpersonal difficulties. In addition, job discrimination and potential problems with acquiring and/or maintaining health insurance coverage are perceived as other risks associated with loss of confidentiality. At present, there are a number of state and federal laws, such as the 2008 Genetic Information Non-Discrimination Act (GINA), which protect individuals from the use of their genetic information by health insurance providers for the determination of coverage eligibility or the setting of insurance premiums. With regard to genodermatoses, additional issues could include the discovery of different parentage than expected or consanguineous family members.

In conclusion, regulatory, legal, billing, coding, reimbursement, and ethical issues may pose some obstacles to the broader implementation of molecular diagnostic tests for dermatologic disorders. However, there are various resources available to provide support to physicians and laboratories willing to embrace these challenges. Professional guidance documents and recommendations serve to ensure the quality of molecular testing and improve the management of patients with skin diseases.

Acknowledgments I would like to acknowledge the helpful suggestions and comments of Karen Babish.

References

1. Maticic M, Poljak M, Lunder T, et al. Lichen planus and other cutaneous manifestations in chronic hepatitis C: pre- and post-interferon-based treatment prevalence vary in a cohort of patients from low hepatitis C virus endemic area. J Eur Acad Dermatol Venereol. 2008;22:779–88.
2. Galan A, McNiff JM, Nam Choi J, et al. Fatal HHV6 infection in an immunocompromised patient presenting with skin involvement. J Cutan Pathol. 2010;37:277–81.
3. Clinical Laboratory Improvement Act. 2004. http://wwwn.cdc.gov/clia/regs/subpart_m.aspx. Accessed Jan 2010.

4. American Academy of Dermatology. CLIA Quick Facts. 2003. http://www.aad.org/pm/compliance/clia/facts.html. Accessed Jan 2010.
5. College of American Pathology. 2010. http://www.cap.org/. Accessed Jan 2010.
6. International Standards Organization. 2010. http://www.iso.org/. Accessed Jan 2010.
7. Clinical Laboratory Improvement Act, Health and Human Service approved Board Certifications. 2010. http://www.cms.hhs.gov/CLIA/16_Certification_Boards_Laboratory_Directors.asp#TopOfPage. Accessed Jan 2010.
8. Chen B, Gagnon M, Shahangian S, et al. Good laboratory practices for molecular genetic testing for heritable diseases and conditions. Morb Mortal Wkly Rep. 2009;58:1–29.
9. Jennings L, Van Deerlin VM, Gulley M. Recommended principles and practices for validating clinical molecular pathology tests. Arch Pathol Lab Med. 2009;133:743–55.
10. Gulley ML, Braziel R, Halling KC, et al. Clinical laboratory reports in molecular pathology. Arch Pathol Lab Med. 2007;6:852–63.
11. Maddalena A, Bale S, Das S, et al. Technical standards and guidelines: molecular genetic testing for ultra-rare disorders (URDs). Genet Med. 2005;7:571–83.
12. Ogino S, Gulley ML, den Dunnen JT, et al. Standard mutation nomenclature in molecular diagnostics. J Mol Diagn. 2007;9:1–6.
13. HUGO Gene Nomenclature Committee. 2010. www.genenames.org/. Accessed Jan 2010.
14. United States Patent and Trademark Office Department of Commerce. 2010. http://patft.uspto.gov/. Accessed Jan 2010.
15. Current Procedural Terminology. CPT 2010. Professional Edition. American Medical Association. ISBN 978-1-60359-119-5.
16. Weinberg JW, Koestenblatt EK, Jennings MB. Utility of histopathologic analysis in the evaluation of onychomycosis. J Am Podiatr Med Assoc. 2005;95:258–63.
17. Bergmans AMC, Schouls LM, van der Ent Klaassen MA, et al. Validation of PCR–reverse line blot, a method for rapid detection and identification of nine dermatophyte species in nail, skin and hair samples. Clin Microbiol Infect. 2008;14:778–88.
18. Thurber SE, Zhang B, Kim YH, et al. T-cell clonality analysis in biopsy specimens from two different skin sites shows high specificity in the diagnosis of patients with suggested mycosis fungoides. J Am Acad Dermatol. 2007;57:782–90.
19. Kardjeva V, Summerbell R, Kantardjiev T, et al. Forty-eight-hour diagnosis of onychomycosis with subtyping of *Trichophyton rubrum* strains. J Clin Microbiol. 2006;44:1419–27.
20. Ross LF, Moon MR. Ethical issues in genetic testing of children. Arch Pediatr Adolesc Med. 2000;154:873–9.
21. Freund CL, Sugarman J. Genetic testing: ethical issues. 2010. http://www.answers.com/topic/genetic-testing-ethical-issues. Accessed Jan 2010.
22. Office of Biotechnology Activities. Secretary's Advisory Committee on Genetics, Health, and Society (SACGHS). 2010. http://oba.od.nih.gov/SACGHS/sacghs_documents.html. Accessed Jan 2010.

Additional Resources

- **Association for Molecular Pathology** (http://www.amp.org/). An association that provides guidance in the field of molecular diagnostics. Website includes a molecular test directory for infectious agents, solid tumors and hematopathology.
- **GeneTests** (http://www.ncbi.nlm.nih.gov/sites/GeneTests). A medical genetics information resource for physicians and researchers. Website includes an introduction to genetic counseling and testing, in addition to a genetics laboratory directory.
- **American College of Medical Genetics** (http://www.acmg.net/). Provides education, resources and a voice for the medical genetics profession.
- **College of American Pathologists** (http://www.cap.org/). A leading organization of pathologists. Website includes resources related to molecular diagnostic testing.
- **Online Mendelian Inheritance in Man (OMIM)** (www.ncbi.nlm.nih.gov/omim). National Center for Biotechnology Information database of human genes and genetic phenotypes. Website includes information on all known Mendelian disorders and more than 12,000 genes.
- **The SNP Consortium Database** (www.ncbi.nlm.nih.gov/SNP/). Comprehensive database containing information on all known single nucleotide polymorphisms.
- **SNPedia** (http://www.snpedia.com/). A wiki discussing human genetics. Shares information on the effects of DNA variations, citing peer-reviewed scientific publications.
- **Human HapMap Consortium** (http://hapmap.ncbi.nlm.nih.gov/index.html.en). A public resource, which was developed by scientists in partnership with funding agencies, to aid researchers identify genes associated with human disease.
- **Office of Population Genetics** (www.genome.gov/GWAStudies/). Catalogue of published genome-wide association studies.
- **Human Genetics Commission** (http://www.hgc.gov.uk/Client/index.asp?ContentId=1). The UK Government's advisory body on developments in human genetics and their impact on individuals.
- **GenoMEL, the Melanoma Genetics Consortium** (http://www.genomel.org/). Melanoma research consortium. Website includes interactive materials for medical professionals, patients and their families, and the general public.

M.J. Murphy (ed.), *Molecular Diagnostics in Dermatology and Dermatopathology*,
Current Clinical Pathology, DOI 10.1007/978-1-60761-171-4,

Index

D

N