

Gregory A. Hosler · Kathleen M. Murphy

# Molecular Diagnostics for Dermatology

Practical Applications of  
Molecular Testing for the  
Diagnosis and Management of  
the Dermatology Patient

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Practical Applications of Molecular  
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Patient



Springer

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ISBN 978-3-642-54065-3      ISBN 978-3-642-54066-0 (eBook)  
DOI 10.1007/978-3-642-54066-0  
Springer Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014938358

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## Acknowledgments

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### Clinical Images

Dr. Stephen Weis  
Dr. Alan Menter  
Dr. Ern Loh  
Dr. Elaine Miller  
Dr. Jennifer Dharamsi  
Dr. Travis Vandergriff

---

### Graphics

Aneliza Jones and her graphics team: Andrew Jenkins, Bronson Ma, Julie Robinson Gillies, Meetu Chawla, Jennifer Nielsen, Jonathan Seales

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## **Other Support**

Julie and the boys (C, Q, S)

ProPath Dermatopathology:

Dr. Terry Barrett

Dr. Jeffrey Detweiler

Dr. Ryan Hick

Dr. Imrana Khalid

Dr. Robert Law

Dr. Marc Lewin

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## Abbreviations

A	Adenine
ACGH	Array-based comparative genomic hybridization
AD	Autosomal dominant
ADCC	Antibody-dependent cell cytotoxicity
ADE	Adverse drug event
AFB	Acid-fast bacilli
AFH	Angiomatoid fibrous histiocytoma
AIN	Anal intraepithelial neoplasia
AJCC	American Joint Committee on Cancer
AKT1	v-akt murine thymoma viral oncogene homologue 1
ALCL	Anaplastic large cell lymphoma
ALL	Acute lymphoblastic leukemia
AMA	American Medical Association
AML	Acute myeloid leukemia
AMP	Association for Molecular Pathology
APL	Acute promyelocytic leukemia
AR	Autosomal recessive
ARMS	Amplification refractory mutation system
ATRA	All-trans retinoic acid
AVL	Atypical vascular lesion
BA	Bacillary angiomatosis
BAC	Bacterial artificial chromosomes
BAP1	BRCA1-associated protein 1
BCL-2	B-cell lymphoma 2
BCL-6	B-cell lymphoma 6
bDNA	Branched deoxyribonucleic acid amplification
BP	Base pair
BRAF	v-raf murine sarcoma viral oncogene homologue B1
BRIM	BRAF-in-melanoma
C	Cytosine <i>or</i> constant (domain)
CADMA	Competitive amplification of differentially melting amplicons
CAMTA1	Calmodulin-binding transcription activator 1
CAP	College of American Pathologists
CCS	Clear cell sarcoma
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention <i>or</i> complement-dependent cytotoxicity

CDK4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase N2A
CE	Capillary electrophoresis
CEA	Carcinoembryonic antigen
CF	Cystic fibrosis
CGH	Comparative genomic hybridization
CISH	Chromogenic in situ hybridization
CLIA	Clinical Lab Improvement Act
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CMML	Chronic myelomonocytic leukemia
COSMIC	Catalogue of Somatic Mutations in Cancer
CPE	Cytopathic effect
CPT	Current procedural terminology
CR	Conserved region (domain)
CREB	cAMP response element binding protein
CSD	Cat scratch disease
CSF	Cerebrospinal fluid
CTCL	Cutaneous T-cell lymphoma
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CVS	Chorionic villus sampling
CYP	Cytochrome p450
D	Diversity (as in V-D-J)
DAPI	4',6-Diamidino-2-phenylindole
ddNTP	dideoxynucleotide triphosphate
DFA	Direct fluorescent antibody
DFSP	Dermatofibrosarcoma protuberans
DGGE	Denaturing gradient gel electrophoresis
DIHS	Drug-induced hypersensitivity syndrome
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DOE	Department of Energy
DRESS	Drug rash with eosinophilia and systemic symptoms
DTIC	Dacarbazine
EBV	Epstein-Barr virus
EDV	Epidermodysplasia verruciformis
EGFR	Epidermal growth factor receptor
EHE	Epithelioid hemangioendothelioma
EHK	Epidermolytic hyperkeratosis
EORTC	European Organization for Research and Treatment of Cancer
EPCAM	Epithelial cell adhesion molecule
ERK	(aka MAPK) mitogen-activated protein kinase
ETS	E-twenty-six (gene family)
EWS	Ewing sarcoma
FAMM	Familial atypical mole melanoma (syndrome)
FDA	United States Food and Drug Administration
FET	Fus-Ewsr1-Taf15 (gene family)
FFPE	Formalin fixed and paraffin embedded

FISH	Fluorescence in situ hybridization
FR	Framework region (domain)
FRET	Fluorescence resonance energy transfer
G	Guanine
GCF	Giant cell fibroblastoma
GIST	Gastrointestinal stromal tumor
GMS	Gömöri methenamine silver
GNA11	Guanine nucleotide-binding protein subunit $\alpha$ -11
GNAQ	Guanine nucleotide-binding protein G(q) subunit $\alpha$
GWAS	Genome-wide association studies
H&E	Hematoxylin and eosin
HCCC	Hyalinizing clear cell carcinoma
HCV	Hepatitis C virus
HHV-8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HNPPCC	Hereditary nonpolyposis colon cancer
HPV	Human papillomavirus
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homologue
HRSA	Health Resources and Services Administration (US Department of Health)
HSP	Heat shock protein
HSV	Herpes simplex virus
HTLV-1	Human T-cell leukemia virus type 1
ICD	International Statistical Classification of Diseases and Related Health Problems (codes)
Ig	Immunoglobulin
IGH	Immunoglobulin heavy chain
IGK	Immunoglobulin light chain kappa
IGL	Immunoglobulin light chain lambda
IHC	Immunohistochemistry
ISCL	International Society for Cutaneous Lymphoma
ISCN	International System for Human Cytogenetic Nomenclature
ISH	In situ hybridization
IVD	In vitro diagnostic
J	Joining (as in V-D-J)
JBAIDS	Joint Biological Agent Identification and Diagnostic System (anthrax detection)
JM	Juxtamembrane (domain)
JMML	Juvenile myelomonocytic leukemia
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue
KOH	Potassium hydroxide
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue
KS	Kaposi sarcoma
KSHV	Kaposi sarcoma herpesvirus
LANA-1	Latency-associated nuclear antigen 1
LCA	Leukocyte common antigen

LCH	Langerhans cell histiocytosis
LCR	Ligase chain reaction
LDT	Lab-developed test
LGFMS	Low-grade fibromyxoid sarcoma
LYP	Lymphomatoid papulosis
MALT	Mucosa-associated lymphoid tissue (lymphoma)
MAP	MUTYH-associated polyposis
MAPK	Mitogen-activated protein kinase (pathway)
MART-1	Melanoma antigen recognized by T cells 1
MC1R	Melanocortin-1 receptor
MCC	Merkel cell carcinoma
MCV	Merkel cell polyomavirus (or MCPyV)
MDM2	Mouse double minute 2 (gene/protein) (aka MAP2K) mitogen-activated protein kinase kinase
MEK	(aka HGFR) hepatocyte growth factor receptor
MET	Mycosis fungoides
MF	Malignant fibrous histiocytoma
MFH	O(6)-methylguanine DNA methyltransferase
MGMT	microribonucleic acid
miRNA	Microphtalmia transcription factor
MiTF	Human homologue of <i>E. coli</i> MutL 1
MLH1	Multiplex ligation-dependent probe amplification
MLPA	Mismatch repair
MMR	Mycobacteria other than tuberculosis
MOTT	messenger ribonucleic acid
mRNA	Methicillin-resistant <i>Staphylococcus aureus</i>
MRSA	Melanocyte-stimulating hormone
MSH	Human homologue of <i>E. coli</i> MutS 2
MSH2	Human homologue of <i>E. coli</i> MutS 6
MSH6	Microsatellite instability
MSI	Mendelian susceptibility to mycobacterial diseases
MSMD	Microsatellite stable
MSS	mtDNA
mtDNA	Mitochondrial deoxyribonucleic acid
MTOR	Mechanistic target of rapamycin (gene/protein)
MTS	Muir-Torre syndrome
MUTYH	mutY homologue (gene/protein)
N	Nucleotide
NCI	National Cancer Institute
NER	Nucleotide-excision repair
NGS	Next-generation sequencing
NIH	National Institutes of Health
NK	Natural killer (cells)
NPV	Negative predictive value
NRAS	Neuroblastoma rat sarcoma viral oncogene homologue
NSCLC	Non-small cell lung cancer
NSE	Neuron-specific enolase
NTM	Nontuberculous mycobacteria
OMIM	Online Mendelian Inheritance in Man

PAS	Periodic acid-Schiff
PBP	Penicillin binding protein
PCFCL	Primary cutaneous follicle center cell lymphoma
PCMZL	Primary cutaneous marginal zone B-cell lymphoma
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PEL	Primary effusion lymphoma
PET-FISH	Paraffin-embedded tissue fluorescence in situ hybridization
PGDFR	Platelet-derived growth factor receptor
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLC	Pityriasis lichenoides chronica
PLEVA	Pityriasis lichenoides et varioliformis acuta
PMS-2	Postmeiotic segregation increased, <i>S. cerevisiae</i> , 2 (gene/protein)
PNET	Primitive neuroectodermal tumor
PPK	Palmoplantar keratoderma
PPV	Positive predictive value
PTEN	Phosphatase and tensin homologue
RAF	Rapidly accelerated fibrosarcoma (gene family)
RAPID	Ruggedized advanced pathogen identification device
RAS	Rat sarcoma (gene family)
RB	Retinoblastoma (gene/protein)
RFLP	Restriction fragment length polymorphism
RMSF	Rocky Mountain spotted fever
RNA	Ribonucleic acid
ROC	Receiver operating characteristic (curve)
ROS	Reactive oxygen species
RR	Relative risk
RSS	Recombination signal sequences
RSV	Respiratory syncytial virus
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SALT	Skin-associated lymphoid tissue (lymphoma)
SCC	Squamous cell carcinoma
SCCmec	Staphylococcal cassette chromosome
SCF	Stem cell factor
SCLC	Small cell lung carcinoma
SCPLTCL	Subcutaneous panniculitis-like T-cell lymphoma
SDA	Strand displacement amplification
siRNA	Small interfering ribonucleic acids
SJS	Stevens-Johnson syndrome
SLL	Small lymphocytic lymphoma
SMRT	Single molecule real time
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SPA	Staphylococcal protein A
SS	Sézary syndrome
SSCP	Single-strand conformation polymorphism
T	Thymine

TB	Tuberculosis
TCR	T-cell receptor
TEN	Toxic epidermal necrolysis
TERT	Telomerase reverse transcriptase
TM	Transmembrane (domain)
TMA	Transcription-mediated amplification
TMZ	Temozolomide
TNF	Tumor necrosis factor
TNM	Tumor-node-metastasis (staging)
TNMB	Tumor-node-metastasis-blood (staging)
tRNA	Transfer ribonucleic acid
TFI	Thyroid transcription factor 1
Tyrp-1	Tyrosinase-related protein 1
U	Uracil
V	Variable (as in V-D-J)
VEGFR	Vascular endothelial growth factor receptor
VIN	Vulvar intraepithelial neoplasia
VZV	Varicella zoster virus
WGS	Whole-genome sequencing
WHO	World Health Organization
XLD	X-linked dominant
XLR	X-linked recessive
XP	Xeroderma pigmentosum
YAC	Yeast artificial chromosomes

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## Contents

<b>1</b>	<b>Introduction</b>	1
	Reference .....	4
<b>2</b>	<b>Basics of Nucleic Acids and Molecular Biology</b>	5
2.1	Introduction .....	6
2.2	DNA (Deoxyribonucleic Acid) .....	7
2.2.1	Structure.....	7
2.2.2	Genes .....	8
2.2.3	Replication.....	8
2.2.4	The Human Genome .....	9
2.3	The Human Genome Project.....	11
2.4	RNA (Ribonucleic Acid) .....	12
2.4.1	Structure.....	12
2.4.2	Function .....	13
2.5	Transcription and Translation .....	13
2.5.1	Gene Expression .....	15
2.5.2	Reverse Transcription .....	15
2.6	Nucleic Acid Alterations .....	16
2.6.1	Types of DNA Alterations .....	16
2.6.2	Causes of DNA Alterations .....	18
2.6.3	Repair of DNA Alterations .....	18
2.7	Nucleic Alterations and Disease .....	20
2.7.1	Germline Alterations .....	20
2.7.2	Benign Genetic Variants .....	20
2.7.3	Somatic Alterations and Neoplasia .....	23
2.8	Genomes of Infectious Agents.....	25
2.9	Summary .....	25
	References.....	26
<b>3</b>	<b>Molecular Methods</b> .....	27
3.1	Introduction .....	28
3.2	General Considerations for Assay Design and Implementation .....	30
3.2.1	Types of Genetic Alterations and Performance Requirements .....	30
3.2.2	Specimen Type and Composition .....	30
3.2.3	Lab-Developed Tests (LDT) Versus FDA-Approved In Vitro Diagnostic (IVD) Tests ...	33

3.3	The Basics of a Molecular Test . . . . .	34
3.3.1	Hybridization: Virtually All Molecular Tests Are Based on the Principle of Hybridization . . . . .	34
3.3.2	Enzymes . . . . .	35
3.4	Non-amplification Nucleic Acid Analysis Methods . . . . .	36
3.4.1	Karyotyping (Cytogenetic Analysis) . . . . .	36
3.4.2	In Situ Hybridization (ISH): Chromogenic In Situ Hybridization (CISH) and Fluorescent In Situ Hybridization (FISH) . . . . .	38
3.4.3	Southern Blot . . . . .	40
3.4.4	Microarrays and Comparative Genomic Hybridization (CGH) . . . . .	41
3.5	Amplification Methods . . . . .	43
3.5.1	Polymerase Chain Reaction (PCR) . . . . .	44
3.5.2	Microsatellite Instability Analysis (MSI) . . . . .	47
3.5.3	T-Cell and B-Cell Gene Rearrangement Analysis . . . . .	47
3.5.4	Real-Time PCR . . . . .	49
3.5.5	Other Amplification Methods . . . . .	53
3.6	Sequencing . . . . .	53
3.6.1	Sanger Sequencing . . . . .	53
3.6.2	Pyrosequencing . . . . .	54
3.6.3	Next-Generation Sequencing . . . . .	55
3.7	Practical Considerations . . . . .	58
3.7.1	What to Look for in a Laboratory and/or Test Result . . . . .	58
3.7.2	Costs and Reimbursement . . . . .	59
3.8	Summary and Looking Ahead . . . . .	60
	References . . . . .	60
4	<b>Melanoma. Part I. Risk Assessment, Diagnosis, and Prognosis: Using Molecular Tools to Diagnose Melanoma, Predict Its Behavior, and Evaluate for Inheritable Forms . . . . .</b>	63
4.1	Introduction . . . . .	64
4.2	The Genetics of Melanoma: Assessing Risk . . . . .	66
4.2.1	Loci Associated with Melanoma Risk . . . . .	67
4.2.2	Testing for Germline Mutations . . . . .	70
4.3	Diagnosis . . . . .	71
4.3.1	Comparative Genomic Hybridization (CGH) . . . . .	75
4.3.2	Fluorescence In Situ Hybridization (FISH) . . . . .	77
4.3.3	Mutational Analysis . . . . .	81
4.3.4	Gene Expression Profiling . . . . .	82
4.4	Prognosis . . . . .	82
4.4.1	Molecular Evaluation of the Sentinel Lymph Node . . . . .	84
4.4.2	Chromosomal Aberrations by FISH . . . . .	84
4.4.3	Ocular Melanoma . . . . .	85
4.4.4	Other Molecular Prognostic Biomarkers . . . . .	85
4.5	Practical Considerations for Ordering and Performing Molecular Tests . . . . .	85

---

4.5.1	Genetic Testing for Familial Melanoma . . . . .	85
4.5.2	CGH Versus FISH . . . . .	86
4.5.3	Mutational Analysis of Melanoma Signaling Molecules and Gene Expression Profiling . . . . .	89
4.5.4	Prognostic Testing . . . . .	89
4.6	Classification of Melanoma: Current and Near-Future Perspectives . . . . .	90
	References . . . . .	92
<b>5</b>	<b>Melanoma. Part II. Personalized Medicine: Using Molecular Tools to Guide Targeted Therapy . . . . .</b>	<b>97</b>
5.1	Introduction . . . . .	98
5.2	Melanoma Tumor Progression. . . . .	99
5.3	Melanoma Signaling Pathways and the Biology of Melanoma . . . . .	100
5.3.1	MAP Kinase Pathway . . . . .	102
5.3.2	KIT . . . . .	104
5.3.3	PI3K/AKT/mTOR Pathway . . . . .	105
5.3.4	Others. . . . .	106
5.4	Clinical Trials and Therapeutic Strategies. . . . .	106
5.4.1	Signaling Molecule and Pathway Inhibition . . . . .	107
5.4.2	Immunotherapy . . . . .	113
5.4.3	Resistance to Therapy and Clinical Relapse . . . . .	114
5.4.4	Combination Therapy and Emerging Therapeutic Strategies . . . . .	115
5.5	Practical Considerations for Ordering and Performing Molecular Tests . . . . .	117
5.5.1	Targeted Mutation-Specific Molecular Assays. . . . .	117
5.5.2	Immunohistochemistry . . . . .	124
5.5.3	Companion Testing: The New Reality? . . . . .	125
5.6	Summary . . . . .	126
	References . . . . .	127
<b>6</b>	<b>Leukemia and Lymphoma. Part I. Mycosis Fungoides and Sézary Syndrome: Using Molecular Tools to Aid in the Diagnosis, Staging, and Therapy for Mycosis Fungoides and Sézary Syndrome . . . . .</b>	<b>133</b>
6.1	Introduction . . . . .	134
6.2	Diagnosis . . . . .	135
6.2.1	Clinical Features . . . . .	135
6.2.2	Histology . . . . .	135
6.2.3	Immunohistochemistry . . . . .	135
6.2.4	The Need for Molecular Testing . . . . .	138
6.2.5	Molecular Studies . . . . .	138
6.2.6	Diagnostic Algorithms for MF/ SS . . . . .	147
6.3	Staging and Prognosis . . . . .	148
6.3.1	Assessing Prognosis by PCR . . . . .	148
6.3.2	Assessing Prognosis by FISH and aCGH . . . . .	151
6.4	Therapy . . . . .	151

6.5	Practical Considerations for Ordering, Performing, and Interpreting Molecular Tests . . . . .	151
6.5.1	Assay Selection and Design . . . . .	152
6.5.2	Interpretation of the PCR TCR Gene Rearrangement Assay . . . . .	156
6.6	Summary . . . . .	161
	References . . . . .	162
<b>7</b>	<b>Leukemia and Lymphoma. Part II: Primary Cutaneous B-Cell Lymphoma and Other Non-MF/SS</b>	
	<b>Hematopoietic Tumors . . . . .</b>	167
7.1	Introduction . . . . .	168
7.2	Determination of Clonality in B-Cell Infiltrates . . . . .	169
7.2.1	Immunohistochemistry and Flow Cytometry . . . . .	169
7.2.2	Molecular Studies . . . . .	169
7.3	Diagnostic Applications for Molecular Testing . . . . .	173
7.3.1	Primary Cutaneous B-Cell Lymphomas . . . . .	174
7.3.2	Non-MF/SS Primary Cutaneous T-Cell Lymphomas . . . . .	179
7.3.3	B-Cell Versus T-Cell Lymphoma . . . . .	181
7.3.4	Other Hematopoietic Tumors Primarily and Secondarily Involving the Skin . . . . .	181
7.4	Other Applications for Molecular Testing . . . . .	184
7.4.1	Prognosis . . . . .	184
7.4.2	Therapy . . . . .	186
7.5	Practical Considerations for Ordering, Performing, and Interpreting Molecular Tests . . . . .	186
7.5.1	Gene Rearrangement Assays . . . . .	186
7.5.2	Other Molecular Methods for the Diagnosis and Management of the Cutaneous Leukemia/Lymphoma Patient . . . . .	193
7.6	Summary . . . . .	195
	References . . . . .	195
<b>8</b>	<b>Tumors of the Soft Tissue: Using Molecular Tools to Aid in the Diagnosis of Soft Tissue Tumors and the Management of the Sarcoma Patient . . . . .</b>	199
8.1	Introduction . . . . .	200
8.2	Diagnosis . . . . .	200
8.2.1	Genetic Aberrations in Soft Tissue Pathology . . . . .	202
8.2.2	Examples of Soft Tissue Tumors with Characteristic Molecular Defects . . . . .	204
8.3	Prognosis . . . . .	215
8.3.1	Translocations and Fusion Genes . . . . .	216
8.3.2	Gene Amplification . . . . .	216
8.4	Therapy . . . . .	216
8.4.1	Fusion-Gene Targeted Therapy . . . . .	217
8.4.2	Mutation-Specific and Other Signaling Pathway-Directed Therapies . . . . .	217

---

8.5	Molecular Tests Performed on Soft Tissue Tumors and Practical Considerations . . . . .	218
8.5.1	FISH. . . . .	218
8.5.2	RT-PCR . . . . .	220
8.5.3	Others. . . . .	222
8.6	Summary . . . . .	224
	References. . . . .	224
<b>9</b>	<b>Genodermatoses. Part I: Muir-Torre Syndrome . . . . .</b>	<b>231</b>
9.1	Introduction . . . . .	232
9.2	Pathophysiology of MMR-Defective MTS . . . . .	233
9.3	Clinical Features . . . . .	236
9.4	Histologic Features . . . . .	237
9.5	Immunohistochemical Features . . . . .	238
9.6	Assessing MMR Defects: Immunohistochemistry and PCR-Based Assays . . . . .	239
9.6.1	Immunohistochemistry for MMR . . . . .	239
9.6.2	Molecular MSI Testing . . . . .	239
9.6.3	IHC Versus MSI. . . . .	241
9.6.4	Genetic Testing . . . . .	243
9.7	Approach to the Suspected MTS Patient. . . . .	245
9.7.1	Defining MTS . . . . .	245
9.7.2	An Algorithmic Approach to the Diagnosis of MTS. . . . .	246
9.8	Summary . . . . .	250
	References. . . . .	250
<b>10</b>	<b>Genodermatoses. Part II: Other Hereditary Dermatologic Disease . . . . .</b>	<b>253</b>
10.1	Introduction . . . . .	254
10.2	Genodermatoses Associated with Cutaneous and/or Visceral Tumors (Inheritable Tumor Disorders) . . . . .	257
10.3	Inheritable Vascular Disorders. . . . .	263
10.4	Inheritable Bullous Disorders . . . . .	263
10.5	Inheritable Keratinization Disorders . . . . .	270
10.6	Ectodermal Dysplasias and Other Inheritable Disorders of the Sweat Glands, Hair, Nails, and/or Teeth. . . . .	278
10.7	Inheritable Connective Tissue Disorders. . . . .	278
10.8	Inheritable Disorders of Pigmentation. . . . .	278
10.9	Inheritable Metabolic Disorders . . . . .	278
10.10	Miscellaneous Disorders . . . . .	301
10.11	Practical Issues of Testing . . . . .	301
10.11.1	Testing Strategy . . . . .	301
10.11.2	Interpretation . . . . .	309
10.11.3	Cost and CPT Coding . . . . .	309
10.12	Summary . . . . .	311
	References. . . . .	312

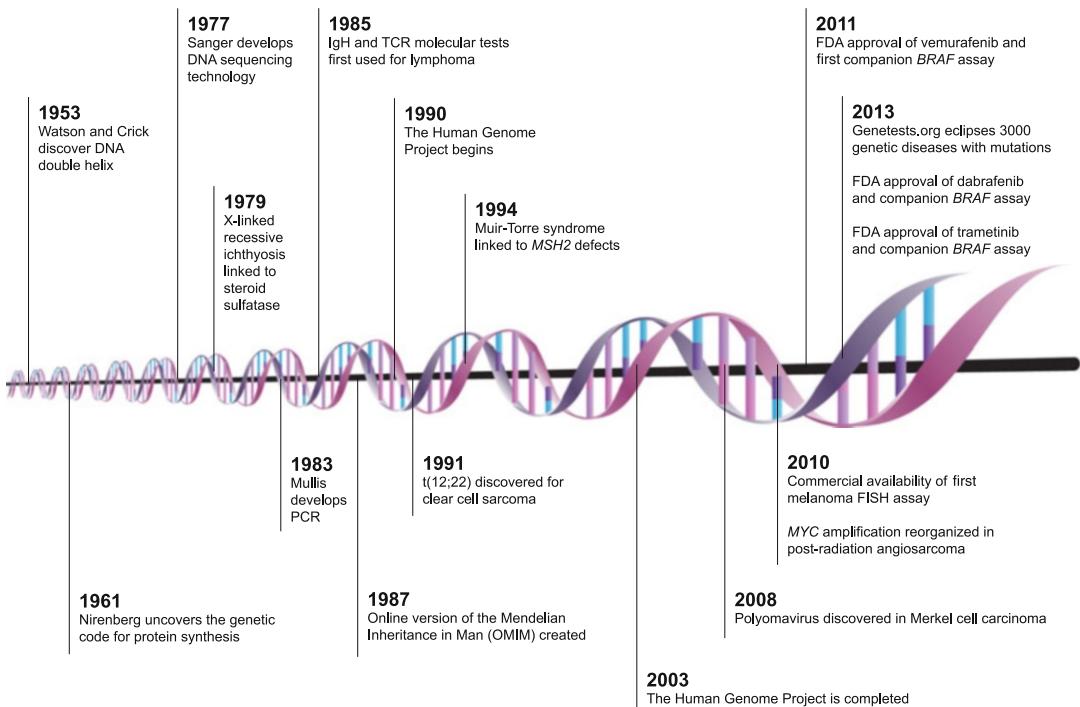
<b>11 Infectious Disease Testing . . . . .</b>	313
11.1 Introduction . . . . .	314
11.2 Assay Design and Testing Strategies . . . . .	316
11.3 Clinical Molecular Infectious Disease Testing . . . . .	318
11.4 Viruses . . . . .	319
11.5 Viral Infections Associated with Neoplasia . . . . .	320
11.5.1 Human Papillomavirus (HPV) . . . . .	320
11.5.2 Human Herpesvirus 8 (HHV-8) . . . . .	322
11.5.3 Merkel Cell Polyomavirus (MCV or MCPyV) . . . . .	323
11.6 Herpesvirus . . . . .	324
11.7 Fungi . . . . .	324
11.8 Parasites . . . . .	326
11.8.1 Leishmania . . . . .	326
11.9 Bacteria . . . . .	327
11.9.1 Mycobacteria . . . . .	327
11.9.2 Rickettsia . . . . .	330
11.9.3 Lyme Disease . . . . .	331
11.9.4 Syphilis . . . . .	331
11.9.5 Bartonella . . . . .	332
11.9.6 Cutaneous Anthrax . . . . .	333
11.10 Drug Resistance Testing . . . . .	334
11.10.1 Methicillin-Resistant Staphylococcus aureus . . . . .	334
11.11 Genetic Factors That Influence Susceptibility/ Resistance to Infectious Agents . . . . .	335
11.12 Practical Considerations . . . . .	335
11.12.1 External Controls (Positive, Negative, and No-Template) . . . . .	336
11.12.2 Sensitivity Control . . . . .	337
11.12.3 Internal Control . . . . .	337
11.12.4 Inhibition Control . . . . .	337
11.13 Summary . . . . .	337
References . . . . .	338
<b>12 Emerging Molecular Applications and Summary . . . . .</b>	341
12.1 Molecular Testing in Current Clinical Practice . . . . .	342
12.1.1 Clinically Significant Targets . . . . .	344
12.1.2 New Technologies . . . . .	345
12.2 Looking Ahead . . . . .	346
12.2.1 Theranostics . . . . .	346
12.2.2 Pharmacogenetics . . . . .	347
12.3 Summary . . . . .	352
References . . . . .	353
<b>Appendix . . . . .</b>	355

## Content

Reference .....	4
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For many, understanding molecular medicine is like standing at the tip of a long oceanic pier, gazing out. This vast, boundless body of information is enticing to some, overwhelming to most. If we choose to ignore it, at the very least, we will be lesser providers of care. We can choose to accept it or, better, embrace it, and we will not only benefit our patients but elevate the quality of modern medicine, entering new diagnostic and treatment frontiers.

Over a century of research on nucleic acids has led to step-by-step advancements in the understanding of their role in inheritance and disease. The uncovering of the double helix structure of DNA by James Watson and Francis Crick in 1953 was instrumental, beginning an era of manipulating these genetic building blocks to predict, diagnose, and manage disease, spawning the discipline of molecular diagnostics (Fig. 1.1). The completion of the Human Genome Project in 2003 was another notable leap. As part of this project, the entire 3.2 gigabase human genome was sequenced [1]. Since then, more genomes have been sequenced, including those from research organisms such as *Drosophila melanogaster* (fruit fly) and *Caenorhabditis elegans* (roundworm), pathogens such as *Haemophilus influenzae*, and, of course, more humans, including James Watson himself. Out of the Human Genome Project, we learned of the approximately 25,000 human genes, a surprisingly low total capable of orchestrating our development and every menial and complex task. We confirmed that all humans are >99.9 % genetically alike,



**Fig. 1.1** Timeline of significant events in molecular diagnostics. There have been innumerable impactful events in the history of molecular diagnostics over the past half

century. Several, including some in the field of dermatology, are highlighted here

even at the base pair level, with the other <0.1 % holding the mystery to all of our individual differences and genetic sources of disease. And, perhaps most importantly, the human genome became accessible to the entire investigative world, providing an unprecedented template for molecular research. The field of molecular medicine became poised to explode. Molecular diagnostics has captivated medicine in a “Gangnam Style” fashion—fresh, new, and unavoidable. But unlike the popular song, molecular diagnostics has staying power.

In contrast to more conventional diagnostic tools such as histology, cultures, and biochemical assays, molecular diagnostics is traditionally defined by the use of DNA-based (or RNA-based) tests for the diagnosis of human disease. The field has evolved, however. Molecular diagnostics is no longer limited to mere *diagnostics*, separating itself from other ancillary tests in its ability to

predict disease behavior and a patient’s response to therapeutic targets. In colon cancer, for example, the diagnosis is usually not in question, but molecular testing—*KRAS* mutational analysis, for example—is ordered to predict whether or not the tumor will respond to a specific therapy—cetuximab. Now, the trifecta of “molecular diagnostics” includes *diagnostics* (identifying and classifying disease), *prognostics* (predicting disease course), and *theranostics* (predicting response to therapy), with the latter arguably the most rapidly growing area. And the field refuses to stay stagnant, as applications continue to reach new areas, such as risk assessment and therapeutic monitoring.

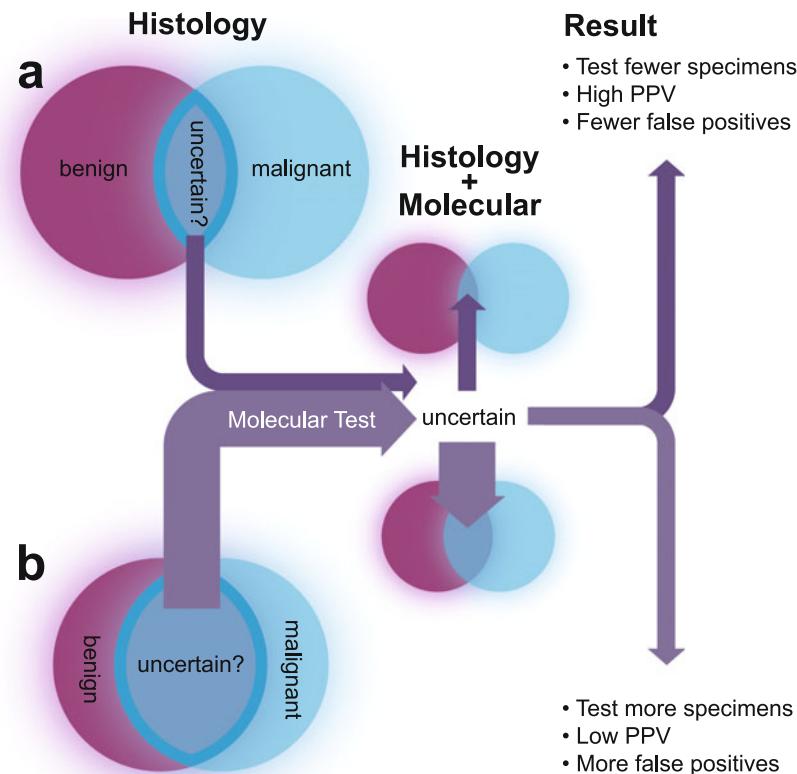
In dermatology, the incorporation of molecular diagnostics has admittedly lagged behind other disciplines, with only few and focused practical applications. This narrative is beginning to change, however, with recent important advancements and exciting new applications, touching all

areas of the above italicized trifecta. As examples, molecular tests are now used to help identify germline mutations in the genodermatoses, somatic mutations in tumors such as melanoma and various sarcomas, and the presence of certain cutaneous infectious agents, just to name a few. For melanoma and lymphoma, testing can potentially predict tumor behavior and modify patient staging. And, regarding theranostics, there is no better impactful example in dermatology than the recent observation that targeted therapy to the mutated B-Raf<sup>V600E</sup> in a subset of melanoma patients dramatically reduces tumor burden and, in rare cases, leads to apparent cure. The entire treatment paradigm for melanoma and other cancers is evolving. “Excision and pray” approaches are being replaced by personalized medicine. Treatment regimens are now being tailored to the individual based on their genome and their tumor’s genome. In cases of relapse, second and third rounds of targeted therapy may induce second and third rounds of remission, respectively. Ultimately, in patients unable to achieve a cure, therapy may evolve to constant tumor genome surveillance with molecularly based fine-tuning of treatments, transforming cancer, as we currently know it, into a chronic illness not unlike HIV and diabetes.

With every new test comes hope for revolutionizing applications. In their wake, however, we often struggle with how to implement them. For example, there is a great tendency to overuse new diagnostic tests, supplanting conventional means. Molecular diagnostic tests are like any other ancillary test, dependent on the prevalence of the disease in the population tested. Testing a large number of samples in a population of low disease prevalence will increase the number of false positives and result in a poor predictive value for the assay. Molecular testing is designed to shape a diagnosis for the pathologist, not be a crutch for the “parapathologist” (see Fig. 1.2 for further development of this concept). New tests may also introduce unanticipated practical or ethical problems. We are now able to generate immense patient and/or tumor genetic data,

most of which we do not understand. We must resist the temptation of testing just because we can, without an evidence-based infrastructure. A recent Supreme Court decision on gene patenting and the new practice of linking specific molecular tests to the FDA approval of therapy have opened avenues and introduced new wrinkles, respectively, for laboratories interested in test development.

Indeed, this is an exciting time in dermatology, and our goal as authors is to present this current (and near-future) state of affairs of molecular testing as it pertains to the dermatology patient, recognizing that this is in constant flux. In the following chapters, we begin with a basic introduction to molecular biology and commonly used methods for molecular diagnostics. We continue by covering practical applications of molecular diagnostics over a cross section of dermatologic disease, including melanoma, lymphoma, soft tissue tumors, genodermatoses, and infectious disease. Throughout the text, we emphasize the role of the dermatopathologist in test selection, preparing the sample, and interpreting results. And as molecular assays trend toward the generation of thousands of data points in a single reaction, we underline the importance of critically evaluating data in the context of the individual patient, often requiring input by the entire care team. We offer some practical advice, to those ordering molecular tests as well as to those considering performing such tests, with the following chapters serving as a potential template for a comprehensive dermatologic molecular diagnostic test menu. Our focus is on current, practical applications, but we also take several opportunities to look ahead, exploring the future of molecular diagnostics in dermatology and its potential impact on later generations. So as we pull off the fresh seal of the molecular peanut butter jar, exposing its contents with that initial scoop, we hope that all readers—clinicians, pathologists, laboratorians, or other inquisitive minds—*independent* of their level of molecular expertise, can find some nugget that will provoke thought or perhaps even change their practice.



**Fig. 1.2** Conceptual schematic of the role of a new diagnostic test. With every new diagnostic test, there is a positive or negative result. The power of the test, or its ability to distinguish the presence or absence of disease, is dependent on its performance characteristics, including but not limited to sensitivity and specificity. This concept can be applied to a biochemical assay, a molecular test, or even looking through the microscope. Using melanoma as an example, the experienced pathologist may look at an H&E section through the microscope and be able to distinguish melanoma from nevus in most cases, with a small but significant overlapping area corresponding to ambiguous lesions or lesions with indeterminate biology (**a**). The “parapathologist” will have a different starting point, less

able to distinguish benign from malignant, with virtually overlapping *circles* (**b**). With the use of a molecular or other ancillary test, the goal is to pull those circles apart, minimizing the overlapping area. The *blue bold lines* along the edges of the overlapping *circles* represent a narrow population of cases with the highest (positive and negative) pretest probability. In (**b**), there is overutilization (more area in intersection of *circles* leading to additional testing) with many of the tested cases having a low pretest probability and, thus, higher numbers of false-positive and false-negative results. Ancillary tests are designed to supplement conventional tests and rarely completely eliminate interpretive overlap. *PPV* positive predictive value

## Reference

1. Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, et al. Initial sequencing and analysis of the human genome. *Nature*. 2001;409:860–921.

# Basics of Nucleic Acids and Molecular Biology

2

## Contents

2.1	<b>Introduction</b> .....	6
2.2	<b>DNA (Deoxyribonucleic Acid)</b> .....	7
2.2.1	Structure.....	7
2.2.2	Genes .....	8
2.2.3	Replication.....	8
2.2.4	The Human Genome.....	9
2.3	<b>The Human Genome Project</b> .....	11
2.4	<b>RNA (Ribonucleic Acid)</b> .....	12
2.4.1	Structure.....	12
2.4.2	Function .....	13
2.5	<b>Transcription and Translation</b> .....	13
2.5.1	Gene Expression .....	15
2.5.2	Reverse Transcription .....	15
2.6	<b>Nucleic Acid Alterations</b> .....	16
2.6.1	Types of DNA Alterations .....	16
2.6.2	Causes of DNA Alterations .....	18
2.6.3	Repair of DNA Alterations.....	18
2.7	<b>Nucleic Alterations and Disease</b> .....	20
2.7.1	Germline Alterations .....	20
2.7.2	Benign Genetic Variants .....	20
2.7.3	Somatic Alterations and Neoplasia.....	23
2.8	<b>Genomes of Infectious Agents</b> .....	25
2.9	<b>Summary</b> .....	25
	<b>References</b> .....	26

### Key Points

- Nucleic acids are essential for all forms of life.
- The human genome is composed of approximately three billion base pairs of DNA, which are organized into two copies of each of 22 autosomes (non-sex chromosomes) and one pair of sex chromosomes (either XX or XY), for a total of 46 chromosomes.
- In humans, DNA stores the genetic code of life. It is the blueprint, or recipe, for producing all of the proteins needed to carry out cellular functions.
- RNA carries out many diverse and highly specialized cellular functions. These functions primarily involve the processes of transcription and translation, which lead to the production of proteins. RNA functions not only to produce proteins but also to regulate the production process.
- The term “gene expression” is used to indicate the production of RNA and/or protein from a gene. A gene may be silent (no expression) or may be highly expressed. The expression level of genes results in the phenotype of a cell.
- DNA can be altered from normal (wild type) in a wide variety of ways including chromosomal number alterations,

structural alterations, and sequence alterations.

- DNA alterations are either inherited (germline) or acquired (somatic). Germline mutations result in inherited diseases. Somatic mutations are important drivers of neoplasia.
- The structural and chemical properties of nucleic acids can be exploited to develop molecular diagnostic tests with an array of clinical utilities.

the development of modern genetics. Understanding the structure of DNA provided an almost immediate understanding of how DNA was replicated and how it might be passed from one generation to the next. In their landmark publication, Watson and Crick wrote “It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material” [2]. The discovery of the double-helix structure of DNA also laid the foundation for the development of molecular biology methods and tools, further accelerating research and discovery.

It is now well established that nucleic acids are found in all living cells and in viruses and are essential for all forms of life. Also well established is the concept that while the structures of DNA and RNA are similar, their function and some important chemical characteristics are very different. The sequencing of the entire human genome and the rapid advances in the fields of genetics and molecular biology have set the stage for a much greater understanding of human disease. Application of this knowledge is leading to improvements in making diagnoses and identifying effective treatments. The concept that nucleic acid alterations resulted in inherited diseases was obvious early on. It was not until the early 1990s, however, that researchers began to appreciate the genetic nature of cancer [3].

This discussion relates specifically to the structure and function of human DNA and RNA. It is not the purpose of this chapter to provide a comprehensive review of nucleic acids and molecular biology. There are entire textbooks devoted to these topics. Rather, the purpose of this chapter is to review basic concepts in molecular biology and nucleic acid chemistry to provide an understanding of the nomenclature and vocabulary required to comprehend molecular testing and its impact on patient care. The normal structure and cellular functions of nucleic acids are reviewed, providing a foundation for the discussion of molecular methods used in the clinical molecular laboratory (Chap. 3). In addition, this chapter begins the discussion of how deviations from normal structure and function result in disease, with special attention given to dermatologic disease.

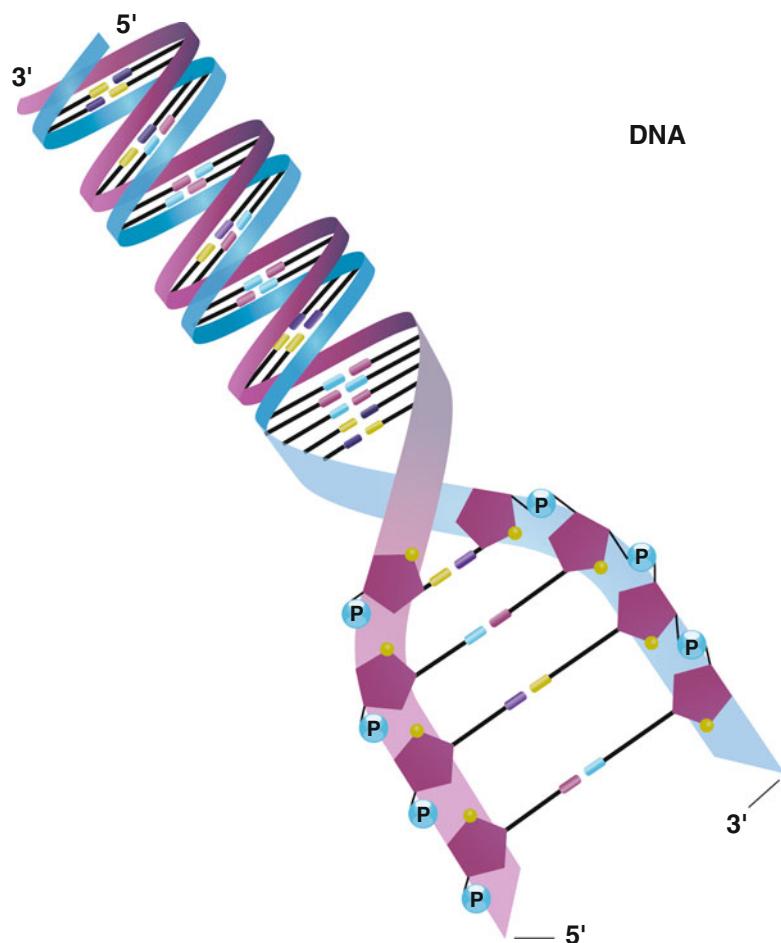
## 2.1 Introduction

Many think of Watson and Crick’s description of the double-stranded helix as the beginning of nucleic acid research, while in fact, nucleic acids were first discovered almost 100 years prior (1869) by Swiss scientist Friedrich Miescher. As indicated by the name nucleic acid, initial work discovered these molecules in the nucleus of cells and determined that they had acidic properties. Early work also determined that there are two basic types of nucleic acids, *deoxyribonucleic acid* (DNA) and *ribonucleic acid* (RNA). Although these basic properties were understood, it would take decades to reveal the structure and function of these molecules. Around the same time (1865), the Austrian monk Gregor Mendel established the idea that physical characteristics are passed from one generation to the next by discrete units, later to be called genes. Over the next several decades, the parallel research into the function of nucleic acids and the mechanism of inheritance started to converge. The microbiologist Oswald Avery and his colleagues at the Rockefeller Institute in New York are largely credited with the collision of these two areas, establishing that DNA, not proteins as many had hypothesized, was the carrier of genetic information [1].

James Watson and Francis Crick, along with significant contributions from Rosalind Franklin, determined the structure of DNA in 1953. This historic discovery is considered the beginning of

**Fig. 2.1** Structure of DNA.

Each ribbon of the double strand represents the sugar (deoxyribose)-phosphate backbone of DNA. The strands are antiparallel (oriented in opposite direction), with complementary base pairing between the two strands



## 2.2 DNA (Deoxyribonucleic Acid)

DNA contains the genetic code of life. It is the blueprint, or recipe, for producing all of the proteins needed to carry out cellular functions. DNA is a very stable molecule, obviously a desired characteristic for a molecule that stores the genetic code for life. The stability of DNA is evidenced by the fact that DNA has been recovered from ancient Egyptian mummies and extinct species many thousands of years after their deaths.

### 2.2.1 Structure

Everyone is familiar with the image of the double-stranded DNA helix, but what exactly

does that image depict? Each of the two strands of DNA is composed of alternating sugar (deoxyribose) and phosphate molecules (Fig. 2.1). This “sugar-phosphate backbone” is linked together through phosphodiester bonds at the number 3' and number 5' carbon positions of the sugar molecule. The two strands that make up the DNA double helix are antiparallel and are complementary. *Antiparallel:* The two strands are oriented in opposite directions. The sugar molecules on the two strands “point” in opposite directions, giving each strand its orientation. The ends of each strand are described as being either 3' (spoken three prime), indicating that the 3 position of the sugar molecule is not linked to another dNTP, or five prime (5'), indicating that there are no additional dNTPs linked at the 5 position of the sugar

molecule. By convention, DNA sequence is always written in the 5'→3' direction so that the orientation is clear. *Complementary*: The first carbon position of each sugar molecule is covalently linked to one of four nitrogenous bases: adenine (A), guanine (G), cytosine (C), and thymine (T). Two of the nitrogenous bases, adenine (A) and guanine (G), are purines, which are double-ring molecules. The other two bases, cytosine (C) and thymine (T), are pyrimidines, which are single-ring molecules. Each purine specifically “base pairs” with its complementary pyrimidine on the opposite strand: adenine pairs with thymine and guanine pairs with cytosine. The specificity of this pairing is essential to maintaining the structure and accurate replication of DNA. Mismatches distort the double helix and can result in mutations. Because the specificity of the pairing is absolute, one can deduce the sequence of a DNA strand if the sequence of the complementary strand is known. The hydrogen bonds that form between the complementary base pairs are responsible for holding the two strands of DNA together. Adenine forms two hydrogen bonds with thymine, and cytosine forms three hydrogen bonds with guanine. Thus, a DNA sequence that contains many Gs and Cs is held together more strongly than a sequence containing many As and Ts. *In vivo*, human DNA remains double stranded except during the process of DNA replication, during which the strands must be separated (see Sect. 2.2.3). In the laboratory, the complementary nature and hydrogen bonds that hold two DNA strands together are the foundation on which essentially all molecular testing methodologies are based. *In vitro*, specific DNA sequences can be detected and identified by the processes of bringing two single strands of DNA together to form a double strand (*hybridization*) and the process of separating two strands to single-stranded molecules (*denaturing, melting*). See Chap. 3 for a more complete discussion of the role of hybridization and denaturing in laboratory assays.

The length of a DNA sequence is measured in bases (b) if describing a single-stranded DNA molecule and base pairs (bp) if describing a double-stranded DNA molecule. When describing

large regions of DNA, the following units of measurement are used:

- kb=kilo base pairs=1,000 bp
- Mb=mega base pairs=1,000,000 bp
- Gb=giga base pairs=1,000,000,000 bp

## 2.2.2 Genes

Genes are segments of DNA that contain the code for specific proteins. Thus, genes are sometimes called coding regions of DNA, while genomic regions that do not result in production of a protein product are called noncoding. By convention, the sequence of a gene is always written in the direction of 5'→3'. The typical gene structure includes exons, which contain the code for protein sequence, separated by noncoding introns. A gene may have just one exon or over 100, and the size of each exon and intron can range from just a few bases to several thousand. The largest human gene (*DMD*), mutations in which are responsible for Duchenne and Becker muscular dystrophy, spans more than 2,000 kilobases, arranged in 79 exons. In contrast, the human type VII collagen gene (*COL7A1*), mutations in which are responsible for epidermolysis bullosa dystrophica, spans just 31 kilobases, yet has more exons (118), due to the use of smaller exons and introns. The DNA sequence upstream of a gene contains sequence elements such as promoters that are essential for the cellular machinery to recognize the gene-coding sequence and regulate the production of protein from the gene. Sequences downstream of the gene are important for terminating protein production (see Sect. 2.5 ).

## 2.2.3 Replication

With each cell division, the entire genome of an organism must be faithfully replicated in order to preserve the integrity of the genetic code and maintain viability of the species. DNA is replicated by a DNA-dependent DNA polymerase enzyme. “DNA dependent” indicates that DNA is the template, but this phrase is often omitted, and the enzyme is generally referred to as simply a

DNA polymerase. Additional enzymes and other molecules are required to unwind and separate the two strands. The complementary nature of the two DNA strands allows for each to serve as the template to synthesize the other strand. This process of semiconservative replication results in two new strands of DNA, each of which consists of one of the original strands and a newly synthesized complementary strand.

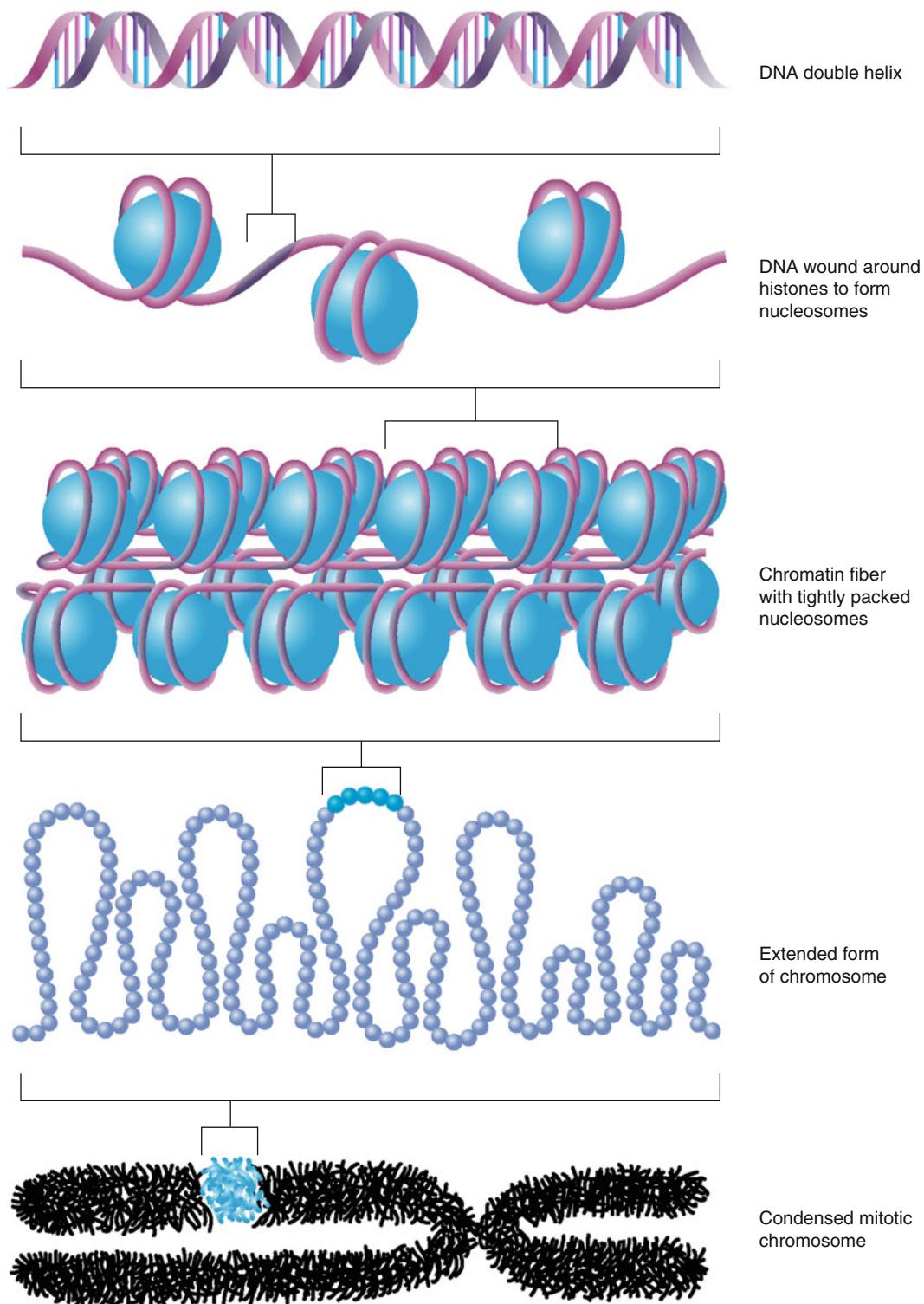
#### 2.2.4 The Human Genome

The human genome is composed of approximately three billion base pairs of DNA, which are maintained in highly organized structures within the nucleus of cells (Fig. 2.2). The DNA double helix is wrapped around histone proteins, resulting in bead-like structures called nucleosomes. The nucleosomes are coiled into chromatin fibers. When cells are in a “resting,” nondividing state (interphase), their DNA is arranged in a relatively diffuse and extended structure comprised of loops of chromatin fibers. As a cell prepares to go through division, the DNA is replicated and packaged tightly in order to ensure proper segregation of the genetic material to each daughter cell. It is in this compact state (metaphase) that chromosomes can be stained and analyzed microscopically to generate the familiar images of chromosomes. Figure 2.3 depicts a generic chromosome and describes important chromosomal structures such as the centromere and telomeres. The term “locus” is used to describe a position or location in the genome, which may or may not code for a gene. For each locus, there are two alleles, one maternally inherited and one paternally inherited. If the sequence of allele 1 and allele 2 is identical, it is said to be homozygous at that locus. If the sequence of allele 1 and allele 2 is not identical, it is described as heterozygous at the locus.

The three billion base pairs of the human genome are arranged into two copies of each of 22 autosomes (non-sex chromosomes) and one pair of sex chromosomes (either XX or XY), for a total of 46 chromosomes. A karyotype is a description of the chromosomal constitution of a

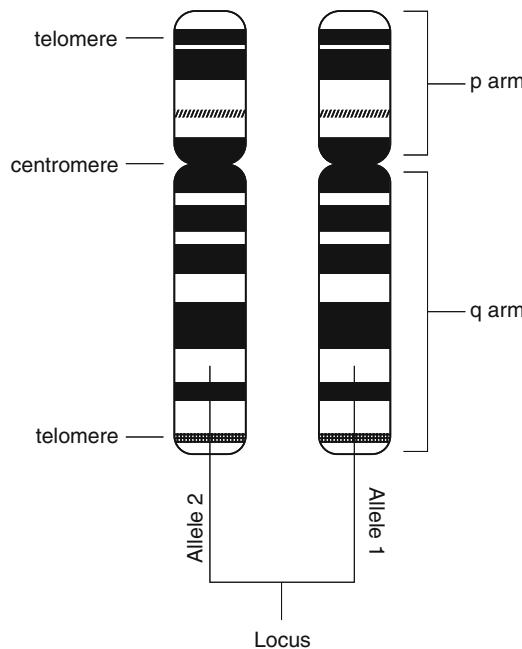
specimen. A normal human karyotype is 46,XX or 46,XY (Fig. 2.4). Individual chromosomes are identified by their size, centromere position, and banding pattern [4]. By convention, chromosomes are numbered sequentially based on size, from largest to smallest. The shorter arm of a chromosome is designated “p,” and the longer arm, “q.” On each arm of a chromosome, the bands are numbered consecutively beginning at the centromere and extending outward along each chromosomal arm. A particular region is designated by the chromosome number, the arm symbol, the region number, and the band number within the region. For example, 1p36 indicates chromosome 1, short arm, region 3, band 6. Because the region and band numbers are distinct, 1p36 is spoken as “one p three six,” not “one p thirty-six.” If an existing band has been subdivided, a decimal point is placed after the original band designation, followed by the sub-band number, for example, 1p36.2.

With two exceptions, normal human cells contain two copies of all DNA sequences (diploidy), one maternally and the other paternally inherited. The first exception is gametes, which contain a single-copy genome (haploidy). Gametes must be haploid in order for fertilization of a haploid egg with a haploid sperm to result in a diploid cell. The second exception is mitochondrial DNA. Although the vast majority of DNA is located in the nucleus of cells, mitochondria, which are located in the cytoplasm, contain a limited amount of DNA that codes for a small number of genes. Similar to nuclear DNA, defects in mitochondrial DNA (mtDNA) can result in disease including dermatologic diseases such as Leigh syndrome and palmar-plantar keratoderma with deafness. In contrast to nuclear DNA, mtDNA is strictly maternally inherited and is present in varying amounts in different types of cells. Human cells have multiple mitochondria, and each mitochondrion contains several copies of mtDNA. Thus, while cells carry just two copies of nuclear genes, they carry hundreds to thousands of copies of each mitochondrial gene. For the purpose of this publication, DNA refers to nuclear DNA unless otherwise specified.



**Fig. 2.2** Packaging of DNA within the nucleus. In mammals, the length of double-stranded DNA in its primary form corresponds to approximately 1 m. In order to contain the entire genome within the nuclei of cells, DNA

must be efficiently folded and packaged. This process includes the winding of DNA around histone proteins to form nucleosomes and further packaging of nucleosomes to form chromatin



**Fig. 2.3** Diagram of chromosomal structure. Telomeres are repetitive sequences at each end of chromosomes. These sequences protect the DNA from damage and loss. The centromere contains repetitive sequences and plays an important role in accurately segregating chromosomes during cell division. The centromere divides the chromosome into two arms. The shorter arm of the chromosome is referred to as the p arm, and the longer arm is the q arm. A locus is a position or location on the chromosome. For each locus there are two alleles

Only a small percentage of our genome codes for proteins. This was one of the most surprising findings from the Human Genome Sequencing Project. Of the three billion base pairs in our genome, fewer than 5 % code for proteins [5]. Although the remaining 95 % was originally thought of as “junk DNA” because it did not directly code for proteins, we now know that these noncoding regions in DNA play essential cellular roles. Repetitive regions in noncoding DNA help to maintain the stability of DNA and protect it from damage and loss. Other noncoding regions function to ensure proper segregation of newly synthesized DNA into daughter cells during cell division. In addition, noncoding regions play important roles in regulating gene expression of the coding regions. This area of study is still in its infancy, and discoveries will likely have major impacts on science and medicine.

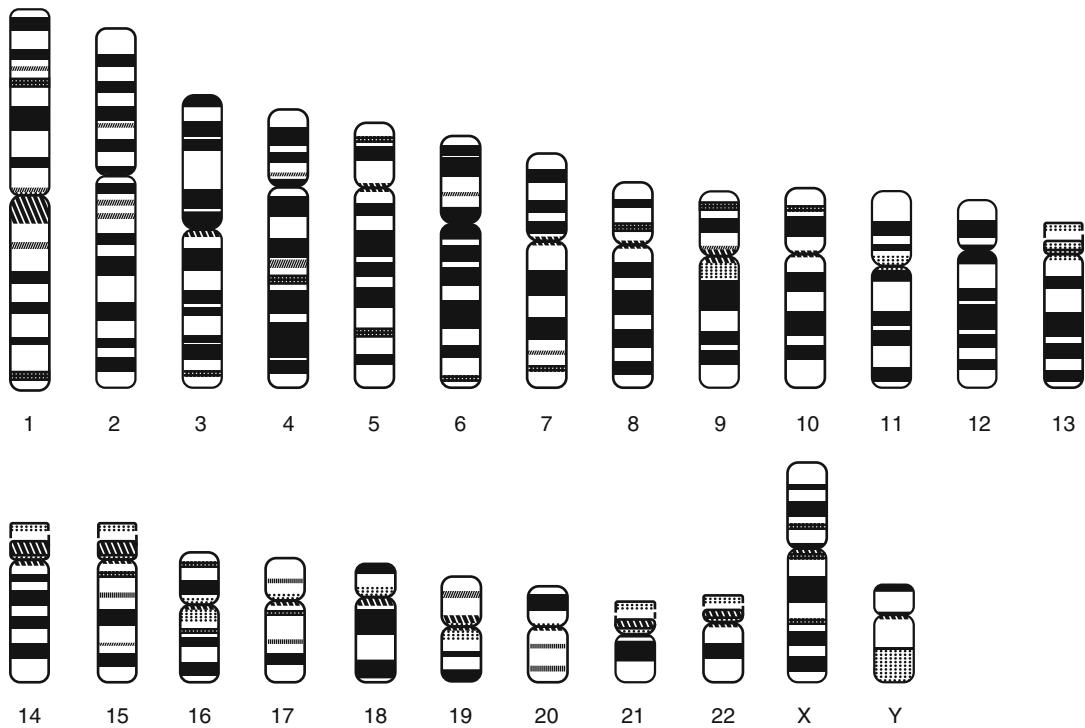
The sequence of the human genome is readily available from the NIH’s genetic sequence database, GenBank, [www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/), which contains all publicly available DNA sequences. The Human Genome Sequencing Project sequenced the genome of an unknown individual, which is approximately 99.9 % identical to the sequence in all human beings. Human genetic differences occur at approximately 1 out of every thousand bases of DNA. It is these minute differences that make us individuals. This small number of genetic differences determines skin, eye, and hair color, predisposition for disease, and response to medications and likely has significant influence on more subtle characteristics such as personality and other traits.

### 2.3 The Human Genome Project

The Human Genome Project was an international, collaborative research program with the goal of complete mapping and understanding of all the genes in the human genome. In the United States, the program was coordinated and funded by a joint effort between the Department of Energy (DOE) and the National Institutes of Health (NIH). The project was launched in 1990, with full-scale sequencing production commencing in 1999. Amazingly, the project was completed ahead of schedule and under budget (approximately \$2.7B). The project remains one of the largest single investigative projects in modern science.

The International Human Genome Sequencing Consortium published the first draft of the human genome in the journal *Nature* in February 2001, which included the sequence of approximately 90 % of the entire genome’s three billion base pairs [5]. The full sequence was completed and published in April 2003. One of the most surprising findings from the project was that the human genome coded for approximately 20,000–30,000 genes, which was significantly fewer than what was estimated by previous studies.

The Human Genome Project produced detailed information about the structure, organization, and function of the complete set of human genes. This resource is freely available worldwide and continues



**Fig. 2.4** Schematic of a normal human karyotype. Chromosomes are identified by their size, centromere position, and banding pattern. They are numbered sequentially based on size, from largest to smallest

to be an important resource for ongoing studies of the structure and function of the genome as well as studies of individual genes. Because the Human Genome Project emphasized the development and pilot testing of new technologies, it helped to drive innovations in technologies such as yeast artificial chromosomes (YAC), bacterial artificial chromosomes (BAC), polymerase chain reaction (PCR) amplification, electrophoresis, and data management. The tools created as part of the project continue to inform and support large-scale scientific discoveries.

## 2.4 RNA (Ribonucleic Acid)

### 2.4.1 Structure

The structure of RNA is very similar to DNA, consisting of a backbone of alternating sugar and phosphate molecules, with a nitrogenous base attached to the first carbon position of each sugar

**Table 2.1** Comparison of DNA and RNA

	RNA	DNA
Sugar molecule	Ribose	Deoxyribose
Nitrogenous bases	guanine (G), cytosine (C), adenine (A), and <i>uracil</i> (U)	guanine (G), cytosine (C), adenine (A), and <i>thymine</i> (T)
Conformation	Generally single stranded	Generally double stranded
Stability	Highly unstable	Highly stable

molecule. Structural differences between RNA and DNA include the use of ribose as the sugar molecule rather than deoxyribose and the use of uracil (U) in place of thymine (T) as one of the four nitrogenous bases (Table 2.1). Generally, RNA is a single-stranded molecule, compared to DNA, which is typically double stranded. As opposed to DNA, RNA is a very unstable molecule, largely due to the ubiquitous presence of RNase enzymes, which rapidly degrade RNA molecules. This instability makes RNA a very

**Table 2.2** Function of RNA

Type of RNA	Function
mRNA	Messenger RNA
tRNA	Transfer RNA
rRNA	Ribosomal RNA
miRNA	Micro RNA
siRNA	Small interfering RNA
lncRNA	Long noncoding RNA

challenging molecule to work with in the laboratory. Special precautionary measures to avoid degradation, and additional testing controls, are required to ensure accurate test results.

#### 2.4.2 Function

RNA carries out many diverse and highly specialized cellular functions. RNA molecules are subdivided and named based on the function(s) they perform (Table 2.2). These functions primarily involve the processes of transcription and translation, which lead to the production of proteins (see below). RNA functions not only to produce proteins but also to regulate the production process. Currently, most clinical RNA-based studies involve the analysis of messenger RNA (mRNA). While much research continues on some of the more newly described RNA molecules such as microRNA (miRNA), analysis of these molecules has yet to find clinical utility.

## 2.5 Transcription and Translation

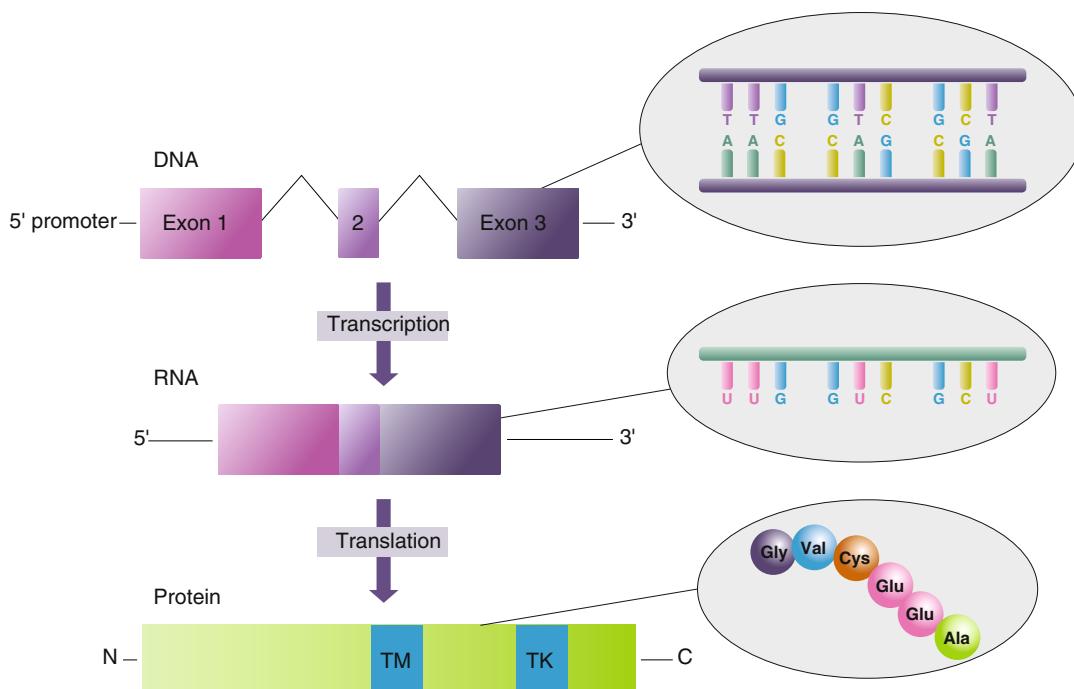
DNA is the code of life, but without a system for “breaking the code,” there is no life. The code is broken by the processes of transcription and translation. The end product of these processes is the

production of a protein molecule composed of amino acids. The amino acid composition of the protein is determined by the starting DNA sequence.

Transcription is the process of generating mRNA molecules using DNA as the template. The process requires a DNA-dependent RNA polymerase, often referred to simply as an RNA polymerase. During the process of transcription, the coding exons in the gene are spliced together, eliminating the noncoding intron regions. One gene can be used as a template to rapidly produce many mRNA transcripts.

Translation is the process of generating proteins using mRNA as the template. A single mRNA molecule can go through the process of translation multiple times to make multiple protein molecules. The translation process involves ribosomes and transfer RNA (tRNA) molecules in addition to the mRNA template. This flow of DNA → RNA → protein has been called the “central dogma of molecular biology” (Fig. 2.5).

Transcription is a one-to-one process, with each base of DNA being transcribed to a base of RNA. Translation is a three-to-one process. Three bases of RNA code for one amino acid, which are the building blocks of proteins. The three-base unit is termed a “codon” because it codes for one amino acid. There are 64 possible three-base combinations of the four bases. These three-base combinations code for 20 amino acids, and “stop codons,” which tell the cellular machinery that it



**Fig. 2.5** Central dogma of molecular biology. Schematic of a gene with the coding regions (exons) as boxes and the intervening noncoding regions (introns) as lines. Transcription is the process of using the DNA template to create a single-stranded RNA molecule which contains only coding regions. DNA and RNA are always written or illustrated in the 5' → 3' direction. Translation is the pro-

cess of generating protein molecules from an RNA template. Protein sequence is always displayed in the left to right direction as amino (*N*) terminus to the carboxy (*C*) terminus. Schematics of proteins typically depict specific regions of the protein such as the transmembrane (TM) and/or tyrosine kinase (TK) domains

has reached the end of the coding portion of the sequence. Figure 2.6 is the key to the genetic code. It deciphers which amino acid is coded by each three-base combination. Obviously there are more three-base combinations (64) than are needed to code for 20 amino acids and a stop signal. This is due to the fact that in some cases, multiple three-base combinations code for the same amino acid. This redundancy, also called degeneracy, in the genetic code primarily occurs with changes in the third position of the codon, which is often referred to as the “wobble position.” For example, GGT, GGC, GGA, and GGG all code for the amino acid glycine (Gly). The redundancy of the genetic code allows the genome to tolerate many single base DNA alterations without any phenotypic effect because there is no change to the amino acid sequence of the protein that is produced.

		Second letter					
		T	C	A	G		
		Phe Phe Leu Leu	Ser Ser Ser Ser	Tyr Try STOP STOP	Cys Cys STOP Trp	T C A G	
First letter	C	Leu Leu Leu Leu	Pro Pro Pro Pro	His His Gln Gln	Arg Arg Arg Arg	T C A G	
	A	Ile Ile Ile Met	Thr Thr Thr Thr	Asn Asn Lys Lys	Ser Ser Arg Arg	T C A G	
	G	Val Val Val Val	Ala Ala Ala Ala	Asp Asp Glu Glu	Gly Gly Gly Gly	T C A G	

**Fig. 2.6** The genetic code

### 2.5.1 Gene Expression

The term “gene expression” can be used to indicate the production of RNA and/or protein from a gene. A gene may be silent (no expression of RNA or protein) or may be highly expressed. The expression level of genes results in the phenotype of the cell. Although all cells in the body have the same DNA, different subsets of genes are expressed (transcribed and translated) in different cell types. Thus, while an individual’s melanocytes, keratinocytes, and white blood cells all carry the exact same genetic information (DNA sequence), they express different subsets of genes that allow them to carry out their specialized functions. Both transcription and translation are highly regulated to ensure that each cell has the correct type and quantity of the proteins it needs to carry out its functions. Some genes code for proteins that are needed at all times in particular cell types. For example, the keratin (*KRT*) gene family codes for intermediate filament proteins that are crucial for maintaining the structure of the skin, hair, and nails [6]. Other genes encode proteins that are only needed at specific times. For example, proteins that are needed to carry out cell division are expressed during mitosis but are turned off for the remainder of the cell’s life cycle.

For the vast majority of genes, protein products are expressed from both the maternal and paternal gene copies. The redundancy of having two gene copies provides some protection for humans from the effects of genetic alterations. When one copy of a gene is “bad” (nonfunctional or altered function), the “normal” copy is often, but not always, sufficient to maintain normal cellular functions. For a few genes, only one of the two gene copies is used to produce proteins. In females, genes on the X chromosome are expressed from only one of the two copies. Inactivation of one of the X chromosomes occurs essentially randomly in each cell such that approximately half of the cells in a female use the maternal copy and half use the paternal copy of the X chromosome as the template for gene expression. Genetic imprinting is another example of when only one gene copy is used as the

template for protein production. A small portion of human genes are imprinted (e.g., the parent of origin is marked or “stamped” on the gene), resulting in protein expression from only the maternal or paternal copy. The reason for imprinting is not entirely clear, but what is clear is that inheritance of a “bad” copy of the gene that is expressed results in disease since the other copy, although normal in sequence, is not expressed. The inheritance pattern of diseases related to imprinted genes does not follow typical inheritance patterns (see below) since the presence of one altered gene copy may or may not result in disease depending on from which parent it was inherited.

### 2.5.2 Reverse Transcription

In humans, the processes of transcription (DNA → RNA) and translation (RNA → protein) are unidirectional. For some viruses, however, that is not the case. Some viruses with an RNA genome, rather than a DNA genome, are able to replicate their RNA genome into a DNA copy. Since the process of making an RNA molecule from a DNA template was already referred to as transcription, the process of synthesizing DNA from an RNA template was termed “reverse transcription.” As opposed to the DNA-dependent DNA polymerase that carries out the DNA replication, reverse transcription requires an RNA-dependent DNA polymerase, commonly referred to as reverse transcriptase.

In the laboratory, reverse transcription is used to convert RNA to the more stable DNA molecule, which can then be subjected to PCR and other laboratory techniques. The DNA produced is termed complementary, or cDNA, to differentiate it from genomic, gDNA. gDNA sequence contains intronic and other noncoding sequences; cDNA contains only coding sequence. The development of reverse transcription greatly facilitated gene cloning, in a process whereby an mRNA sequence is converted to cDNA, which can be inserted into vectors, such as plasmids, and then transferred into cells in culture in the laboratory [7]. This results in expression of the gene in cells that

would not otherwise express it and allows researchers to study the effects of expression of that particular gene.

## 2.6 Nucleic Acid Alterations

### 2.6.1 Types of DNA Alterations

The un-mutated or “normal” DNA sequence is referred to as the “wild-type” sequence, indicating that it is considered to be the normal sequence found in nature. For the purpose of this publication, the terms “alteration” and “variant” are used interchangeably to denote a change or difference from the wild-type sequence, without regard to whether the effect of the alteration/variant is

benign or disease causing. Variants that are known to be disease causing are referred to as mutations or aberrations. DNA can be altered from the wild-type sequence in a wide variety of ways (Table 2.3). DNA alterations are either inherited (germline) or acquired (somatic). Germline mutations can be inherited from either or both parents and are present in essentially every cell in the body. Somatic mutations are not inherited but are acquired in specific cells during one’s lifetime. These mutations are important drivers of neoplasia.

DNA alterations can be segregated into three broad categories: chromosomal number alterations, structural alterations, and sequence alterations (see Table 2.3). Chromosomal number alterations result in cells with too many or too few total chromosomes. This genetic composition is

**Table 2.3** Types of DNA alterations

Type of alteration	Description
<i>Chromosome number alterations</i>	
Aneuploidy	One or more individual chromosomes are gained or lost Trisomy: gain of a single chromosome Monosomy: loss of a single chromosome
Polyploidy	Having >2 chromosomal sets Triploidy: 3 chromosomal sets Tetraploidy: 4 chromosomal sets
<i>Structural alterations</i>	
Gain of genetic material	Gain of additional copies (>2) of a single gene (gene amplification) or a larger chromosomal region. Generally this type of alteration results in overexpression of a gene(s) and a “gain of function” due to overexpression
Loss of genetic material	This includes loss of a single gene or a larger chromosomal region. Loss of genetic material may be referred to as <i>loss of heterozygosity</i> (LOH), indicating the reduction from two alleles to one. Generally this type of alteration results in “loss of function” due to reduced or completely abolished gene expression
Inversions and translocations	These involve the exchange of material between one or more chromosomes. These are described as “balanced” if there is no overall gain or loss of genetic material or “unbalanced” if there is a net gain or loss. The breakpoints may involve gene-coding and/or noncoding genomic regions
<i>Sequence alterations</i>	
Single base substitutions	The substitution of one base for another may have no effect if the substitution occurs in a noncoding region of the genome, or if it occurs in a coding region, but does not result in an amino acid substitution due to the redundancy of the genetic code. Substitutions that result in an amino acid sequence change can have varying effects on the protein function <i>Single nucleotide polymorphisms (SNPs)</i> are single base substitutions that have no or very little effect on protein production and function. SNPs are largely responsible for the polymorphic differences between individuals <i>Point mutation</i> is a term that refers to a single base substitution that has a deleterious effect
Insertions and deletions	Similar to single base substitutions, insertions and deletions of one or a few bases can result in benign polymorphisms or deleterious mutations. When insertions and deletions occur in gene-coding regions, they generally alter or destroy protein function

referred to as aneuploidy, to differentiate it from normal diploidy composition. The gain or loss can affect a single or a few chromosomes resulting in trisomy (three copies of one chromosome) or monosomy (one copy of one chromosome). These single chromosome number alterations can occur in the germline (e.g., Down syndrome, trisomy 21) or result from somatic events during neoplasia. In contrast, gains of an entire chromosomal complement, termed polyploidy, only occur in neoplastic tumors. In contrast to normal diploid (46 chromosomes) cells, these tumor cells typically have three (triploid, 69 chromosomes) or four (tetraploid, 92 chromosomes) copies of each chromosome.

Structural alterations include gains and losses of regions of chromosomes, such as the loss of 9p21 in melanoma. Structural alterations can also involve breaks and rearrangement of genetic material such as in translocations and inversions. Translocations and inversions can be relatively simple such as breaks on two chromosomes with improper repair resulting in a “swap” producing two chromosomes that are “hybrids.” In tumors, translocations can be very complex resulting in swapping of material that involves multiple chromosomes. Nomenclature for chromosomal number and structural alterations is discussed in Chap. 3.

Finally, there are sequence alterations. Sequence alterations that occur in gene-coding regions can have three different outcomes. The nucleic acid change may (1) result in no change to the amino acid sequence of a protein (silent), (2) result in a change to the amino acid sequence of the protein (missense), or (3) result in the formation of a stop codon, which prematurely terminates protein production (nonsense). Silent alterations are unlikely to have a phenotypic

effect. Nonsense mutations often result in a non-functional protein due to the fact that the protein production is terminated early resulting in a truncated protein. These types of alterations are likely to have a phenotypic effect. The outcomes of missense mutations are highly variable. If the amino acid substitution is conservative (the wild-type amino acid and the substituted amino acid have similar structural and chemical properties), the substitution may have little to no effect on the protein function. If the substituted amino acid has different properties from the wild type (nonconservative substitution), the single change can result in a nonfunctional protein, a protein with normal function but functioning at increased or decreased levels relative to wild type, or a protein with novel functions.

In the germline, benign single base changes are referred to as single nucleotide polymorphisms, SNPs. Both in the germline and in tumors, deleterious single base alterations are referred to as point mutations. Sequence alterations can also involve substitutions of multiple bases and deletions and insertions of bases. Insertions and deletions within protein-coding DNA sequence typically result in significant changes to the protein function.

Standard nomenclature to describe sequence variants uses either the coding DNA sequence position, preceded by “c.”, or the protein amino acid position, preceded by “p.” [8]. Protein alterations can be described using either the three- or one-letter amino acid abbreviation. Table 2.4 demonstrates the standard and common nomenclature used for two different *BRAF* mutations found in melanoma.

In addition to alterations that change the DNA sequence and/or structure, DNA can undergo epigenetic alterations, which can either increase or

**Table 2.4** Mutation nomenclature: *BRAF* mutation examples

DNA alteration nomenclature	Protein alteration nomenclature	Commonly used nomenclature	Description of alteration at codon 600
c.1799T>A	p.Val600Glu	V600E	DNA: GTG → GAG Protein: valine → glutamic acid (V, Val) → (E, Glu)
c.1798_1799GT>AA	p.Val600Lys	V600K	DNA: GTG → AAG Protein: valine → lysine (V, Val) → (K, Lys)

decrease gene expression. The most widely studied epigenetic alteration is *methylation*. Methylation of cytosine residues in the 5' untranslated promoter region of a gene can silence expression of that gene. In the germline, a relatively small number of genes have epigenetic modifications that result in parent-specific gene expression, rather than codominant expression (gene imprinting). Similar to somatic mutations, somatic epigenetic modifications are seen in tumors, including melanoma. In tumors, somatic mutation or somatic epigenetic modification of genes that regulate growth signaling pathways results in the same net outcome, unregulated and/or constitutive activation of the signaling pathway.

### 2.6.2 Causes of DNA Alterations

There are many causes of nucleic acid alterations. Chromosome number alterations occur when chromosome pairs fail to separate correctly into daughter cells during meiosis or mitosis. Chromosomal structural alterations often result from chromosome breaks that are improperly repaired. Sequence alterations are caused by several different mechanisms. Sequence alterations can result from errors that occur during DNA replication. Each time a cell divides, the entire genome must be accurately replicated in a very short period of time. There are at least a dozen human DNA polymerases with different but overlapping functions [9]. Although their error rates vary, it is estimated that the error rate during DNA replication is approximately 1 per every 100,000 nucleotides. While this may not seem like a high error rate, it corresponds to making approximately 120,000 mistakes every time a cell divides. Many of the errors that occur during replication are repaired by highly sophisticated DNA repair mechanisms (see below). However, a few mutations will escape repair or be repaired incorrectly, which can have devastating results.

Sequence alterations can also be caused by endogenous and exogenous mutagens (Fig. 2.7a). Endogenous mutagens are primarily the by-products of oxidative metabolism. Normal oxidative metabolism pathways in the mitochondria

can generate reactive oxygen species (ROS) such as superoxide anions, peroxide, and hydroxyl radicals. These reactive molecules can bind to the nitrogenous bases of DNA molecules, resulting in damaged DNA, single-strand breaks, and mutations [10, 11]. It is estimated that each cell's genomic DNA undergoes thousands of oxidative hits per day. The body has an innate antioxidant defense system composed of enzymes and other molecules such as superoxide dismutase (SOD) and glutathione (GSH) peroxidase. These mechanisms can neutralize radicals, preventing their binding to DNA, and therefore mutagenesis (see further discussion under Sect. 2.7.3.1).

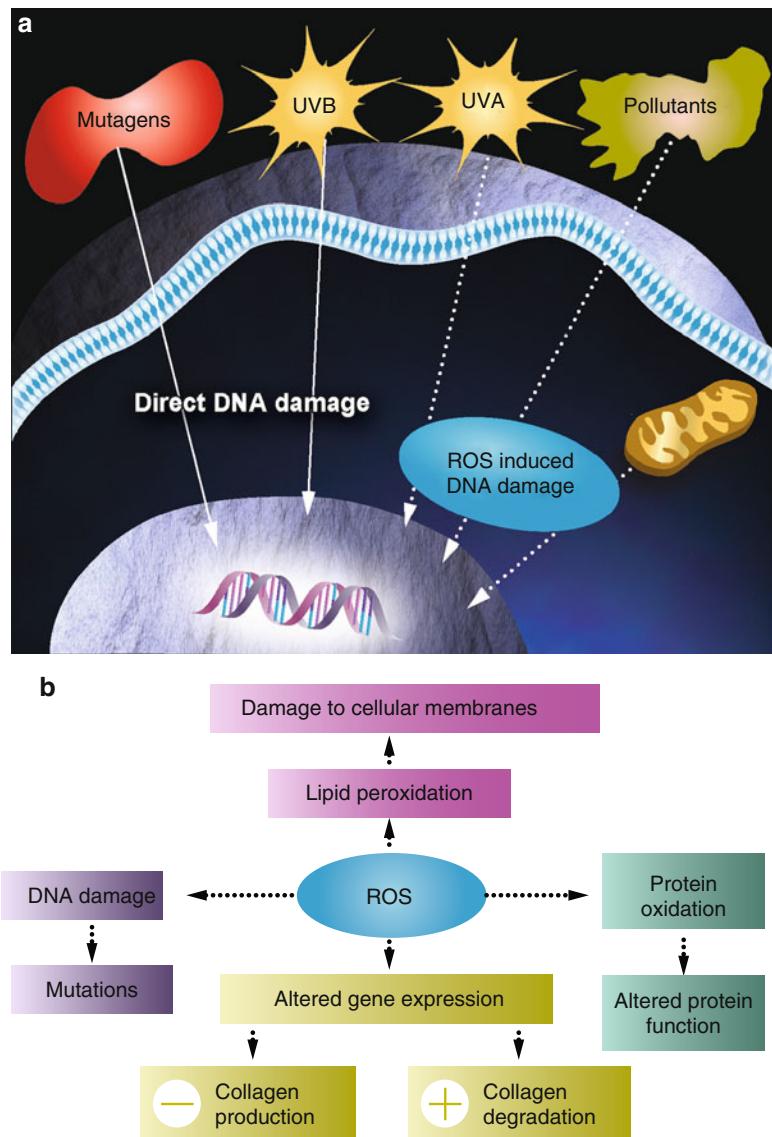
The final cause of sequence alterations is exposure to exogenous mutagens. Exogenous mutagens include chemicals, infectious agents, and radiation. Our genome must contend with constant exposures to a wide variety of exogenous mutagens in our food and water sources, air pollution, and, of course, sunlight. There are frequent popular and scientific reports of some new item that has been linked to cancer: caffeine, hot dogs, popcorn, antiperspirants/deodorants, etc. While some of these agents have gone through the rigorous scientific process of demonstrating cause and effect, it is important to note that many of the studies covered by the popular media are association studies, and a direct cause-and-effect relationship has not been demonstrated.

### 2.6.3 Repair of DNA Alterations

Humans have multiple DNA repair mechanisms to combat the frequent and at times complex DNA alterations that occur on a regular basis. The number of cellular genes devoted to DNA repair and the redundancy in the systems provides evidence that maintaining the correct genetic sequence is a crucial cellular function. These repair mechanisms are overlapping and complex. Table 2.5 provides a simplified description of four of the DNA repair pathways. Germline mutations in genes in these pathways can result in a nonfunctioning DNA repair system and familial cancer syndromes.

Germline mutations in mismatch repair (MMR) genes result in Muir-Torre and Lynch

**Fig. 2.7** Exogenous and endogenous sources of DNA damage (a). Exogenous sources of DNA damage include those that directly damage nucleic acids [ultraviolet B light (*UVB*) and mutagens] and those that cause reactive oxygen species (*ROS*)-mediated DNA damage [ultraviolet A light (*UVA*) and pollutants]. The primary endogenous source of DNA damage is through ROS generated during oxidative metabolism in mitochondria. Cellular effects of ROS (b). In addition to causing DNA damage, ROS can alter or damage other cellular molecules including proteins and lipids



**Table 2.5** DNA repair mechanisms

Repair pathway	Function
Mismatch repair (MMR)	Primarily repairs mutations that occur during replication
Nucleotide-excision repair (NER)	Primarily repairs alterations that occur from exposure to exogenous sources
Base-excision repair (BER)	Primarily repairs alterations that occur from exposure to endogenous sources
Double-strand break repair	Primarily involved in repairing chromosomal breaks

syndromes (see Chap. 9) [12]. The MMR system is primarily responsible for repairing mutations that occur during replication. When the MMR system is nonfunctional, mutations accumulate during each cell division. Germline mutations in nucleotide-excision repair (NER) genes result in xeroderma pigmentosum [13]. NER is primarily responsible for repairing alterations that occur from exogenous sources such as sunlight. In this disorder, the inability to repair DNA damage caused by sunlight results in extreme photosensitivity and a greater than thousandfold increased

risk for skin cancer (see Chap. 10 for overview of the genodermatoses, including those with DNA repair defects).

## 2.7 Nucleic Alterations and Disease

Alterations in the quantity and/or sequence of DNA or RNA can result in human disease. Disease-causing DNA alterations are either *inherited (germline)* or *acquired (somatic)*. Somatic mutations occur in neoplastic tumors. Clinical testing can be helpful for the diagnosis or management of the neoplastic disease only. Germline testing can have much broader implications including the risk of developing disease and the risk of having an offspring with disease. Although testing for somatic mutations is technically “genetic testing,” it is not subject to the same requirements, such as patient consent, as germline genetic testing.

### 2.7.1 Germline Alterations

Inherited alterations are generally found in every cell in the body. Thus, germline DNA testing using a peripheral blood specimen, a buccal swab, or a skin biopsy will all yield the same result. Germline alterations that clearly cause disease are referred to as mutations. Germline alterations that clearly do not cause disease are referred to as polymorphisms. Despite the large amounts of genetic data that have been generated, many genetic variants are not clearly benign (polymorphism) or disease causing (mutation). There are still a large number of alterations for which the clinical significance is unknown. For example, the hereditary breast and ovarian cancer genes, *BRCA1* and *BRCA2*, were discovered approximately 20 years ago, and clinical testing for germline alterations has been available for over 10 years, yet approximately 1 in 10 to 1 in 20 women who undergo genetic testing of *BRCA1* and *BRCA2* receive a result of “variant of unknown significance” [14].

In addition, some genetic variants can have deleterious effects, but only under certain conditions.

For example, variants in drug-metabolizing genes can cause significant morbidity and mortality, but only if the individual receives a drug that is metabolized by the affected pathway. Is this variant a mutation or a polymorphism? When in doubt, it is best to refer to it as an alteration or a variant rather than a mutation or polymorphism.

### 2.7.2 Benign Genetic Variants

An example of testing for benign polymorphisms is the analysis of human leukocyte antigen (HLA) variants, most frequently tested in the setting of organ transplantation. HLA testing continues to evolve away from serologic methods to DNA-based methods that can have higher precision for some applications [15, 16]. Another example of testing for benign polymorphisms is the analysis of genetic variants that are able to discriminate one individual from another. This testing was initially optimized for forensic and criminal purposes as well as parentage/paternity testing. It is now widely used in clinical labs in the setting of bone marrow transplantation to determine the origin (patient or donor) of cells present in post-transplant specimens [17]. A final application involves clinical testing for genetic variants that are associated with response or resistance to drugs and/or adverse drug events. Although our current understanding of the effects of gene sequences on drug metabolism is relatively limited, this area of testing has the potential to have a significant impact on healthcare. Relatively simple tests from blood or buccal swab specimens could increase the safety and efficacy of many, if not all, pharmacologic agents by identifying individuals who are likely to react adversely to the drug, as well as those who are likely to gain no benefit from the drug [18]. This newly emerging field of pharmacogenetics is discussed in Chap. 12.

#### 2.7.2.1 Inherited Diseases

Because disease-causing inherited mutations occur in every cell of the body and must be compatible with life, they tend to involve a relatively small area of the genome, often affecting a single

gene, sometimes just a single base pair. There are a relatively limited number of germline mutations that can occur and still allow fairly normal development and function of most organ systems. Many inherited diseases affect a single organ system: neuromuscular, hematologic, dermatologic, etc.

The inheritance patterns for germline disorders differ based on whether the gene involved is located on one of the 22 non-sex chromosomes (autosomes) or on a sex chromosome (most frequently the X chromosome) and whether one mutated copy is sufficient to cause disease (dominant) or if both copies of the gene must be affected to cause disease (recessive) (Table 2.6). Thus, autosomal dominant disorders are caused by genes located on one of the 22 autosomes and result from inheritance of a single mutated copy of the gene. Because a single mutated copy is sufficient to cause disease, often a parent of an affected offspring has the disorder. In contrast, autosomal recessive disorders are caused by genes located on one of the 22 autosomes, but require inheritance of two mutated gene copies to result in the disease phenotype. Typically both parents are unaffected carriers, each with one mutated and one wild-type gene copy. Offspring of two carriers have a 25 % chance of inheriting a mutated copy from each parent and therefore having disease. Conversely, there is a 75 % chance that an offspring will inherit only one or no mutated gene copy and therefore will not have

disease. Many individuals that carry mutations that cause autosomal recessive disorders are unaware they carry, or have the potential to carry, a mutation because there is no family history of the disease. In contrast, families with autosomal dominant or x-linked diseases are much more likely to be aware of a risk because there are often affected individuals in the family.

Disorders caused by genes on the X chromosome (X linked) create a unique situation since males have only a single X chromosome. Thus, in males, the inheritance pattern of diseases caused by genes on the X chromosome is essentially always dominant since they do not have a second X chromosome to “cover” for one with a gene defect. In X-linked recessive disorders, females are generally carriers of the trait, and males are most commonly affected. Female carriers have one affected and one unaffected X chromosome. Their male children have a 50 % chance of having disease depending on which X chromosome they inherit (by definition, male children inherit a Y chromosome from their father). X-linked dominant inheritance indicates that a single copy of an affected gene on the X chromosome is sufficient to cause disease. This inheritance pattern is not as common as X-linked recessive inheritance and does not necessarily affect males more than females. Only the female offspring, not the male offspring of an affected male, will be affected since his male children do

**Table 2.6** Inheritance patterns of genetic disorders

Inheritance pattern	Characteristics
Autosomal dominant (AD)	Gene involved is located on one of the 22 non-sex chromosomes A single aberrant gene copy is sufficient to cause disease
Autosomal recessive (AR)	Gene involved is located on one of the 22 non-sex chromosomes Both copies of the gene must be abnormal to cause disease
X-linked dominant	Gene involved is located on the X chromosome A single aberrant gene copy is sufficient to cause disease Males and females may be affected equally Is relatively uncommon
X-linked recessive	Gene involved is located on the X chromosome In males, inheritance of an affected X chromosome results in disease. In females, inheritance of two affected X chromosomes is required for disease Typically, males are affected and females are carriers
Mitochondrial	Maternal inheritance All offspring of an affected female are affected

not inherit his X chromosome. Affected females generally have one affected and one unaffected X chromosome. Their offspring, regardless of gender, have a 50 % chance of inheriting the affected X chromosome and therefore having disease.

The following are general concepts related to inherited diseases.

### One Gene Can Cause Different Clinical Syndromes

Example: Mutations in Keratin 5 (*KRT5*) cause epidermolysis bullosa simplex. Different mutations in *KRT5* can result in different phenotypes including Dowling-Meara (severe, clustered/herpetiform blistering, palmar-plantar hyperkeratosis), Weber-Cockayne (acrocentric, mild), and Koebner (generalized, mild) types (see Chap. 10).

### Mutations in Different Genes Can Cause the Same Clinical Syndrome

Examples: Muir-Torre/Lynch syndrome and xeroderma pigmentosum are caused by mutations in one of several genes, which function in DNA MMR and DNA NER pathways, respectively. Mutations in any one of the genes in the pathway can dismantle the entire pathway, resulting in a similar phenotype.

### Not All Disease-Causing Mutations Are 100 % Penetrant

Penetrance refers to the proportion of individuals carrying a particular genetic variant that have the associated disease/phenotype. It is often expressed as risk by a particular age. For example, *MLH1* mutations, responsible for Lynch and Muir-Torre syndromes, are highly penetrant, carrying a risk of colon cancer of approximately 80 % by age 70 years, at least for males [19].

### Development of Some Inherited Diseases Is Likely Multifactorial

In addition to gene sequence, other factors such as environmental exposures are likely to play a role in the development of some inherited diseases. The absence of the necessary environmental exposure may explain incomplete penetrance for some disorders. For example, the melanocortin-1 receptor (*MC1R*) is considered

a moderate-penetrance risk gene for melanoma. *MC1R* is a key regulator of skin pigmentation [20]. Some *MC1R* variants have reduced receptor function resulting in increased pheomelanin (photosensitive) production relative to eumelanin (photoprotective). Development of melanoma in individuals with *MC1R* variants likely requires additional factors such as UV exposure.

### Inheritance of Some Genetic Disorders Is Complex and Likely Involves Multiple Genes (Polygenic)

For some genetic disorders, a gene has been identified that appears to be important for disease development, but the inheritance pattern does not follow one of the patterns discussed above. And for some disorders which clearly have a genetic component, such as eczema and psoriasis, no specific disease-causing genes have yet been identified. In these cases, it is likely that sequence alterations in multiple genes and/or so-called modifier genes may increase or decrease risk of disease development. Modifier genes may function in the same pathway as the defective gene, play a role in immune response, or be involved in metabolic pathways. Completion of the human genome sequencing project will hopefully expedite research into these complex diseases.

### Not All Germline Diseases Are Due to Inheritance of a Variant That Occurs in a Parental Germline

It is worth noting that diseases that result from additions or losses of entire chromosomes are generally due to improper segregation of chromosomes during egg or sperm production. These chromosomal number abnormalities are not present in every cell of the parent, only the gamete that was generated during meiosis. These disorders are relatively common and include Down syndrome (47 XX or XY, +21 = trisomy 21), Klinefelter syndrome (47, XXY), and Turner syndrome (45, X). Although these alterations are in the germline of the affected individual, they are not indicative of a germline alteration in a parent, and thus, they do not follow any of the inheritance patterns discussed above.

A compendium of human genes and genetic phenotypes is available at the Online Mendelian Inheritance in Man (OMIM) website [www.ncbi.nlm.nih.gov/omim](http://www.ncbi.nlm.nih.gov/omim).

### 2.7.2.2 Clinical Genetic Testing

Genetic testing is often not required to make a diagnosis of an inherited genetic disorder. For example, diseases such as sickle cell anemia and hemophilia can be diagnosed based on the analysis of the protein product of the affected gene. This is an important concept when considering what constitutes a genetic test and which tests should be governed by regulations such as requirements for informed consent and access to genetic counselors. Although genetic tests are used at times to make diagnoses, they are more frequently used to identify individuals at risk for a particular disease or to determine the risk of having offspring with a particular disease. The inheritance pattern for a particular disorder is very important, as it has implications for other family members including offspring. This type of testing generally requires education of the patient and their informed consent prior to testing. It is the job of genetic counselors to clearly convey to patients what information the testing will and will not provide and the implications of that information for the individual and their family members.

### 2.7.3 Somatic Alterations and Neoplasia

Neoplasias are defined by three characteristics:

1. The cells are clonal. All of the tumor cells are derived from a single progenitor cell.
2. Cellular growth and division are deregulated/unregulated relative to normal tissue at the same site.
3. The cells contain somatic mutations.

Somatic gene alterations are universally found in neoplasias. They are typically not found in other tissues in the body, and they are not passed on to offspring unless they occur in a gamete. In contrast to germline mutations, somatic mutations can be more complex and involve larger

areas of the genome. The genetic constitution of tumor cells can be wildly abnormal with multiple chromosomal gains and losses, translocations, and sequence alterations.

Multiple somatic alterations are required for a cell to become fully malignant. Comprehensive sequencing efforts over the past decade have revealed that typical tumors contain two to eight mutations that promote or “drive” mutagenesis [21, 22]. These driver mutations occur in genes that function in cellular pathways involved in cell fate, growth, proliferation, survival, and genome maintenance. A database of somatic alterations identified in human cancers has been compiled in the Catalogue of Somatic Mutations in Cancer (COSMIC, available at [www.sanger.ac.uk/resources/databases/cosmic.html](http://www.sanger.ac.uk/resources/databases/cosmic.html)).

Genetic alterations in cancers result in quantitative and qualitative changes in gene expression. Quantitative changes include increased expression of genes that promote cell growth and replication and/or expression of genes that are normally not expressed (silent) such as carcinoembryonic antigen (CEA), a protein that is normally only expressed during fetal development but is expressed by several different tumor types. In addition, tumor cells may decrease or eliminate expression of genes, particularly if their function is to negatively regulate cell growth, or if they are involved in DNA repair and maintenance. Qualitative changes to proteins are due to mutations that alter their amino acid sequence and therefore their function. The altered protein product may have increased activity (B-Raf), decreased activity (PTEN), or novel activity (EWSR1-ATF1 fusion) compared to its wild-type counterpart. These alterations lead to the cellular and histologic phenotypic changes that can be identified microscopically.

Oncogenic mutations typically result in increased genetic instability, inhibition of cell death, and/or growth promotion. Growth-promoting cellular signaling pathways are normally highly regulated and are generally only turned on in the presence of a signal for growth, such as the presence of a growth factor. Mutations in genes that function in these pathways can cause the pathway to become constitutively activated,

even in the absence of the growth factor. The genes in these pathways fall into two broad categories: oncogenes and tumor suppressors. In general, *oncogenes* are drivers of the pathway. Mutations in these genes turn the pathway on. *Tumor suppressor genes* are negative regulators of these pathways. Oncogenic mutations in tumor suppressors result in inactivation of the gene, resulting in reduced or eliminated negative regulation. In both scenarios, the outcome is the same; the pathway is constitutively activated.

Although mutations can occur anywhere in the genome, similar types of oncogenic mutations are seen in various cancers. This is not because these genes are mutated more frequently than other areas of the genome, but because of the growth-promoting effect of the mutations. Mutations that confer no growth advantage, or result in a growth disadvantage, will not out-compete other cells and will likely be lost over time. Conversely, there is a selection pressure for cells with mutations that confer a growth advantage. These cells will “out-compete” their unmutated counterparts and will become present at increasing numbers over time. Thus, oncogenic mutations are “selected for” because of their growth-promoting properties.

Specific types of mutations may be consistently observed in particular tumor types. This likely reflects the specific mutagen(s) to which the tissue has been exposed. Mutagens exert their effects on DNA through several different mechanisms, resulting in specific types of DNA alterations and creating a “signature” of DNA damage. The tumor suppressor TP53 provides a model for identifying likely carcinogens for specific tumor types [23]. Inactivating mutations in TP53 occur in most human cancers. Although many different mutations have been reported, certain types of mutations are associated with specific tissues and the mutagens to which they are exposed. For example, in skin cancer, over half of the TP53 mutations observed are C → T and CC → TT, supporting the importance of sunlight as a major carcinogen (see below). In contrast, the most common TP53 mutations in lung cancer involve G → T substitutions, most likely driven by tobacco and other mutagen exposure.

### 2.7.3.1 Mutations and Neoplasia in the Skin

The genome of skin cells is under constant assault by both exogenous and endogenous exposures (see Fig. 2.7a). The primary exogenous exposure is ultraviolet (UV) radiation, although certainly the skin is exposed to other chemicals and mutagens such as cigarette smoke and other pollutants in the air. Sun exposure likely promotes carcinogenesis through several mechanisms including direct DNA damage (primarily caused by UVB), secondary DNA damage caused by oxidative stress and the production of reactive oxygen species (ROS) (primarily caused by UVA), and immune dysregulation resulting in inflammatory responses and immune suppression [10, 24, 25].

The majority of DNA damage appears to occur through direct photochemical pathways rather than through indirect damage induced by oxidative stress [10, 24]. The most common photochemical effects involve adjacent pyrimidines (cytosine and thymine) on the same DNA strand. Ultraviolet light exposure at these dipyrimidine sites causes the formation of a cyclobutane pyrimidine dimer or a pyrimidine (6–4) pyrimidine product. Although the formation of the former is more common, the formation of the latter results in considerable distortion to the sugar-phosphate backbone of DNA and likely plays a significant biologic role. Inaccurate repair of these alterations can result in C → T and CC → TT substitutions, particularly when the cytosine residue(s) is methylated. The presence of a large number of these types of alterations is considered a UV signature.

Endogenous mutagens are primarily the by-products of normal oxidative metabolism, such as reactive oxygen species (ROS). ROS, generated by both endogenous and exogenous pathways, result in a variety of adverse cellular effects (see Fig. 2.7b). In addition to their ability to directly damage DNA, ROS can trigger activation of transcription factors that cause altered gene expression. The result is increased expression of enzymes that damage and degrade collagen and a decrease in production of collagen itself. ROS also cause lipid peroxidation resulting in damage to cellular membranes and protein oxidation resulting in altered chemical properties.

To combat the effects of ROS, our bodies have a complex antioxidant system that can inactivate these reactive molecules. The innate system can be overwhelmed leading to a state of oxidative stress. In theory, oral and topical supplementation of antioxidants could provide additional protection from ROS and may be cancer protective. While there are a wide variety of over-the-counter antioxidant oral supplements and topical treatments available, there are relatively little data on the ability of most of these products to prevent cancer. Delivery of topical antioxidants is particularly challenging as they must be in high concentrations, stable, and able to penetrate the stratum corneum [11, 26]. Although many sunscreen products claim to provide antioxidants, they likely have little or no capacity to achieve any meaningful protection from ROS.

### 2.7.3.2 Clinical Molecular Oncology Testing

Molecular oncology tests are useful in diagnostic and prognostic applications, monitoring disease, and therapy selection. Often cancer can be diagnosed based on histologic features in combination with assessing specific protein expression detected by immunohistochemistry or flow cytometry. However, molecular testing can be useful in some cases when these are not definitive. Examples include the use of fluorescence *in situ* hybridization (FISH) to identify chromosomal alterations that support a diagnosis of melanoma and the detection of T-cell gene rearrangements that support the diagnoses of mycosis fungoides and other cutaneous T-cell lymphomas (see Chaps. 4 and 6, respectively).

Although useful for diagnostic and in some cases prognostic and disease monitoring purposes, by far, the most promising aspect of molecular testing is in its utility for therapy selection. With the advent of molecularly targeted therapies comes the need to understand the molecular constitution of individual tumors in order to select the therapy most likely to be beneficial. The ability to tailor therapy to an individual based on the molecular constitution of their tumor, so-called personalized medicine, is becoming a reality.

## 2.8 Genomes of Infectious Agents

All living organisms contain DNA genomes. Viruses can have RNA or DNA genomes which can be single stranded or double stranded. The structure and properties of nucleic acids in infectious agents are essentially identical to that of human DNA, allowing common testing methods to be used regardless of whether the nucleic acid target is human, bacterial, or viral. Clearly the genomic sequence of infectious agents differs significantly from that of humans, allowing for specifically targeted tests to be designed. Molecular testing is now standard for many viral targets and continues to replace standard culture methods for bacteria and other infectious agents. Although molecular methods have many advantages over traditional culture and serology, they also have challenges. The advantages and disadvantages of molecular methods targeting infectious agents are discussed in detail in Chap. 11.

## 2.9 Summary

Nucleic acids are essential for all forms of life. Although the structure of the human genome is relatively simple, its function is incredibly complex. The genome codes for the entire blueprint for an individual. This code includes the instructions for manufacturing the right proteins at the right time in the right cells to carry out all of the functions necessary to maintain life.

Alterations in nucleic acids can result in human disease. The structural and chemical properties of nucleic acids can be exploited to develop molecular diagnostic tests with an array of clinical utilities. Inherited genetic disorders and cancer are two areas with immediate molecular testing applications. A clear picture has emerged for some inherited disorders caused by mutations in a single, highly penetrant gene. Much research is still needed to understand multifactorial and polygenic inherited diseases.

The rapid advances in molecular oncology, including the sequencing of entire “cancer genomes,” have identified key mutations that

drive tumorigenesis [21, 22, 27]. This work has already translated into a variety of molecular oncology tests with clinical utility for many aspects of patient management including diagnosis, prognosis, disease monitoring, and therapy selection. This work has also laid the foundation for the development of new therapies and new companion diagnostic assays.

A basic understanding of molecular biology and nucleic acid chemistry is required to fully understand virtually all human diseases. The sequencing of the entire human genome was the first step toward being able to provide medical treatment tailored to an individual rather than treating based on statistical analysis of a disease. As additional progress is made, a familiarity with the language and nomenclature associated with nucleic acids and molecular biology will become essential for ordering appropriate tests and interpreting their results. The basic principles discussed in this chapter provide a foundation for the discussion of molecular testing methods and their applications for dermatologic diseases in the following chapters.

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## Contents

3.1	<b>Introduction</b> .....	28	3.7	<b>Practical Considerations</b> .....	58
3.2	<b>General Considerations for Assay Design and Implementation</b> .....	30	3.7.1	What to Look for in a Laboratory and/or Test Result .....	58
3.2.1	Types of Genetic Alterations and Performance Requirements .....	30	3.7.2	Costs and Reimbursement.....	59
3.2.2	Specimen Type and Composition .....	30	3.8	<b>Summary and Looking Ahead</b> .....	60
3.2.3	Lab-Developed Tests (LDT) Versus FDA-Approved In Vitro Diagnostic (IVD) Tests.....	33		<b>References</b> .....	60
3.3	<b>The Basics of a Molecular Test</b> .....	34			
3.3.1	Hybridization: Virtually All Molecular Tests Are Based on the Principle of Hybridization.....	34			
3.3.2	Enzymes.....	35			
3.4	<b>Non-amplification Nucleic Acid Analysis Methods</b> .....	36			
3.4.1	Karyotyping (Cytogenetic Analysis) .....	36			
3.4.2	In Situ Hybridization (ISH): Chromogenic In Situ Hybridization (CISH) and Fluorescent In Situ Hybridization (FISH) .....	38			
3.4.3	Southern Blot .....	40			
3.4.4	Microarrays and Comparative Genomic Hybridization (CGH) .....	41			
3.5	<b>Amplification Methods</b> .....	43			
3.5.1	Polymerase Chain Reaction (PCR).....	44			
3.5.2	Microsatellite Instability Analysis (MSI).....	47			
3.5.3	T-Cell and B-Cell Gene Rearrangement Analysis.....	47			
3.5.4	Real-Time PCR.....	49			
3.5.5	Other Amplification Methods .....	53			
3.6	<b>Sequencing</b> .....	53			
3.6.1	Sanger Sequencing.....	53			
3.6.2	Pyrosequencing .....	54			
3.6.3	Next-Generation Sequencing .....	55			

## Key Points

- Although a wide variety of molecular methods are readily available, all are founded on a relatively few basic molecular principles such as hybridization and enzymatic reactions.
- The method used for a specific application is largely driven by the type of alteration to be detected and the performance characteristics required for clinical utility.
- Specimens must be evaluated prior to testing to ensure that the tissue is appropriate and sufficient for the test requested. Regardless of the method, the composition and quality of the specimen can affect test results.
- Non-amplification methods are useful for assessing the entire genome for large structural alterations.
- Amplification methods can detect very low amounts of a specific sequence,  $\leq 0.01\%$ . They are used in essentially

all applications that identify sequence variations.

- Both amplification and non-amplification methods are used in the diagnosis and management of the dermatology patient. These include FISH for the diagnosis of melanoma, B-cell and T-cell gene rearrangement for the diagnosis of cutaneous lymphoma, and MSI and germline sequencing for the diagnosis of genodermatoses, and the detection of mutations in *BRAF* and other signaling molecules for therapy selection.
- Next-generation sequencing technologies are poised to replace many of the current technologies because of their ability to detect a wide range of genetic variants simultaneously and their superior limit of detection.

tedious. Although the Southern blot method is no longer commonly used, it remains the foundation from which all nucleic acid “microarray” testing methods have been developed.

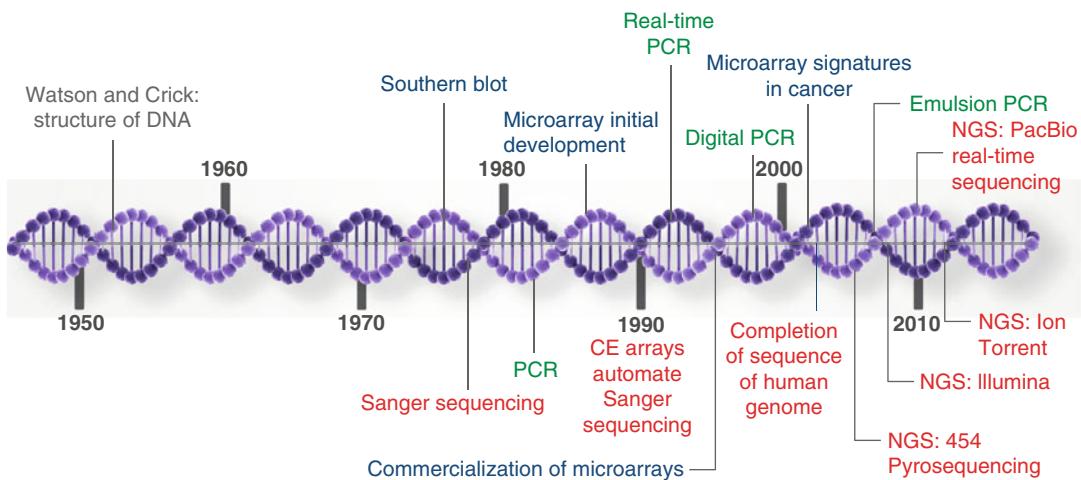
In the mid- to late 1970s, the first DNA sequencing techniques were developed. Although two different methods, Maxam and Gilbert sequencing and Sanger sequencing, were described at the time, Sanger sequencing quickly became the method of choice [2–4]. Developed by Frederick Sanger, the original technique of Sanger sequencing was a manual process that involved separating radioactive DNA fragments on large gels that were fragile and difficult to manipulate. Advances in Sanger sequencing have made it relatively easy and rapid to perform. One of the major advances was the development of fluorescently labeled deoxynucleotide triphosphate molecules (dNTPs), which allowed all four bases to be analyzed simultaneously, rather than separately, thus reducing the workload to one-fourth [5, 6]. A second major advance was the development of automated capillary electrophoresis instrumentation. These instruments transformed the laborious process of manual electrophoresis into an automated process that greatly increased the speed at which DNA sequence data could be generated. These breakthroughs fueled the human genome sequencing project and are largely responsible for the project being completed ahead of the anticipated completion date. Sanger sequencing is still widely used in many clinical laboratories. Although its departure from clinical testing is not imminent, it may be inevitable due to the development of newer, “next-generation” sequencing methodologies, which have much higher-throughput capacities and are more cost-effective [7].

DNA sequencing required amplification of the DNA region of interest prior to performing a sequencing reaction. Until the late 1980s, amplification was performed primarily by cloning the DNA sequence of interest into a plasmid or other vector that could be introduced into bacteria such as *E. coli*. During each cell division, the bacteria replicated not only its genome but also the cloned DNA sequence. Because of the rapid doubling time of the bacteria, an overnight incubation could result in millions of copies of the

### 3.1 Introduction

Molecular biology and nucleic acid technologies have evolved rapidly over the last 40 years (Fig. 3.1). Although chromosome analysis (karyotyping) was advancing and improving during the 1960s, it was more than 20 years after Watson and Crick’s first description of the structure of DNA that the first major molecular method was developed. In 1975, Edwin Southern published a method for detecting specific DNA sequences using electrophoresis and hybridization of specific DNA probes [1]. This technique, termed Southern blot, gave scientists a tool to map specific genes and entire genomes of some species. The technique was widely used in the late 1970s and 1980s and is largely responsible for initiating the molecular era.

Although the Southern blot was a major breakthrough that facilitated significant advancements in molecular biology, it was a cumbersome process that involved radioactivity and could take weeks to perform a single experiment. Improvements brought the dot blot, slot blot, and reverse dot blot, although all were still rather



**Fig. 3.1** History of advances in molecular methods. Advances relating to hybridization methodologies are in blue, sequencing advances are in red, and PCR advances

cloned DNA segment, which then had to be isolated and purified. Clearly, this was a laborious process. In 1983, a method was invented using an enzymatic reaction to specifically amplify a region of DNA. The method, called polymerase chain reaction (PCR), remains one of the most commonly used methods in laboratories around the world. Similar to other landmark discoveries, PCR was invented somewhat “by accident.” The inventor of PCR, Kary Mullis, was not trying to create a DNA amplification method, but was trying to solve another problem when he realized that with the right ingredients and temperature conditions, one copy of DNA could be amplified into millions of copies [8–10]. PCR revolutionized many aspects of nucleic acid-based research and testing, and in 1993, Kary Mullis was awarded the Nobel Prize in Chemistry “for contributions to the developments of methods within DNA-based chemistry.” Many iterations and improvements to PCR have been developed, but all are based on the original principles of 30 years ago. It is impossible to underestimate the impact that PCR has had. Hundreds of thousands of scientific publications have employed the power of PCR in fields as diverse as genetics, agriculture, evolution, oncology, and microbiology. Some version of PCR remains a key element in all DNA sequencing applications including the sequencing of the human genome.

are in green. PCR polymerase chain reaction, NGS next generation sequencing

The technical improvements over the last few decades have led to tremendous advancements in the fields of genetics and molecular biology. Although the application of this knowledge to the practice of medicine is in its infancy, nucleic acid-based tests have already transformed three areas of clinical medicine—genetics, oncology, and infectious disease—and additional dramatic changes will occur in the decade that follows. Although the purpose of testing in these diverse fields may be very different, all involve the analysis of nucleic acids and use the same or very similar molecular methods, techniques, and instrumentation. Each application does, however, present specific analytical and interpretive challenges. For example, assays for inherited genetic mutations, like those seen in genodermatoses (Chaps. 9 and 10), must evaluate large expanses of DNA, with little concern for limit of detection. Inherited genetic alterations are essentially present in every cell in the body; therefore, virtually any specimen from an individual will contain a nucleic acid population with the alteration of interest either absent, present at a level of 50 % (present in one of the two gene copies), or present at a level of 100 % (present in both gene copies). Thus, the limit of detection and linearity requirements for this type of assay are generally not difficult to achieve. However, assessment for inherited genetic alterations often requires

analysis of a relatively large DNA sequence (tens of thousands of base pairs), which can create technical challenges and add to the expense of a test. In contrast, some infectious disease applications require an assay that is quantitative over a large dynamic range and has a very sensitive limit of detection, able to detect the presence of minute amounts of an organism's DNA. The limit of detection and linearity requirement for this type of assay are much more difficult to achieve. Fortunately, the nucleic acid target for infectious disease applications is generally a very small genetic sequence (tens to a few hundred bases). These different requirements play a large role in determining the optimal molecular method and assay design for a specific genetic application.

There are many different molecular methods, with “new” ones added every day. “New” is qualified because the vast majority of molecular tests are based on a few common principles, which have been “tweaked,” sometimes to improve assay performance, but sometimes solely to avoid patent and intellectual property infringements. All methods have strengths and weaknesses in terms of sensitivity, specificity, limit of detection, complexity, and cost. The decision about which method to use should be driven primarily by the performance requirements of the assay and secondarily by cost and workflow considerations. For many applications, more than one molecular method can be used. For example, a single base alteration, such as *BRAF* V600E (c.1799T>A), can be detected by allele-specific PCR, real-time PCR, or sequencing, just to name a few. Each of these techniques has strengths and weaknesses that can significantly impact the sensitivity and specificity of the test. For example, allele-specific PCR has a very low limit of detection, down to approximately 1 % mutant alleles. However, it can generally only detect one specific mutation. In contrast, Sanger sequencing has a limit of detection of approximately 20 % mutant alleles, but it is capable of detecting multiple mutations such as *BRAF* V600E, V600K, and V600R in one assay. Thus, a basic understanding of molecular techniques can be critical for accurate and appropriate interpretation of a test result.

The purpose of this chapter is not to provide a complete list of all molecular methods—by the

time of publication, there will be “new” methods, and some older methods will have been abandoned. Rather, the purpose is to discuss key concepts in assay design and performance and compare and contrast some of the most commonly used methods in the clinical laboratory setting, allowing the reader to choose an appropriate test and understand the strengths and limitations of the results generated.

## 3.2 General Considerations for Assay Design and Implementation

### 3.2.1 Types of Genetic Alterations and Performance Requirements

There are a number of factors to be considered to determine, or at least narrow down, what molecular method(s) is most appropriate for a particular clinical application. The primary factor to consider is the type of nucleic acid alteration to be detected. As discussed in Chap. 2, there are many different types of genetic alterations. Some involve hundreds of thousands of base pairs, and others involve a single base pair. Some result in gain of genetic material, some in loss, and others in no change to the total amount of genetic material. Not surprisingly, not all methods are capable of detecting all types of genetic alterations. After defining the type of alteration to be detected, the clinical requirements for the assay must be considered. For example, will the assay be qualitative (the genetic alteration is present or absent) or quantitative (how much of the genetic alteration is present)? What is the analytical sensitivity or limit of detection required to obtain a clinically relevant result? What is the required analytical linearity of the assay? These specifics will also drive the selection of the analysis method.

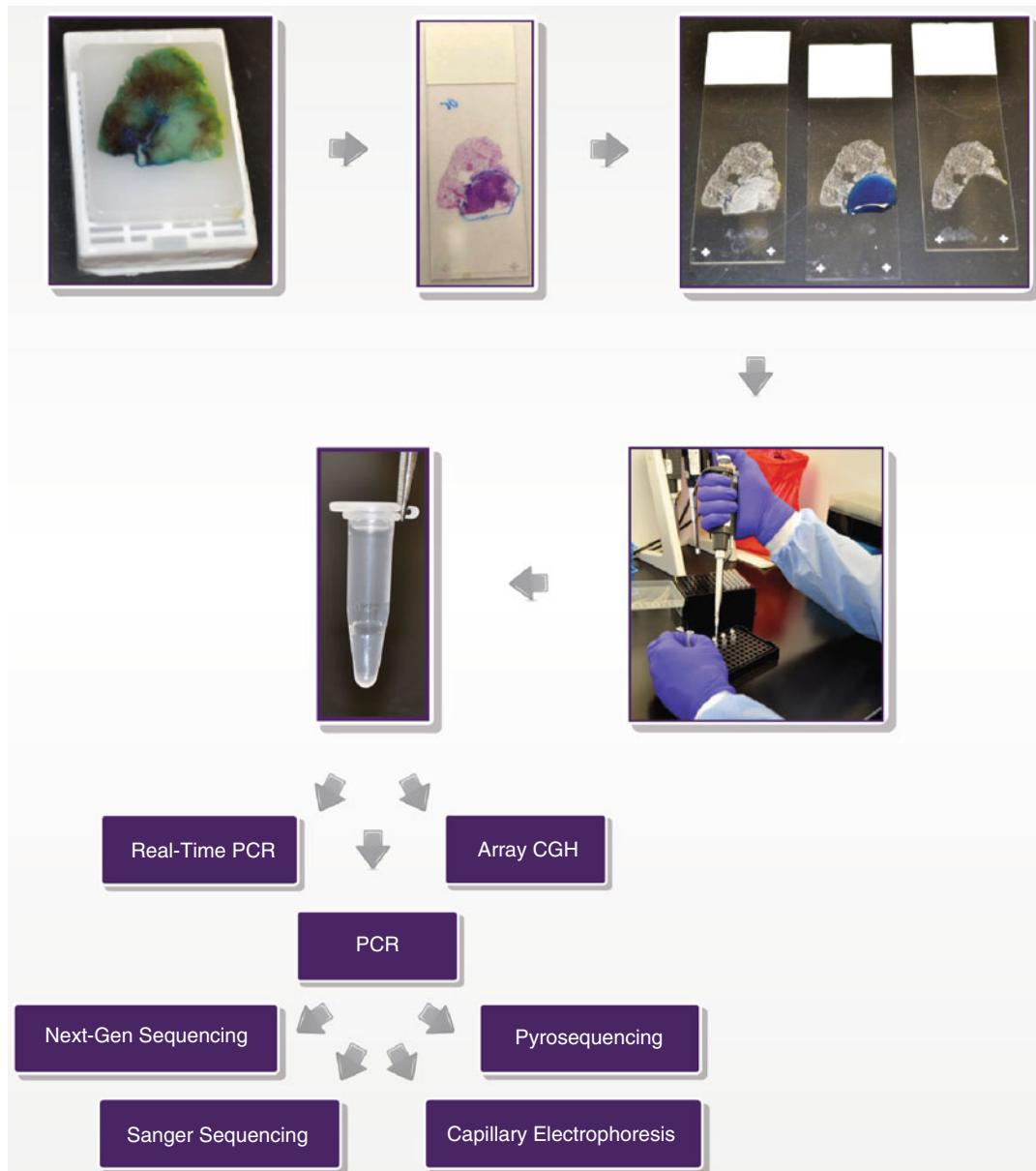
### 3.2.2 Specimen Type and Composition

An important consideration for method selection is the sample type and quality. Pre-analytical

variables such as type and length of time of tissue fixation and other tissue processing variables affect the quality of the nucleic acids within the specimen and therefore influence method selection. Peripheral blood and bone marrow samples are generally received in the lab “fresh” (unfixed and unprocessed) for analysis. Nucleic acid extraction from these types of specimens typically yields a large amount of high-quality nucleic acid for post-extraction analysis. In contrast, most solid tissues such as the skin are processed by formalin fixation and embedded in paraffin (FFPE). Fixation in formalin, tissue processing (dehydration), and embedding in paraffin cause damage and degradation to the nucleic acids in the specimen. Nucleic acid extraction from these types of specimens often yields a lower quantity of lower-quality (damaged and/or fragmented) nucleic acids, limiting the types of molecular techniques that can be performed.

Another important sample consideration is specimen composition. Specimen composition (what cell types are present in the tissue sample) is most important when evaluating a tumor specimen for somatic (acquired) alterations. Tumor specimens always contain normal cellular components including stroma, vessels, infiltrating leukocytes, and adjacent normal tissue. Invariably, some areas of a tissue sample will have significantly more tumor or normal cells than others. DNA isolated from an area of tissue that has a low density of tumor cells can yield a false-negative test result if the amount of tumor is below the limit of detection of the assay. For this reason, tumor tissues to be tested for somatic alterations should always be reviewed by a pathologist familiar with molecular testing to identify and specify the area(s) of highest density of tumor and ensure that the overall tissue is appropriate for testing. Although this sounds relatively straightforward, pathologists not trained in assessing tumor density may fail to accurately assess the percentage of tumor relative to non-tumor cells. This is partly due to the fact that accurate tumor density assessment requires that the pathologist focuses on just the percentage of tumor nuclei, which contain the DNA, rather than the total cellular volume. Another part of the problem stems from the fact that pathologists’

eyes are trained to hone in on abnormal cells and overlook the normal cells. For example, areas of necrosis have numerous, and often overlooked, neutrophils which carry normal genetic material. Thus, pathologists may vastly overestimate the amount of tumor present in a tissue specimen. A recent research experience highlights the importance of accurately assessing tumor tissue for molecular testing. The research project involved DNA sequencing of multiple genes from approximately 30 specimens of a particular tumor type. An H&E-stained slide was reviewed by a pathologist who was an expert in that specific tumor type. He marked areas of tumor that he estimated contained >80 % tumor cells. Sequencing of DNA extracted from these specimens failed to identify any gene mutations, even in genes that had been reported in scientific literature to be mutated at a frequency of ≥20 %. In light of this, the H&E-stained slides were re-reviewed by a pathologist with substantial molecular diagnostic experience. His interpretation was that the tissue identified by the first pathologist contained ≤30 %, not >80 %, tumor density in the vast majority of specimens. This amount of tumor is generally below the limit of detection for Sanger sequencing analysis and likely caused false-negative results to be generated. While this failure to accurately estimate tumor density caused an unfortunate and wasteful research experience, the risk for similar experiences in clinical testing is more worrisome. Had these samples been submitted for clinical testing, the false-negative results that were generated could have resulted in inaccurate diagnoses or failure to use an appropriate therapeutic agent. The CAP Molecular Oncology Committee has repeatedly warned, “It is highly advisable to evaluate an H&E section by a pathologist for tumor content and for estimation of neoplastic cellularity because there is always a risk that the section used for analysis may not contain sufficient number of tumor cells required for a specific molecular assay. It is also important to perform tissue dissection for enrichment of tumor cellularity especially when lower sensitivity assays are used for BRAF mutation, e.g. Sanger Sequencing.” Despite this, recent proficiency testing surveys from the College of American Pathologists (CAP) indicate that



**Fig. 3.2** Workflow for molecular testing. Multiple slides are cut from the tissue block. One slide is H&E stained and reviewed by a pathologist to identify tissue with the highest tumor density. The H&E-stained slide is then superimposed on an unstained slide to guide tissue dissection. A reagent (blue) is placed on the tissue to be removed. This reagent hardens, facilitating accurate removal of the tissue

of interest. If desired, the slide can then be H&E stained and reviewed to ensure that the proper area of tissue was removed. Once the tissue of interest has been isolated, nucleic acids can be extracted by a variety of methods. The resulting DNA specimen can be used for many different downstream molecular tests. *PCR* polymerase chain reaction, *CGH* comparative genomic hybridization

5–10 % of participating clinical labs do not perform any dissection for enrichment of tumor cells and instead use whole tissue sections [11]. Figure 3.2 outlines the workflow of molecular

testing from tumor specimens. The upfront work of identifying the highest density of tumor tissue and dissecting the tissue is critical for generating accurate results. Note that in this case, it

is estimated that the circled area contains 70 % tumor cells. Had dissection not been performed and the entire tissue section used, the DNA specimen would likely contain 40–50 % tumor cells, corresponding to 20–25 % mutant alleles (mutations typically occur on only one of the two gene copies), which is near the limit of detection for mutation detection by Sanger sequencing.

### 3.2.3 Lab-Developed Tests (LDT) Versus FDA-Approved In Vitro Diagnostic (IVD) Tests

Over the last decade, multiple FDA-approved molecular diagnostic kits have become available from several in vitro diagnostic (IVD) companies. Most of the FDA-approved kits are for infectious disease applications. These applications tend to be high-volume tests, which allow the manufacturer to recoup the costs of the FDA approval process. In addition, the claims for FDA approval for infectious disease assays are generally straightforward: A positive test result indicates that the organism is present; a negative indicates the organism is absent. Fewer kits are available for genetic testing and oncology-focused assays. These tests are performed at a much lower volume than infectious disease testing, making it difficult for an IVD company to justify the expense of the FDA approval process, as it knows it will sell a relatively limited number of tests. In addition, the claims for FDA approval for genetic and oncology molecular tests tend to be more complex. For example, over 1,000 mutations in the *CFTR* gene, the gene responsible for cystic fibrosis (CF), have been reported. Most clinical *CFTR* assays do not test for all known mutations, just for the most common ones. Therefore, testing person who has a mutation that is not included in the test panel will result in a false-negative result. Because the mutation frequency and spectrum differ by ethnic groups, determining an assay's sensitivity is further complicated. The rate at which false-negative results occur will be different in different ethnic populations, because the mutation frequency is variable between populations.

An additional problem for IVD companies is that new disease-causing mutations may be identified. If the manufacturer wants to include the new mutation in the kit, they must go through another FDA approval process.

Most IVD manufacturers that produce molecular diagnostic assays own intellectual property rights (patents) around specific molecular methods. For example, Roche (F. Hoffmann-La Roche Ltd.) holds multiple patents around PCR applications. Hologic/Gen-Probe, Inc., owns intellectual property around Invader technology and transcription-mediated amplification (TMA). Qiagen has rights to Hybrid Capture and Scorpion technologies. IVD manufacturing companies generally develop assays using the molecular methods that they "own," as they are relatively unencumbered by licensing costs and are protected from competition by the patent(s). Each of these methods is a solid, well-proven approach to molecular testing, and each has its strengths and weaknesses. There is not one method that is clearly superior to the others.

Laboratory-developed tests (LDTs) are tests that are developed and validated by an individual laboratory. Anatomic pathology labs perform a wide variety of lab-developed tests that are not FDA approved, such as immunohistochemistry and flow cytometry. It is the responsibility of the medical director of a laboratory to ensure that an LDT has gone through a rigorous validation process and that the performance characteristics of the assay are suitable for patient testing. The validation process includes demonstrating the sensitivity, specificity, limit of detection, reproducibility, and linearity of the assay for each type of specimen from which clinical testing will be performed. The laboratory must also demonstrate a clinical utility for the assay and be able to support any claims made on patient reports, such as if the mutation is associated with a specific disease or predicts response to a particular therapy. Regardless of whether an assay is lab developed or FDA-approved, the specific performance characteristics for the assay, including sensitivity, specificity, cross-reactivity, and limit of detection, should be readily available to facilitate accurate interpretation of the test result(s).

As with all new tests, molecular assays are initially developed in a research setting. A significant amount of data must be generated to demonstrate that the test has clinical utility, which has typically taken several years. Once there is sufficient evidence that the test has clinical utility, the test is “translated” into the clinical setting (hence the terms “translational research” or “translational medicine”). One advantage of LDTs is that they can be offered for clinical testing much earlier than an FDA-approved test can be available. A downside to being an early adopter is that as research continues, additional data may bring into question the validity of the original findings and the clinical utility of the test. An example is the use of EGFR immunohistochemistry to predict response to EGFR-targeted monoclonal therapies such as cetuximab (Erbitux) and panitumumab (Vectibix) in patients with colon cancer. Although EGFR immunohistochemistry was originally used to determine eligibility for these newly developed drugs, subsequent studies demonstrated that IHC techniques cannot effectively predict who will respond to these colon cancer treatments and therefore it should not be used to restrict treatment or reimbursement [12].

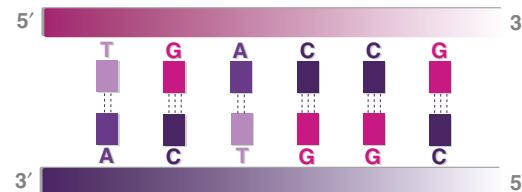
Although many of the oncology-focused clinical molecular diagnostic tests currently available are LDTs, the number of FDA-approved assays is increasing, particularly for tests that predict response to therapy, so-called theranostic testing. The FDA has pushed for “companion diagnostics,” CDx, to be developed and approved in parallel with newly developed targeted cancer therapies [13]. There are now several new targeted cancer therapies labeled to indicate that an FDA-approved test should be performed to determine drug eligibility. This creates challenges for oncologists and molecular diagnostic laboratories. For some gene mutation assays, there is only one FDA-approved test, and thus there is no market competition and no pressure to provide competitive pricing. The cost to perform an FDA-approved test can be prohibitively expensive unless the lab performs a significant number of tests to offset the cost of controls and other quality control materials. This also reduces the availability of the test and further reduces competition. In addition, FDA

approval does not indicate that the test method and performance are superior to any LDT. In fact, it is the opinion of the authors that some FDA-approved tests may be inferior to several lab-developed tests for targets such as *BRAF*.

### 3.3 The Basics of a Molecular Test

#### 3.3.1 Hybridization: Virtually All Molecular Tests Are Based on the Principle of Hybridization

Hybridization involves the use of a “primer” or “probe,” which is a nucleic acid molecule designed to be complementary to a specific nucleic acid sequence of interest. The primer/probe may be large, tens of thousands of base pairs, or smaller, 10–50 base pairs (often called oligonucleotides). Complementary bases between the target sequence and the primer/probe form hydrogen bonds, which bind the two strands to each other (Fig. 3.3). For some assays, such as FISH and Southern blot, hybridization is the endpoint, which can be measured by a variety of methods. For other methods, hybridization is coupled with an enzymatic reaction such as polymerization or ligation to generate a test result. In either case, the hybridization of a primer/probe to its target nucleic acid sequence can be regarded as analogous to the binding of an antibody to a specific protein in immunohistochemistry and other immunoassays. Just as the specificity of the antibody and the reaction



**Fig. 3.3** Hybridization. Hybridization between a target DNA sequence (purple strand) and a primer/probe (pink strand) relies on the hydrogen bonds (dashed lines) that form between the complimentary bases. Adenine (A) and thymine (T) form two hydrogen bonds. Cytosine (C) and guanine (G) form three hydrogen bonds

conditions determine the characteristics of an immunoassay, the sequence of the primer/probe and the reaction conditions used to carry out hybridization largely determine the performance characteristics of a molecular assay.

The sequence of the primer/probe will determine to which sequence(s) it will hybridize. The assay conditions will determine how strongly the strands are held together and whether hybridization can occur in the presence of sequence mismatches. The most important factors that determine the characteristics of hybridization reactions are the temperature of the reaction and the ionic strength forces present in the assay. Increasing temperature breaks hydrogen bonds and causes double-stranded DNA to “melt” or “denature” into single strands. Hybridization reactions that occur at high temperatures must have a large amount of sequence homology (a large number of complementary base pairs) in order for two strands to be held together. At lower temperatures, fewer hydrogen bonds are required to bring and hold two DNA strands together. Low-temperature reactions will allow hybridization to occur, even if the probe is not perfectly complementary to the target sequence. Hybridization at a temperature that is just a degree or two too low can allow a probe to hybridize to sequences other than the target sequence, leading to false-positive results. Hybridization at a temperature slightly too high can inhibit binding of the probe to the target sequence, resulting in a false-negative result.

Ionic strength is the second key to determining the characteristics of nucleic acid hybridization. Nucleic acids are negatively charged molecules. Because both the target and primer/probe are negatively charged, there are repulsion forces between the two strands. The presence of positively charged cations in the reaction solution reduces the inherent repulsion between the two DNA strands and allows increased hybridization to occur. When the ionic strength of the solution is low, the repulsion forces between the probe and the target sequence make hybridization difficult. In this scenario, a large number of hydrogen bonds between the probe/primer and the target DNA (and therefore a better match between the target and probe sequences)

are required for hybridization. When the ionic strength of a reaction is high, the large number of positively charged ions facilitates hybridization, even in the presence of base mismatches between the primer/probe and target DNA strand. The term “stringency” is used to indicate the overall conditions of a hybridization reaction. High-stringency reactions typically utilize higher temperatures and/or lower ionic strengths, making hybridization more difficult. For this reason, highly stringent reactions require a high degree of sequence homology and do not tolerate mismatches between the primer/probe and target sequence. Low-stringency conditions, including lower temperature and/or higher ionic strength, allow hybridization to occur even if the primer/probe and target sequence are not perfectly complementary. Low-stringency conditions are more tolerant of mismatches.

Similar to other types of tests, molecular tests are designed to achieve the desired balance of sensitivity (risk of false negatives) and specificity (risk of false positives). A test can be designed to be highly specific, but that usually comes at the price of reduced sensitivity. In contrast, a test that has a high sensitivity may risk reduced specificity. The hybridization conditions are key to balancing the sensitivity and specificity of molecular testing.

### 3.3.2 Enzymes

The enzymes used in molecular biology reactions have been cloned from a variety of organisms and have been genetically modified in order to achieve specific characteristics that allow the enzyme to have improved functionality in laboratory use. Table 3.1 describes the basic functions of several enzyme families commonly used in molecular diagnostic assays. Many varieties of each of the enzymes listed are commercially available. For example, there are over 50 commercially available DNA polymerases, which differ in their error rates, speed, conditions for activation and temperature requirement for optimal performance. Thus, enzyme selection plays a role in the characteristics of an assay.

**Table 3.1** Enzymes commonly used in molecular diagnostic laboratories

Polymerases: Enzymes that synthesize nucleic acids using a nucleic acid template
DNA-dependent DNA polymerase (DNA polymerase): uses a DNA template strand to synthesize DNA
DNA-dependent RNA polymerase (RNA polymerase): uses a DNA template strand to synthesize RNA
RNA-dependent DNA polymerase (reverse transcriptase): uses an RNA template strand to synthesize DNA
Nucleases: Enzymes that break phosphodiester bonds of nucleic acid strands. These enzymes specifically attack either RNA (ribonucleases) or DNA (deoxyribonucleases)
Exonucleases cleave phosphodiester bonds at the end (either the 5' or the 3' end) of a DNA or RNA fragment
Endonucleases (also called restriction endonucleases or restriction enzymes) cleave phosphodiester bonds within a DNA fragment in a sequence-specific manner
DNA ligase: Enzymes that catalyze the formation of a phosphodiester bond between the 3' end of one DNA fragment and the 5' end of an adjacent DNA fragment

**Table 3.2** Advantages and disadvantages of non-amplification methods

Advantages	Disadvantages
Analysis of individual cells (karyotyping and CISH/FISH) rather than pooling DNA from many cells into one specimen	Not able to detect alterations that affect a small genetic region
Analyzes the entire genome for relatively large structural alterations (karyotyping, Southern blot, aCGH)	Not able to identify sequence variants
Minimal risk for carryover contamination	Limit of detection using formalin-fixed paraffin-embedded tissues may be relatively poor, 20–30 %

induced to go through cell division while in culture in the lab. Dividing cells are then arrested at metaphase of the cell division cycle, allowing for collection of metaphase chromosomes, which can be stained and examined microscopically. Individual chromosomes are identified by their size, centromere position, and pattern of bands that result from the staining process. By convention, chromosomes are numbered sequentially based on size, from largest to smallest. The shorter arm of a chromosome is designated “p,” and the longer arm, “q.” On each arm of a chromosome, the bands are numbered consecutively beginning at the centromere and extending outward along each chromosomal arm. A particular region is designated by the chromosome number, the arm symbol, the region number, and the band number within the region. For example, 1p36 indicates chromosome 1, short arm, region 3, band 6. Because the region and band numbers are distinct, 1p36 is spoken as “one p three six,” not “one p thirty-six.” If an existing band has been subdivided, a decimal point is placed after the original band designation, followed by the sub-band number, for example, 1p36.2.

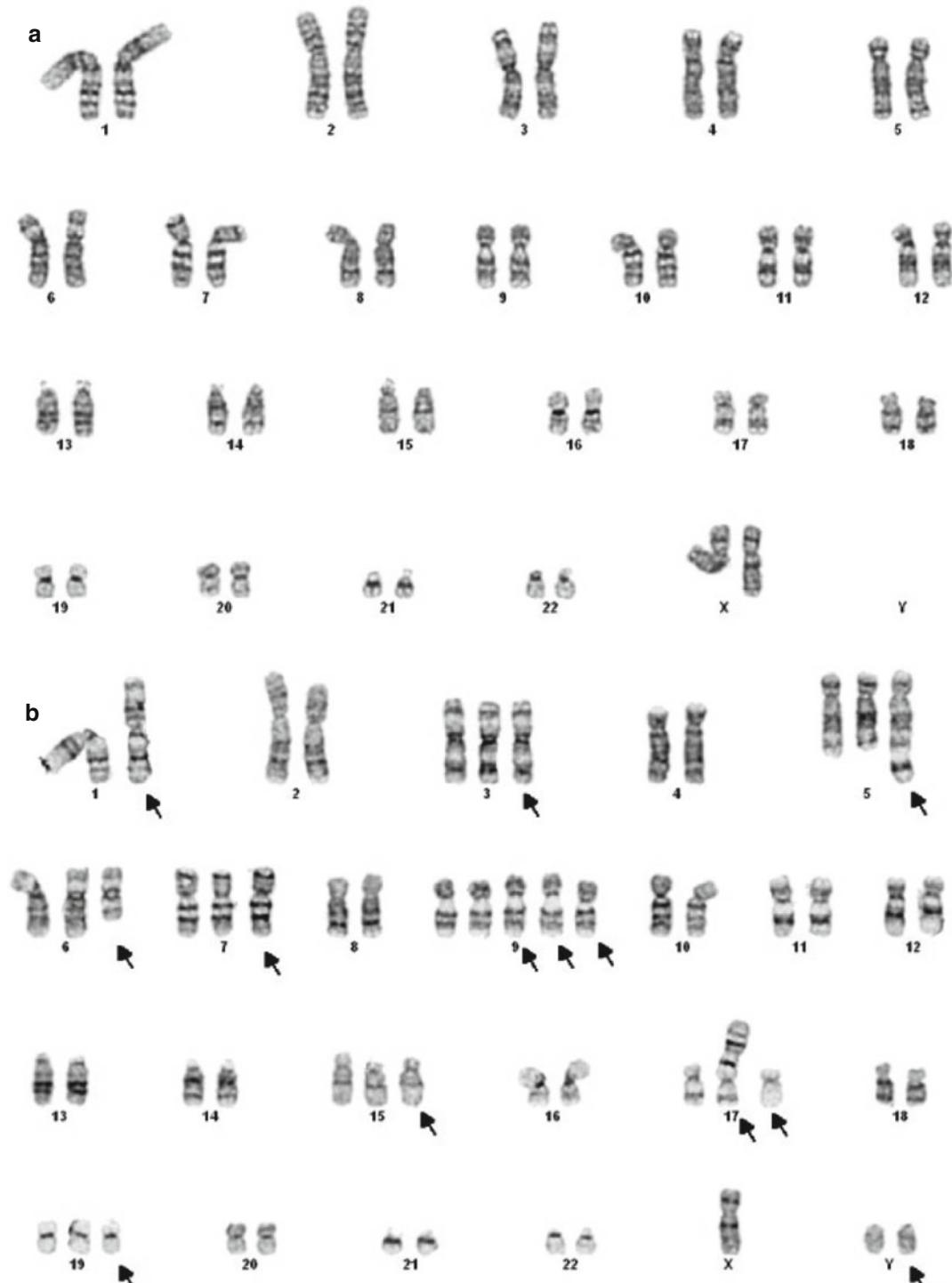
A normal human karyotype is 46,XX or 46,XY (Fig. 3.4a). Chromosomal abnormalities are designated a variety of ways to indicate the location and type of abnormality. For example, 48,XX, +6, +11 describes a genome with an extra copy of chromosomes 6 and 11, making the total number of chromosomes 48. Similarly, 45,XX, -22

## 3.4 Non-amplification Nucleic Acid Analysis Methods

Molecular techniques can be divided into two broad categories: amplification and non-amplification methods. Non-amplification methods generally rely on direct detection of a labeled probe that has hybridized to a nucleic acid target sequence. The probe and target sequences are generally much larger/longer than those analyzed by PCR and other amplification methods. Non-amplification methods can be quantitative but over a relatively limited range. Examples of some of the strengths and weakness of these methods are outlined in Table 3.2.

### 3.4.1 Karyotyping (Cytogenetic Analysis)

A karyotype is a description of the chromosomal constitution of a cellular specimen. Karyotyping involves the collection of live cells, which are



**Fig. 3.4** Karyotypes from human specimens. Normal human karyotype, 46XX (**a**). Abnormal karyotype from a human tumor (**b**), 57,XY,+Y,t(1;17)(q36.1;q21), +3,+5,der(5)(5;11)(q25;q13),+6,i(6)(p10),+7,+9,+9,+9,+15,+17, der(17)t(1;17)(q12;p13),+19[3]/=sl 54~56,sl,

-i(6)(p10),+der(6)t(1;6)(q21;q13),-9[cp2]/56~57,sl,i(6)(p10),der(7)t(7;11)(q36;q13),-9,+17,-der(17)t(1;17)[cp3]/44~58,sl,+6,+6,i(6)(p10),der(6)t(6;9)(q12;q13)x3,-9[cp7]/46,XY[15]. Abnormalities in copy number and structure are identified with arrows

is a karyotype with the loss of chromosome 22, also called monosomy 22. Portions of chromosomes may be gained or lost, and the karyotype would reflect the region(s) involved. For example 46,XX, del(7)(p11.2) describes a karyotype with a loss of a portion of the short arm of chromosome 7. Translocations are indicated by “t” followed by the chromosomes involved in parentheses and then the chromosomal positions involved in a second set of parentheses. For example, 46,XY t(11;22)(q24;q12) describes a translocation between chromosomes 11 and 22. The breakpoint on chromosome 11 is on the long arm at position 24. The breakpoint on chromosome 22 is on the long arm at position 12. Chromosomal abnormalities can become quite complex, particularly in tumor specimens (Fig. 3.4b). For a full description of how to designate particular chromosomal abnormalities, refer to the International System for Human Cytogenetic Nomenclature (ISCN) [14].

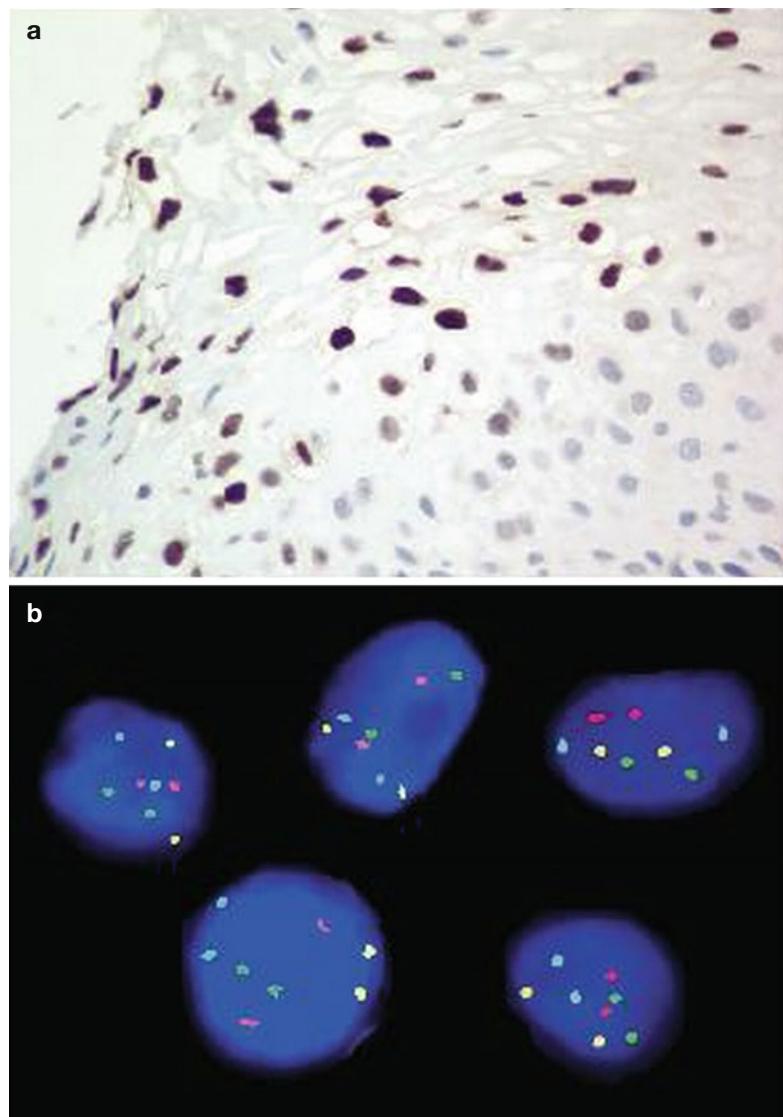
The advantage of cytogenetic analysis is that the entire genome can be analyzed for a wide variety of structural alterations. However, although the resolution of cytogenetic analysis has improved, it is still only useful for detecting alterations involving greater than or equal to 5–10 million base pairs (5–10 Mb) of genetic material; smaller alterations will not be detected. Karyotyping also does not identify sequence alterations. Because chromosomal analysis requires actively dividing cells, its use is relatively restricted. Peripheral blood specimens can be used for germline analysis, often referred to as constitutional chromosomal analysis. Chorionic villus sampling (CVS) and amniocentesis specimens can be used for prenatal analysis. Somatic (neoplastic) karyotyping is largely limited to the use of peripheral blood and bone marrow specimens to identify somatic chromosomal alterations seen in leukemia and other hematopoietic malignancies. Although cytogenetic analysis of solid tumors has been performed for research purposes, it is not commonly performed clinically because of the need for fresh, viable tissue and other technical difficulties encountered with solid tissue.

### 3.4.2 In Situ Hybridization (ISH): Chromogenic In Situ Hybridization (CISH) and Fluorescent In Situ Hybridization (FISH)

CISH and FISH are essentially the same method with different detection strategies. These methods allow for direct detection of nucleic acids in tissue sections on slides. Anatomic pathology labs are generally comfortable with these methods because of their similarity to protein detection by IHC. CISH is commonly used to detect the genomes of infectious agents (see Chap. 11), while FISH is more commonly used to detect human germline and somatic genetic alterations (see Chaps. 4 and 8). Both methods require optimization of assay conditions such as tissue preparation and hybridization conditions in order to generate accurate results. An advantage of these methods is that the histology of the tissue is preserved, which facilitates analysis of the specific cells of interest. Another advantage is that because individual cells are analyzed, the assay can be quantitative or at least semiquantitative. A disadvantage of CISH and FISH compared to karyotyping is that only alterations to which a specific probe is used will be identified.

The process of CISH and FISH involves the use of specific nucleic acid probes that are complementary to the target sequence of interest. The probes are labeled: CISH probes with an enzyme and FISH probes with a fluorescent molecule. The probes are applied to tissue sections under specific hybridization conditions to ensure appropriate sensitivity and specificity of the reaction. If the target sequence of interest is present, the labeled probe will hybridize to it. Unbound probes are washed away before proceeding to the detection step. The probes are then visualized microscopically. Similar to IHC, CISH requires the addition of a substrate that is converted to a colored product if the enzyme attached to the probe is present. The color produced in CISH reactions is visualized using a light microscope (Fig. 3.5a). A limitation of CISH, similar to IHC, is that generally only a single probe can be used

**Fig. 3.5** Examples of CISH and FISH data. CISH using probes specific to human papillomavirus (HPV) types 6 and 11 in a condyloma (a). Note the nuclear staining. FISH using four probes that target different regions of the human genome (b). Each probe is labeled with a fluorescent molecule. Nuclei are stained in blue. Each of the nuclei demonstrates a normal pattern with 2 yellow, 2 red, 2 green, and 2 aqua signals



per slide because the probes are all are labeled with the same enzyme and are therefore indistinguishable. Although there have been advances in CISH to allow for multicolor detection and, therefore, simultaneous analysis of multiple probes, they have not been widely incorporated into clinical use.

In contrast to CISH, FISH uses fluorescent labels on the nucleic acid probes. The probes are visualized using a fluorescent microscope. Because several different fluorescent molecules are available, multiple probes can be used in

combination in a single analysis. The maximum number of probes that can be used simultaneously is typically limited to four, due to technical constraints of the excitation light source and the overlapping emission spectrum of the fluorochromes. FISH is commonly used to detect chromosomal gains, losses, inversions, and translocations that have diagnostic and prognostic significance. Detecting gains and losses is relatively straightforward. Each cell should contain two copies of each genetic target. Gains and losses are detected by the presence of more or fewer

than the normal two copies of a genetic sequence. Figure 3.5b demonstrates four FISH probes used in a single reaction to assess for genetic gains or losses. FISH assays to identify inversions and translocations can be more complicated. These assays are typically designed using one of two different strategies: fusion probes or break-apart probes. Understanding the assay design is absolutely required to accurately interpret the data.

#### 3.4.2.1 Dual-Fusion FISH

This assay design uses a total of four probes, targeting two genetic regions that are known to undergo translocation events. Two probes labeled with the same fluorescent molecule are designed to be complementary to two adjacent regions near each breakpoint locus. The probes for the two different breakpoint loci are labeled with different fluorescent molecules, typically red and green (Fig. 3.6a). In normal cells, the FISH pattern produced is two red and two green signals. Translocation events involve breaking one copy of each chromosome between the two adjacent probes. One green signal will translocate to the chromosome labeled with the red probe, and one red signal will translocate to the chromosome with the green probe. The proximity of the red and green probes involved in the translocation results in a yellow color. The typical pattern for a specimen that contains the translocation is one red, one green, and two yellow signals. An advantage of this strategy is that it definitively identifies both translocation partners. A disadvantage is that if one of the genetic regions has undergone a translocation event that does not involve the genetic region targeted by the other probe, the test may yield a negative result.

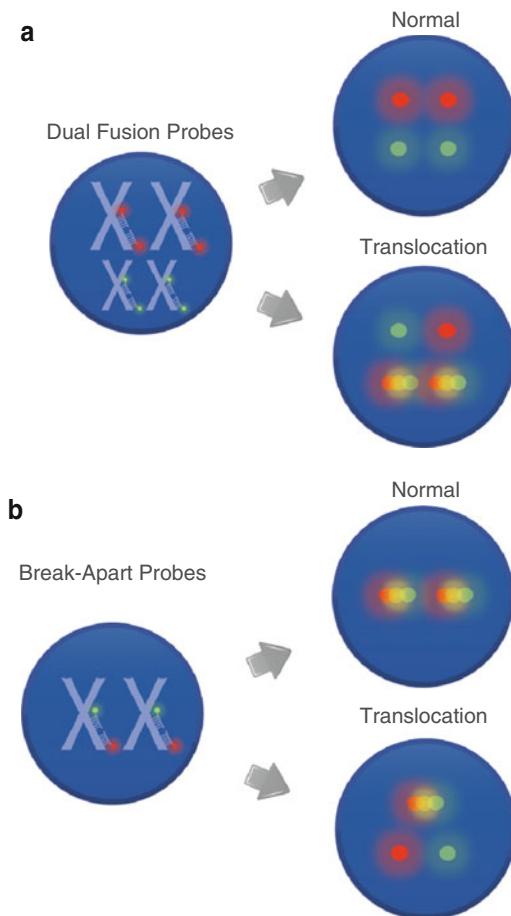
#### 3.4.2.2 Break-Apart FISH

In contrast to the fusion probe design, a break-apart probe strategy uses two probes labeled in different colors that are complementary to adjacent genetic sequences. The normal pattern using this strategy is two yellow signals resulting from co-localization of the green and red probes, Fig. 3.6b. If a translocation or inversion occurs involving the genetic region targeted by the

probes, the two probes will “break apart,” creating a pattern of one fusion (yellow) signal from the normal, uninvolved gene copy, and one red and one green signal resulting from the translocation. The advantage of a break-apart design is that it will detect an inversion or translocation that involves the genetic region of interest, regardless of the fusion partner. This is very useful for promiscuous genes like *EWSR1*, which is known to undergo translocations involving at least a dozen different fusion partners. A disadvantage of this assay design is that the identity of the fusion partner is not known, which may or may not be important clinical information.

#### 3.4.3 Southern Blot

Development of the Southern blot was a landmark innovation that initiated the molecular era. The technique was largely responsible for many significant genetic discoveries including the ability to map genetic diseases. Southern blotting involves the use of restriction enzymes (endonucleases) to cleave genomic DNA into discrete fragments. The fragments are separated by electrophoresis, a process that uses an electric current to separate a mixture of charged molecules. Electrophoresis can be used to separate DNA, RNA, or protein molecules. For Southern blot applications, fragments of DNA are separated primarily based on their size. The process involves applying a DNA sample to a matrix such as an agarose gel, to which an electric current is applied. Because DNA is negatively charged, it migrates toward the positively charged anode. The rate at which it migrates is proportional to the size of the DNA fragment. Small fragments move more quickly through the matrix than larger fragments. The separated DNA fragments are then transferred (“blotted”) to a solid support similar to paper. The fragments are detected by hybridization with specific nucleic acid probes labeled with a radioactive molecule. Southern blotting has several limitations including the requirement for relatively large quantities of high-quality DNA. It cannot be used to analyze



**Fig. 3.6** Schematic of two FISH probe design strategies to detect inversions and translocations. Dual-fusion FISH (a). Two probes labeled with the same fluorescent molecule are complementary to adjacent regions near each breakpoint locus. The probes for the two different breakpoint loci are labeled with different fluorescent molecules. Normal cells demonstrate two red and two green signals. If a translocation involving both genetic regions has occurred, one green signal will translocate to the chromosome labeled with the red probe, and one red signal will translocate to the chromosome with the green probe. The proximity of the red and green probes results in a yellow color. The typical pattern for a specimen that contains the translocation is one red, one green, and two yellow signals. Break-apart FISH (b). Two probes labeled in different colors are complimentary to adjacent genetic sequences. Normal cells demonstrate two yellow signals. The typical pattern for a specimen that contains a translocation is one fusion signal, one red signal, and one green signal.

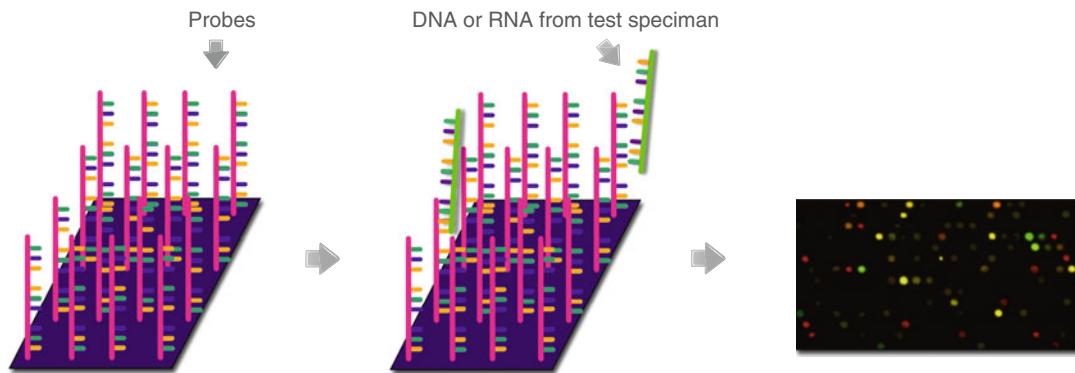
DNA from formalin-fixed paraffin-embedded tissues because the tissue processing results in significant nucleic acid degradation. Although not commonly used today, it is the foundation for many molecular methods currently in use, including microarray analysis.

### 3.4.4 Microarrays and Comparative Genomic Hybridization (CGH)

The term “microarray” is generic, referring to methods that use an “array” of probes to detect multiple targets in a small reaction. Microarrays have evolved as manufacturing developments have allowed the placement of a large number of probes on a small “chip” with high accuracy and reproducibility. An array can contain tens of thousands of probes in the same area that was previously used for a single probe. Microarrays have allowed researchers to gather hundreds of thousands of data points in a single experiment, accelerating discoveries in many areas of research.

Although tissue microarrays and microarrays for protein analysis are available, the following discussion relates only to the use of microarrays to detect nucleic acids (RNA and DNA) targets. Nucleic acid microarrays can be configured in a wide variety of ways. In general, short nucleic acid sequence (oligonucleotide) probes are immobilized to a solid surface often referred to as a “chip” (Fig. 3.7). Nucleic acids from test specimens are allowed to hybridize to the probes on the chip. After removing unbound sequences, the hybridized sequences can be detected by a variety of methods including fluorescence, light emission, and color.

Expression microarrays were developed to detect and quantify specific RNA sequences. Several early publications described “expression profiles” or “expression signatures” for specific tumor types [15–17]. Although the hope was that expression profiles would have diagnostic and/or prognostic utility for neoplastic diseases, there are relatively few applications currently in clinical practice. Lack of clinical adoption stems in part from the inherent instability of RNA,



**Fig. 3.7** Schematic of microarray assay design. DNA probes are tiled onto a solid surface (chip). The probes may target regions across the entire genome or may target specific areas of interest. DNA extracted from a patient specimen is labeled with a fluorescent molecule. The

DNA from the test specimen is allowed to hybridize to the probes on the chip. DNA that does not hybridize to the chip is washed away. Fluorescent signal is detected for each individual probe. The data are compiled by a computer algorithm

which makes it difficult to achieve reproducible data. In addition, there has generally been a failure to demonstrate that the results are clinically actionable.

Microarray methods to detect and quantify DNA were an extension of comparative genomic hybridization (CGH), which had been developed as an alternative to karyotyping. Because karyotyping requires viable cells that can be induced to go through cell division in the lab, the technique cannot be used for a wide variety of specimen types. Although karyotyping is useful for peripheral blood and bone marrow samples, it is difficult to perform on fresh tissue, and it is impossible to perform on formalin-fixed tissue specimens. CGH uses normal chromosomes immobilized to a solid surface such as a slide. DNA from a test specimen is labeled with a specific fluorescent color and mixed with normal control DNA labeled in a different fluorescent color. The mixture is allowed to hybridize to the immobilized metaphase chromosomes, during which the normal and test DNA compete for binding to the immobilized chromosomes. Areas of amplification or deletion in the test specimen are detected by an increase or decrease in the test fluorescent signal compared to the control fluorescent signal. Similar to karyotyping, CGH

allows for interrogation of all 46 chromosomes in a single test; however, it is limited to the detection of abnormalities that result in gains or losses in genetic material that are  $\geq 5\text{--}10$  million bases in size. CGH is not able to detect balanced reciprocal translocations or inversions.

The introduction of microarrays has largely replaced standard CGH with array CGH (aCGH). aCGH uses tens of thousands of probes that are complementary to specific regions of the genome as opposed to using entire intact normal chromosomes. DNA microarrays are now manufactured to address many different scientific questions. For example, an aCGH chip can be designed and produced with tens of thousands of probes to one specific chromosome. In contrast, an aCGH chip can be produced with the same number of probes but targeted to areas spanning the entire genome. The design of the chip determines the size of an alteration that can be confidently detected. Many aCGH designs use probes that target single nucleotide polymorphisms, “SNP chips,” which yield data on heterozygosity as well as copy number. Similar to standard CGH, aCGH is limited to detecting alterations that result in gains or losses in genetic material and are not able to detect balanced translocations and inversions.

aCGH has been used in research applications to identify recurrent gains and losses in many different tumor types (see Chap. 4). In contrast to RNA microarrays, DNA microarrays have found several applications in clinical medicine. Array-based chromosomal analysis methods are replacing standard cytogenetic techniques (karyotyping) for constitutional chromosome analysis [18, 19]. In oncology, aCGH has shown clinical utility for detecting specific gains or losses that have diagnostic and/or prognostic significance in leukemias and some solid tumors [20, 21]. In this setting, aCGH is an attractive alternative to FISH because of its ability to identify essentially all gains and/or losses in a single reaction, its superior throughput capacity, and its potential cost savings.

## 3.5 Amplification Methods

The polymerase chain reaction (PCR) was the first amplification method invented and remains one of the most commonly used methods in laboratories around the world. PCR revolutionized many aspects of nucleic acid–based research and testing. Following the introduction of PCR, a wide range of amplification methods has been developed by using a variety of enzymes and detection strategies. Amplification methods are described as belonging to one of three broad categories: target amplification, signal amplification, and probe amplification (Table 3.3). As the name implies, target amplification involves amplifying the target sequence of interest. In addition to PCR, this category includes TMA. Because target amplification methods result in the production of millions of copies of the target itself, they carry a risk for “carryover” contamination, where just one or a few copied targets contaminate other reagents or equipment in the lab leading to false-positive results in subsequent reactions. Although this was a major problem in the early days of PCR, laboratory precautions and enhanced reagents have greatly reduced the risk of carryover contamination. Signal and probe amplification methods have essentially no risk

**Table 3.3** Amplification-based methods

Target amplification: Very large numbers (millions) of copies of the target sequence are synthesized	Polymerase chain reaction (PCR)
	Transcription-mediated amplification (TMA)
Probe amplification: A synthetic probe that is complimentary to the target sequence is amplified	Ligase chain reaction (LCR)
	Strand displacement amplification (SDA)
	QB replicase
Signal amplification: Signal is generated and amplified in the presence of the target sequence	Branched DNA amplification (bDNA)
	Hybrid Capture
	Cleavage-based amplification (also called Invader)
	Cycling probe

for carryover contamination because the target sequence itself is not amplified. Signal amplification involves the amplification of a signal, often fluorescence, in the presence of target sequence. This group includes cleavage-based amplification (Invader technology) and branched DNA amplification (bDNA). Probe amplification methods rely on the amplification of a nucleic acid probe if the target sequence is present. This category of tests includes the ligase chain reaction (LCR) and strand displacement amplification (SDA).

Why is nucleic acid amplification so important? Because the human genome contains approximately three billion base pairs of DNA sequence. If one is interested in analyzing a specific gene that is composed of ten thousand base pairs, that gene consists of <0.001 % of the bases in a human DNA specimen. It is difficult, if not impossible, to obtain an accurate analysis of the gene when 99.999 % of the DNA in the specimen is not the target sequence of interest. Amplification methods are needed in order to make many copies of a sequence of interest relative to the rest of the genome so that the target sequence can be analyzed.

In contrast to non-amplification methods, amplification methods are generally used to analyze relatively small regions of DNA, up to a few thousand base pairs. One of the major advantages of amplification methods compared

**Table 3.4** Advantages and disadvantages of amplification-based methods

Advantages	Disadvantages
Capable of detecting very low quantities of the target sequence of interest	Risk for false positives due to carryover contamination (target amplification)
Capable of identifying single base alterations and other alterations that affect a small region of DNA (<1,000 bp)	Not able to detect alterations affecting entire chromosomes or large chromosomal regions

to non-amplification methods is their ability to detect very low amounts of a DNA sequence. Advantages and disadvantages of amplification methods are outlined in Table 3.4. Some amplification methods require a separate procedure to detect the amplified products, while other methods have been developed to incorporate amplification and detection into a single reaction.

### 3.5.1 Polymerase Chain Reaction (PCR)

PCR uses two primers, a DNA polymerase, and the building blocks of DNA, dNTPs, to make copies of a specific DNA sequence. The reaction mimics, on a very small scale, replication of the genome, which is required for each cell division. PCR uses a DNA polymerase from thermal-stable organisms, primarily *Thermus aquaticus*, hence the name “Taq polymerase” or just “Taq” that is often associated with PCR reactions. Human DNA polymerases would not withstand the extreme temperatures required for effective PCR amplification. Thermal-stable polymerases allow the reaction to be cycled to high temperatures to achieve separation of the two DNA strands without affecting the function of the polymerase enzyme.

Traditional PCR reactions are cycled multiple times at three different temperatures: a high temperature to separate the double-stranded DNA template into single strands (denature step), a lower temperature to allow hybridization of the PCR primers to the target DNA (annealing step), and a temperature that is optimal for the DNA polymerase to extend the primer, using the

**Table 3.5** Typical thermal cycling conditions

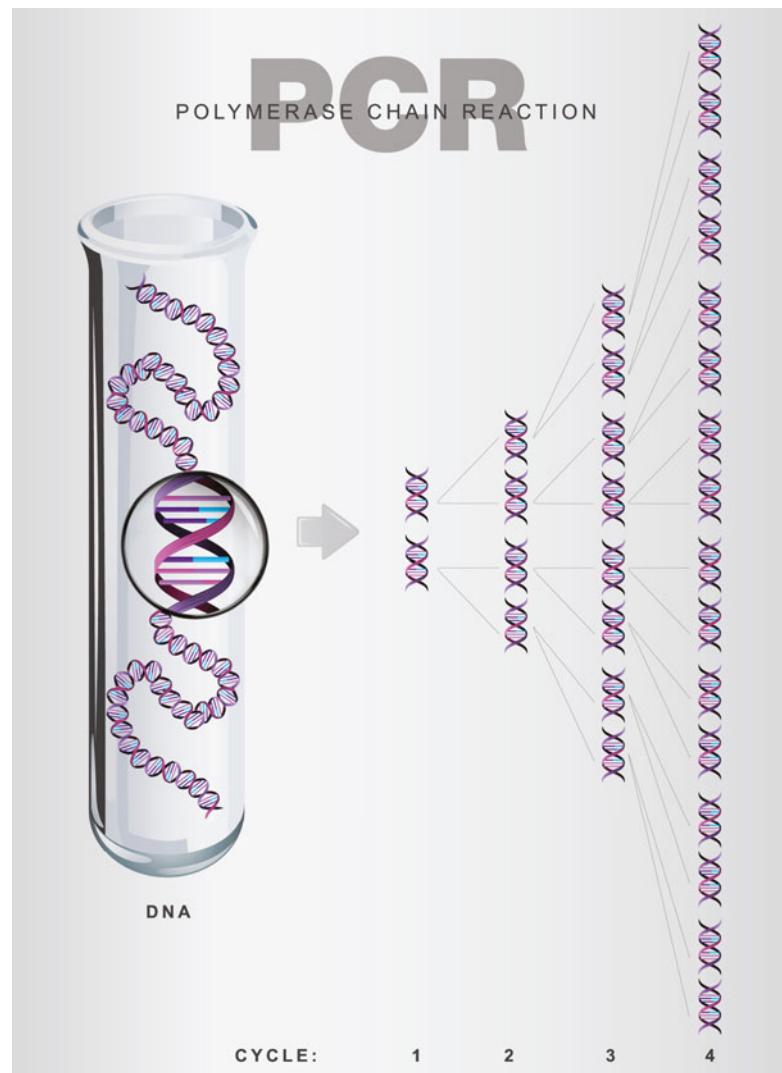
Temperature (°C)	PCR stage	Description
95	Denaturation	High temperature separates the two strands of the target DNA
50–60	Annealing	Primers hybridize to the target sequence Annealing temperature will vary depending on the melting temperature of the primers
60–72	Extension	Using the target DNA as the template, DNA polymerase adds bases (dNTPs) to each of the two primers. From each double-stranded DNA template, two double-stranded PCR products are produced

complementary strand of DNA as the template (extension step). Table 3.5 describes typical thermal cycling conditions. Some of the newer real-time PCR methods allow the annealing and extension steps to be performed at a single temperature. These “two-step” cycling conditions allow for more rapid thermal cycling.

The annealing step of a PCR reaction is a hybridization process. The sequence of the primer and the reaction conditions during the annealing step will determine the robustness and specificity of the PCR reaction. Clearly, in order to achieve a highly specific assay, the sequence of the primer must be complementary to the target sequence and have little homology to other areas of the genome. The temperature used for the annealing step is also critical to assay performance. If the annealing temperature is too high, the primer will not be able to bind to the target sequence, and a false-negative result will be generated. If the annealing temperature is too low, the primer may be able to bind to areas of the genome that have similar, but not exact, complementarity, and a false-positive result may be generated.

During each cycle, the amount of target sequence is doubled (Fig. 3.8). Typically 25–40 cycles are performed. The theoretical amount of

**Fig. 3.8** PCR. During each cycle of PCR, each of the two strands of DNA is used as a template to synthesize the complimentary strand. The amount of the target sequence is doubled during each PCR cycle



the final product can be calculated by  $2^n$ , where  $n$  is the number of cycles. For example, in theory, 30 cycles would generate 1,073,741,824 copies from a single copy of target DNA. In reality, most reactions are not 100 % efficient, so fewer copies are generated, but still many millions, which is plenty for downstream analysis. There are many websites with animations that demonstrate the process of PCR. It is important to choose a reputable source and/or watch several to compare for accuracy.

Since its first description in 1983, many iterations of PCR have been developed. Some PCR variations were developed to address specific

scientific needs. Reverse transcription, for example, allows the PCR method to be used when the starting material is RNA, rather than DNA. Other advancements have improved the ease, speed, and reliability of PCR. Table 3.6 lists common PCR variations that are used in research and clinical laboratories. Real-time PCR is arguably the most significant advancement in this technology (see below).

Standard PCR results in amplification products that require downstream analysis. A common method to assess the products of a PCR reaction is electrophoresis. PCR products are applied to a matrix such as an agarose gel, to

**Table 3.6** Variations of PCR

Assay	Description
PCR	Polymerase chain reaction: Two primers and a DNA polymerase enzyme (often Taq) are used to amplify a specific sequence of interest. Detection of the amplified products is achieved by downstream analysis
RT-PCR	Reverse transcription PCR: Starting material is RNA, which must be converted to DNA by a reverse transcription step before PCR can proceed
Real-time PCR	Amplification products are detected “in real time” by production of a fluorescent signal. The fluorescent signal can be generated by a dye that binds DNA or by a DNA probe that is labeled with a fluorescent molecule. The amount of fluorescent signal generated is proportional to the concentration of the target sequence. There are several different assay and probe designs including TaqMan, FRET, and Scorpions
qPCR	Quantitative PCR: A real-time PCR assay that has been calibrated to yield quantitative results
ASPE/ARMS	Allele-specific primer extension=allele-specific PCR=amplification refractory mutation system: One or both primers are designed such that nucleotide on the 3' end is specific to a mutation or polymorphism site. If the mutation/polymorphism is present, the primer will generate amplification products. If the mutation/polymorphism is not present, the primer will not generate products. ASPE/ARMS has a superior limit of detection for single base variants compared to standard PCR
Nested PCR	Uses two pairs of primers to the same target, usually in two separate reactions. In the first reaction the target is amplified using the “outer” primer set. The products from the first reaction are further amplified in the second reaction using a primer pair that is “inside” of the first primer set and thus generates a smaller-sized product. This approach results in increased sensitivity and specificity but also carries an increased risk for contamination
Multiplex PCR	PCR reaction containing more than one primer set. Amplifies multiple targets simultaneously. Allows multiple results to be generated from a single PCR reaction
Hot start PCR	One or more of the reagents in the reaction are not available or not active until after an initial heating step (“hot start”). This is most commonly achieved by using a hot start DNA polymerase, which is enzymatically inactive until it is heated. The use of hot start reduces nonspecific priming and results in increased specificity of the PCR reaction
COLD PCR	Co-amplification at lower denaturation temperature PCR selectively amplifies mutant alleles based on melting temperature differences between wild-type and mutant amplicons. COLD PCR may result in an improved sensitivity over standard PCR depending on the specific mutation
Digital PCR	DNA specimen is diluted such that single molecules of DNA are amplified individually in multiple, separate PCR reactions.
Emulsion PCR	Emulsion PCR relies on the capture of single DNA molecules within a water-in-oil emulsion “bubble.” Within each emulsion, a single DNA molecule is PCR amplified independently of the others, resulting in parallel amplification of millions of single DNA molecules

which an electric current is applied. Because DNA is negatively charged, it migrates toward the positively charged anode. The rate at which it migrates is proportional to the size of the DNA fragment. Small fragments move more quickly through the matrix than larger fragments. The separated DNA fragments can be visualized by using a fluorescent dye that binds DNA such as ethidium bromide or SYBR green.

Although gel-based electrophoresis has been a workhorse in the laboratory over the last several decades, its use is declining, particularly in clinical labs. One reason for the decline is the development of an automated process of electrophoresis called capillary electrophoresis (CE).

The principle of CE is exactly the same as standard electrophoresis; PCR products are subjected to an electric current, resulting in separation of the nucleic acid fragments based on their size. One requirement for CE that is not needed for gel-based electrophoresis is that the PCR is performed using a primer that has a fluorescent label. The fluorescent label on the primer is incorporated into the PCR products, allowing their detection as they move through the capillary. CE provides significant advantages over standard gel-based electrophoresis including improved sensitivity and specificity of PCR product detection. CE can also size DNA fragments much more accurately than gel-based electrophoresis. Additionally, CE can

be used to analyze multiple PCR products simultaneously due to the use of different-colored fluorescent labels. Multiplex PCR with CE analysis can readily detect as many as 15 DNA targets in a single reaction. Two clinical assays provide great examples of the superiority of CE over traditional electrophoresis: microsatellite instability (MSI) testing and B-cell and T-cell gene rearrangement studies. For both of these assays, the ability to multiplex several primer sets and the single base resolution provided by CE results in superior assay performance characteristics compared to when the PCR products are resolved by standard gel electrophoresis.

### 3.5.2 Microsatellite Instability Analysis (MSI)

MSI testing is used to determine whether the DNA mismatch repair system in a tumor is functional or not. This test is typically performed on tumor tissue to identify individuals that may have Lynch or Muir-Torre syndrome (see Chap. 9). The DNA mismatch repair system repairs errors that are made during DNA replication. Loss of function of the system results in a hypermutable state that is a precursor for tumor development. The DNA mismatch repair system is responsible for repairing a number of different types of replication errors including those that involve short DNA repeat sequences (microsatellites). Because of the repetitive nature of these sequences, the DNA replication machinery often “slips” during replication of these regions resulting in changes in the repeat length, with either too many or too few copies of the repeated unit produced. Normally the DNA mismatch repair system would correct the repeat length in the newly synthesized DNA copy to that in the original DNA template. When the DNA mismatch repair system is not functional, these changes are not repaired, and the tumor demonstrates altered lengths of the repeats when compared to normal tissue.

MSI testing generally involves analysis of several microsatellite markers. Although thousands of microsatellites exist in the genome, a panel of five (called the Bethesda consensus panel) was developed in order to achieve consistency of

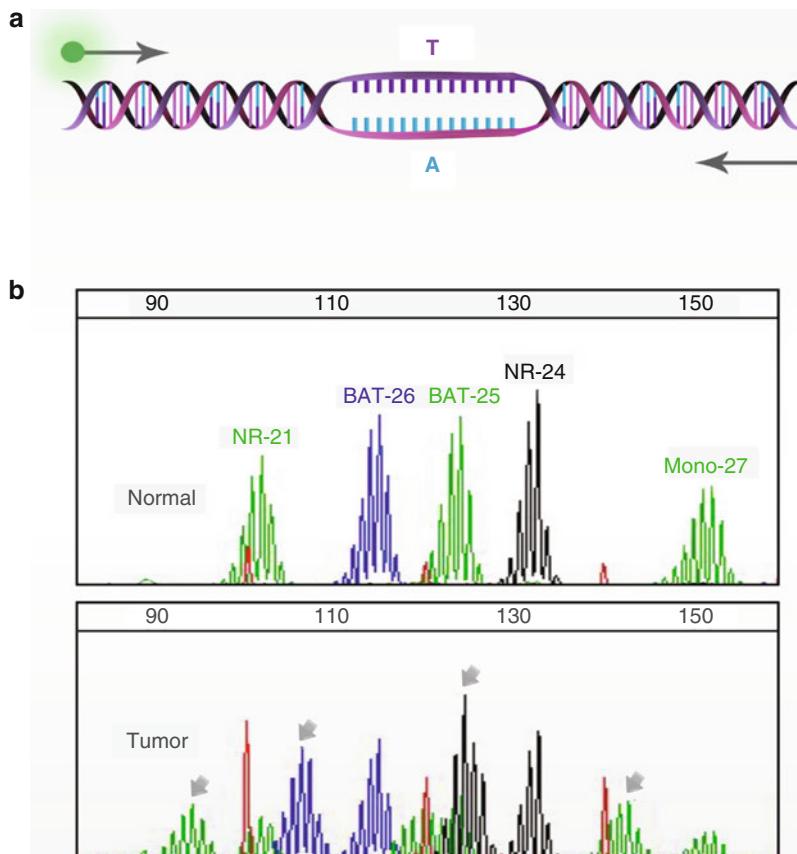
results from different laboratories [22]. This panel consisted of two mononucleotide repeats (single base repeat) and three dinucleotide repeats (repeat of a two base unit). As data developed indicating that the mononucleotide markers were more sensitive and specific for identifying mismatch repair deficiency, newer panels consisting of only mononucleotide markers were developed [23].

Prior to testing, a pathologist should review the case to identify areas of tumor and normal, if available, that are appropriate for testing. DNA is extracted from each and subjected to a PCR reaction that contains primer sets targeting multiple microsatellite regions. One primer in each set is fluorescently labeled, which allows the PCR products to be detected during capillary electrophoresis (Fig. 3.9). The pattern generated from the normal DNA is compared to that generated from tumor DNA. If the patterns are identical, the tumor is microsatellite stable (negative for MSI). If two or more microsatellite markers demonstrate unique PCR products relative to the normal, the tumor is considered MSI high.

### 3.5.3 T-Cell and B-Cell Gene Rearrangement Analysis

T-cell and B-cell clonality assays take advantage of the fact that T and B cells go through a process of gene rearrangement during their normal maturation process (see Chaps. 6 and 7 for applications in cutaneous lymphomas). Rearrangement of the immunoglobulin heavy (IgH) and light (Igκ and Igλ) chains in B cells generates diversity in the antibodies (immunoglobulins) they produce. In T cells, rearrangement of the four T-cell receptor (TCR) peptides ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ) is responsible for generating the T cell diversity that is needed to mount an immune response to numerous diverse agents. Analysis of T-cell and B-cell gene rearrangements differs from many of the other molecular tests in that the target is not an oncogenic mutation, such as *BRAF* V600E, but rather a normal process from which we can assess whether the cell population derives from one progenitor (clonal) or from many progenitors (polyclonal).

The assay design, data generated, and interpretation of T-cell and B-cell gene rearrangement



**Fig. 3.9** Microsatellite instability (MSI) analysis. Schematic of PCR design (a). PCR primers are designed to flank repeat regions, in this case a mononucleotide track of adenines/thymine (A, T). One of the PCR primers is labeled with a fluorescent molecule which will be incorporated into the PCR products and allow for their detection during capillary electrophoresis. Capillary electrophoresis data from matched normal and tumor specimens (b). X-axis is size in bases. Y-axis is fluorescent intensity. The multiplex PCR targets five mononucleotide repeats, three labeled in green (NR21, BAT-25, and Mono-27), one in blue (BAT-26), and one in yellow (NR-24), which is

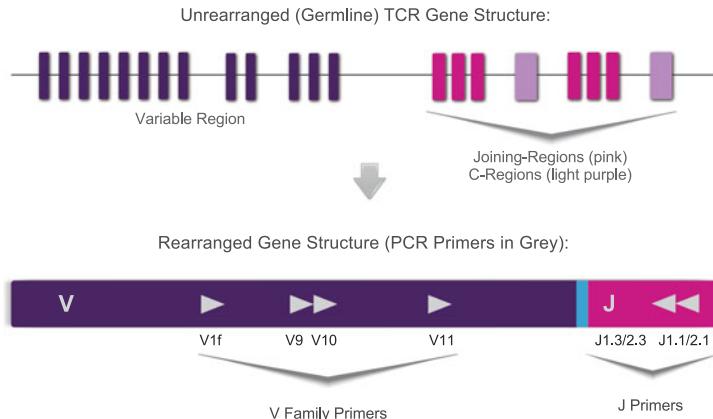
printed in black for better visualization. The five different products can be accurately distinguished from each other based on their color and size of the PCR fragments. Analysis of the normal tissue demonstrates PCR product peaks in a relatively Gaussian distribution for each mononucleotide microsatellite marker. Analysis of the DNA extracted from tumor tissue demonstrates novel peaks not seen in the analysis of the normal. In this case, all of the microsatellite markers are shifted, resulting in PCR products that are smaller in size than the normal (arrows), which is typical. This case would be interpreted as positive for MSI, or MSI high

assays are very similar. The following discussion focuses on T-cell gene rearrangement but is largely applicable to B-cell rearrangement analysis as well. T-cell gene rearrangement assays typically focus on either the  $\beta$ - or the  $\gamma$ -chain for several reasons including easier assay design and a superior sensitivity for detecting clonal populations. During the process of TCR- $\gamma$  rearrangement, variable (V) and joining (J) regions are selected and are joined together to form the

antigen-recognition part of the functional molecule. Because it is not possible to use a single primer set to detect all of the possible V-J combinations, multiple primer sets are typically used in multiplex PCR reactions. Using CE, the products from different primer sets can be differentiated based on the size and color of the PCR product [24, 25] (Fig. 3.10).

Interpretation of CE data for TCR gene rearrangement can be challenging and may differ

**Fig. 3.10** Schematic of TCR assay design. Unrearranged (germline) TCR genes have multiple variable (V, purple) and joining (J, pink) regions. During normal T-cell maturation, the gene undergoes rearrangement in which a single V and J region are brought together. Multiple forward and reverse primers are used to target the different V and J regions, respectively



from one lab to the next [26]. In monoclonal populations, all cells carry the exact same gene rearrangement, producing either one or two distinct peaks of amplification products depending on whether one or both TCR or alleles underwent rearrangement. In polyclonal populations, cells have a variety of gene rearrangements that result in PCR products of varying sizes, roughly distributed in a “normal” or Gaussian distribution. Interpretation of these results is straightforward. But how does one interpret what appears to be a mixture of clonal and polyclonal cells (Fig. 3.11)? Interpretation of these cases is much more challenging. The intensity of the peak relative to the polyclonal population roughly correlates with the percentage of cells in the population that are clonal. This raises the question, what percentage of clonal cells is clinically significant, 10, 20, or 50 %? Different clinical labs will have different interpretation criteria for these types of cases. As one interprets the data, it is important to keep in mind that the function of immune cells is to react and proliferate upon antigen stimulation. Reactive processes can have clonal populations of B and/or T cells [27, 28]. Data should always be interpreted in the context of the specimen type, clinical history, and other laboratory findings.

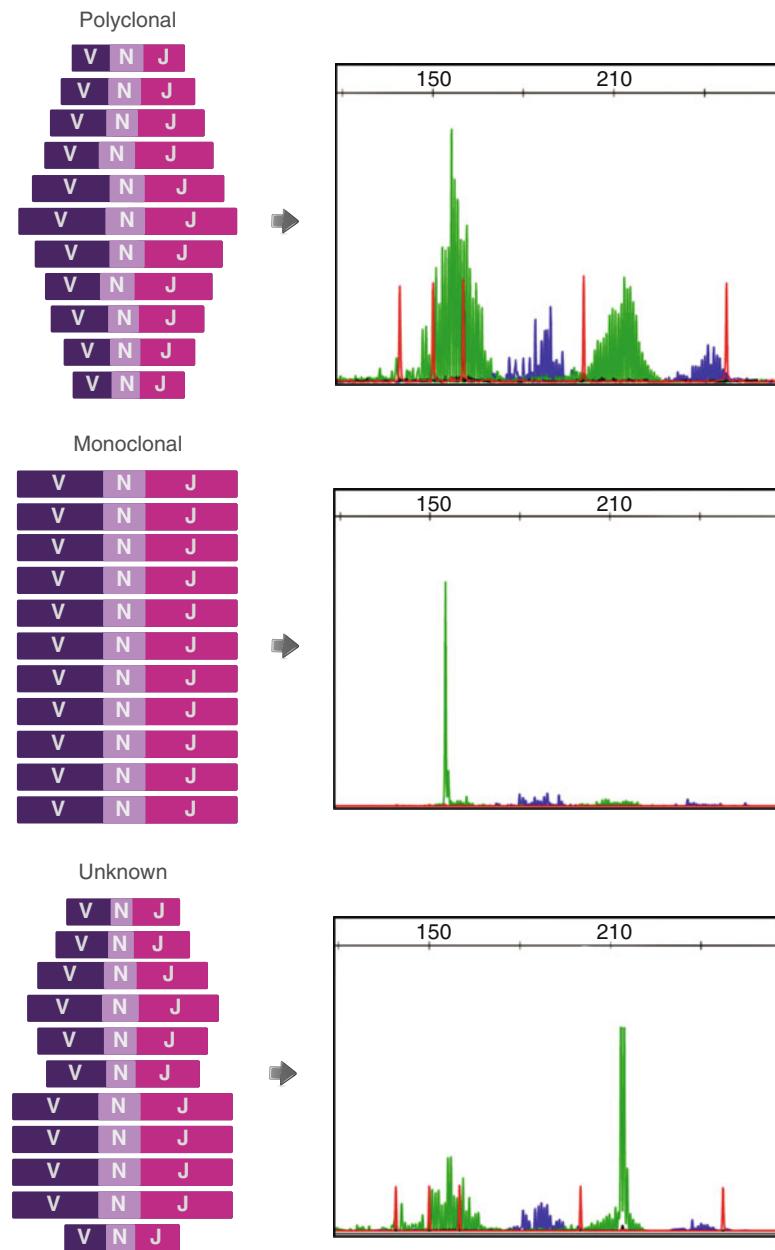
### 3.5.4 Real-Time PCR

Another reason for the decline in use of gel-based electrophoresis is that advancements in PCR,

such as real-time PCR, allow for the PCR product to be detected and analyzed “in real time” (during thermal cycling) rather than requiring a separate analysis step following amplification [29, 30]. Although many variations of PCR have been developed, probably the most significant is real-time PCR. Note that real-time PCR should not be abbreviated as RT-PCR, as that designation is used for reverse transcription PCR. Real-time detection can be achieved by the addition of either a DNA-binding dye or a specific, complementary probe(s) to the PCR reaction. The reaction must be thermal cycled on an instrument that can detect the generation of fluorescent signal during each round of amplification. Although DNA-binding dyes are inexpensive, they are also nonspecific and are therefore not frequently used in clinical laboratories.

Real-time PCR provides several advantages over standard PCR. An important advantage is that it is quantitative, as opposed to standard PCR which can generally only be used for qualitative (positive/negative) applications. By evaluating the PCR products as they are produced, rather than at the endpoint of thermal cycling, the quantity of the starting DNA target can be determined. Quantification across a large dynamic range, such as 5 logs, is readily achievable. In addition, because real-time PCR products are produced and detected simultaneously, it is faster than standard PCR methods. Importantly, real-time PCR reaction tubes are not opened after amplification, reducing the risk for carryover contamination in

**Fig. 3.11** Cartoon and capillary electrophoresis data representing a polyclonal, monoclonal, and questionable population of T cells. During TCR gene rearrangement, variable (V, purple) and joining (J, pink) regions are selected. In the process of joining these two genetic regions, nucleotides may be gained or lost (N region). In the capillary electropherograms, the x-axis is size in bases. Y-axis is fluorescence intensity. Red peaks are size standards. Green and blue peaks are TCR PCR products. Polyclonal populations generate different size PCR products resulting in a Gaussian distribution (top panel). In monoclonal T-cell populations, all of the PCR products are exactly the same yielding a single peak (middle panel). Mixed clonal and polyclonal populations (bottom panel) are more difficult to interpret

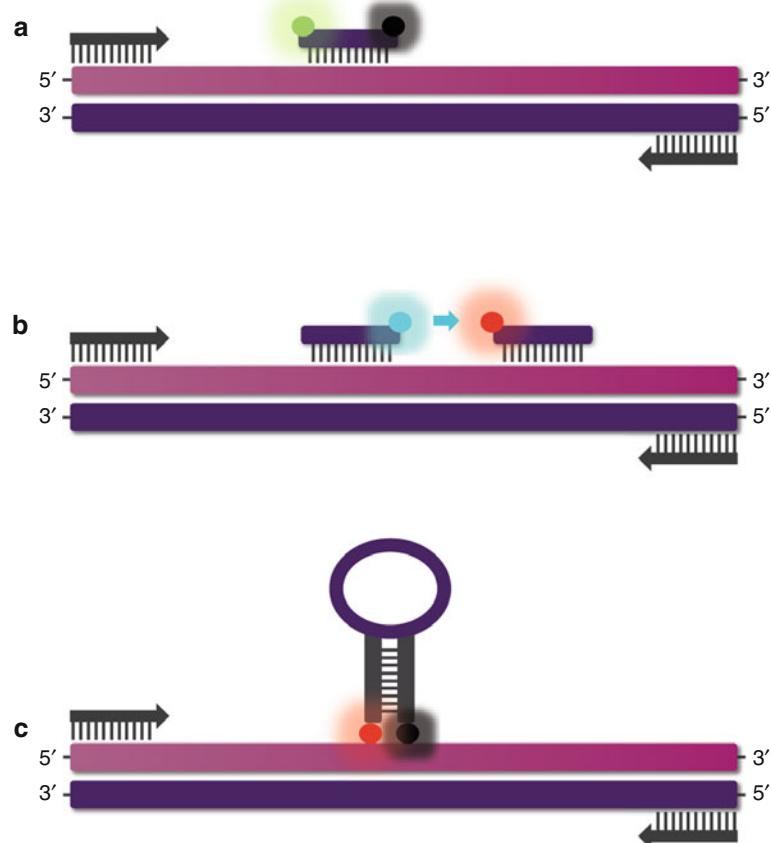


the lab. Even assays not requiring quantification often employ real-time PCR because of its speed, accuracy, and reduced risk for contamination.

The probe design and instruments used for real-time PCR come in several different varieties. TaqMan probes, also called dual-labeled hydrolysis probes, have a fluorescent molecule on one end and a quencher molecule on the other (Fig. 3.12). The DNA sequence is

complementary to DNA sequence within the region being PCR amplified. During each round of PCR amplification, the probe hybridizes to the target and is then degraded as the primers are extended to produce amplification products. Degradation of the probe releases the fluorescent molecule from proximity to the quencher, producing fluorescence. The amount of fluorescence is proportional to the amount of

**Fig. 3.12** Real-time PCR probe design. TaqMan probe (a). While probe is intact, no fluorescence is generated because of proximity of the fluorophore (green) to the quencher molecule (black). During the extension step of thermal cycling, the probe is destroyed, releasing the fluorophore. FRET probes (b). The red fluorophore is excited by light from the blue fluorophore. Only when the two probes are hybridized to the same DNA strand is fluorescence produced from the red fluorophore. Molecular Beacon probe (c). When the probe is not hybridized to the target, the fluorophore (red) is in close proximity to the quencher (black), no fluorescence is generated. When the probe hybridizes to the target, the stem-loop structure is released and the fluorophore is no longer in proximity to the quencher



**Table 3.7** Comparison of two real-time PCR methods for detection of BRAF mutations

Assay	Assay design	Real-time PCR instrument	Mutations detected	Limit of detection
cobas 4800 BRAF V600 Mutation Test, Roche	Real-time PCR using TaqMan probes	cobas 4800	V600E Cross-reacts with V600D, V600K, and V600E2	V600E: ≤5 % V600D: 10–20 % V600K: 30–35 % V600E2: 65–70 %
THxID BRAF kit, bioMérieux, Inc.	Real-time PCR with amplification refractory mutation system (ARMS) primer design (see Table 3.6) and TaqMan probes	ABI 7500 Fast Dx	Detects and differentiates V600E and V600K Cross-reacts with V600E2 and V600D	V600E and V600K: ≤5 % V600E2 and V600D: ≤5 %

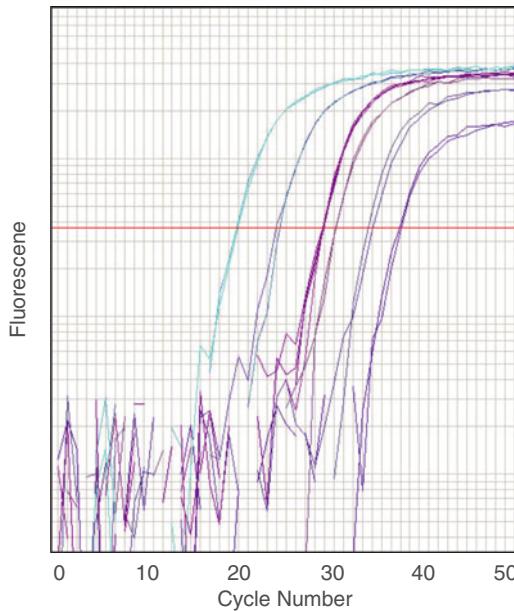
amplification products produced. Table 3.7 compares two FDA-approved tests for the detection of *BRAF* mutations [31, 32]. Note that both methods use TaqMan® probes, but one method uses allele-specific PCR primers that allow V600E and V600K mutations to be distinguished.

Fluorescence resonance energy transfer (FRET) probe designs involve two probes that are complementary to sequences adjacent to one another on the same strand of DNA. One probe has a fluorescent molecule on the 3' end, which is excited by the light source in the real-time PCR instrument. The second probe has a

fluorescent molecule on its 5' end. It is not excited by the instrument light source, but is excited by the wavelength of light that is produced from the fluorescent molecule on the other probe. Thus, fluorescence from the second molecule is only generated when it is in close proximity to the first (i.e., when both probes are hybridized to the same strand of DNA). During each PCR cycle, the probes hybridized, and the amount of fluorescence generated is proportional to the amount of target sequence present. Because the probes are not degraded during PCR thermal cycling, as are TaqMan probes, FRET probes can be used to perform a melt curve analysis after thermal cycling is complete. Melt curve analysis involves starting at a low temperature such that both probes are hybridized to the target, generating fluorescent signal. Fluorescent signal is monitored as the temperature is slowly increased. At the melting temperature of one of the probes, the fluorescent signal will decline due to the probe melting off the target sequence. The temperature at which fluorescent signal is lost provides information about whether the probe was perfectly complementary to the target sequence or if a mismatch(s) was present. This provides additional data about the sequence of the region that is amplified and can be used to ensure specificity of the reaction.

Other types of probes, such as Scorpions and Molecular Beacons, share a similar design strategy. Both involve a fluorescent molecule and a quencher molecule positioned on a DNA probe in close proximity due to a stem-loop structure formed by complementary bases within the probe sequence. While in its native state, there is no signal from the fluorescent molecule because of proximity to the quencher. When the probe hybridizes to its complementary target, the stem-loop structure is released, moving the fluorescent molecule away from proximity to the quencher and producing fluorescence.

Regardless of the probe design, real-time PCR data are typically plotted as shown in Fig. 3.13. Data are presented as PCR cycle number on the x-axis and a measurement of fluorescent intensity on the y-axis. Each reaction is represented by a



**Fig. 3.13** Real-time PCR data. X-axis is cycle number. Y-axis is fluorescence intensity. The red line is the threshold. Each curve (green, blue) represents a single reaction. The results for each reaction are described as the cycle number at which the curve crossed the threshold ( $C_t$  value). The  $C_t$  value is inversely proportional to the starting quantity of the DNA target

line. Specimens that contain the target sequence of interest will demonstrate a typical logarithmic amplification pattern that plateaus when one or more of the reaction constituents (primers, dNTPs, or DNA polymerase) are exhausted. A threshold is set that crosses the curves during the logarithmic phase of amplification. The result for each individual reaction is expressed as the cycle at which the fluorescence crosses the threshold (cycle threshold,  $C_t$ ). The higher the cycle at which the threshold is crossed, the lower the concentration of the target in the starting material. Thus, there is an inverse correlation between the  $C_t$  value and the concentration of the target in the specimen: high  $C_t$  value = low concentration, and low  $C_t$  value = high concentration. Although the software will generate a result for each specimen (positive or negative, and quantity if applicable), the amplification plot should always be evaluated to ensure proper placement of the threshold,

appropriate logarithmic amplification curves, fluorescence within the expected range, and appropriate baseline settings.

### 3.5.5 Other Amplification Methods

Other amplification methods use some combination of hybridization and enzymatic activity (polymerase, nuclease, ligase) to detect target sequences. These methods were developed as alternatives to PCR, and similar to PCR, most of them are highly encumbered by intellectual property. Because the design of these methods can be more tedious and cumbersome than PCR, these methods are not frequently used in research labs or in lab-developed tests. Instead, these methods are often found in commercially available assays, primarily for infectious agent targets.

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## 3.6 Sequencing

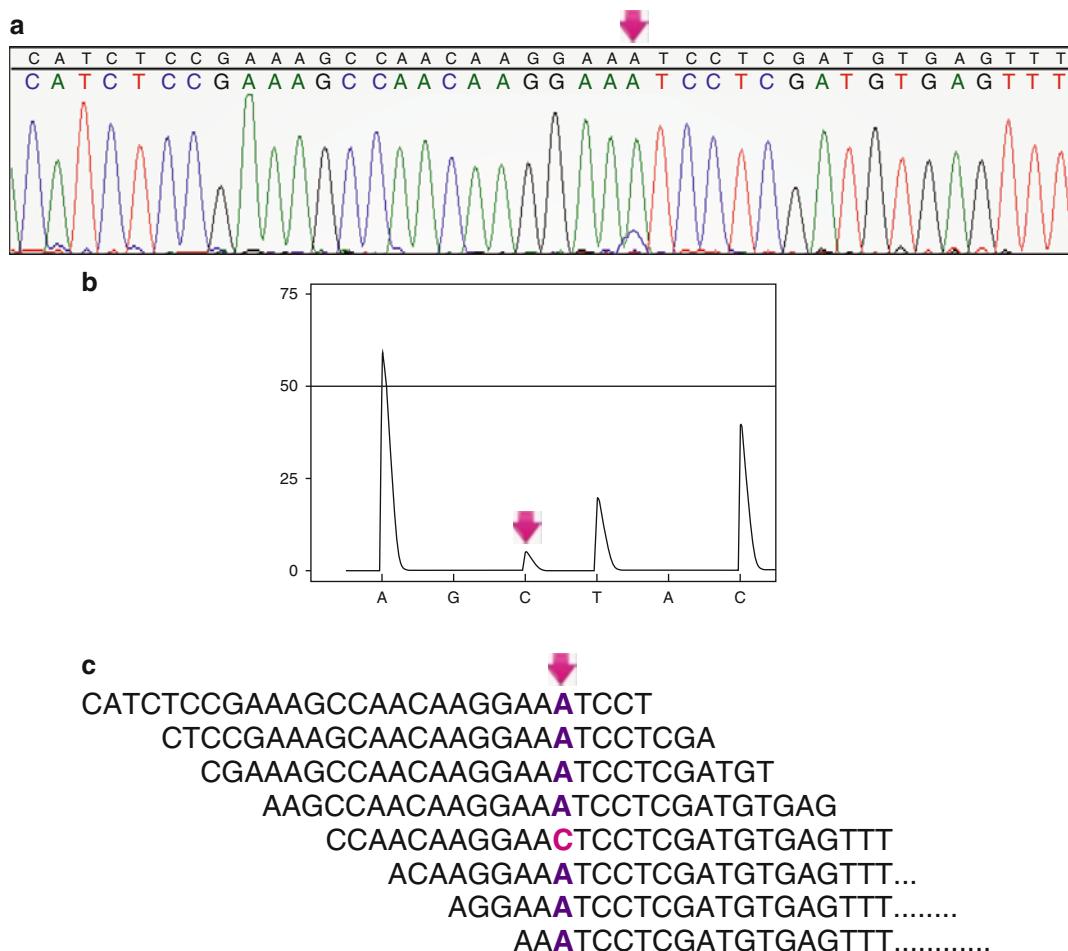
Sequencing DNA generally requires amplification of the target prior to performing a sequencing reaction. Amplification is usually carried out by PCR or a variant of PCR. Sequencing applications are most useful when there is the potential for many different sequence variants. Thus, sequencing is commonly used for “discovery” experiments to identify new mutations and to diagnose genetic diseases caused by many different mutations (see Chap. 10). If the alteration of interest is a known single base substitution, other molecular methods are probably more cost-effective. The limitations of sequencing include its inability to detect large deletions and insertions and a relatively poor limit of detection compared to other methods (Sanger sequencing, specifically).

### 3.6.1 Sanger Sequencing

Sanger sequencing, named for its inventor Frederick Sanger, has been the most widely used sequencing method for approximately 30 years. Sanger sequencing was used to generate the first

sequence of the human genome, and it is still widely used in clinical and research laboratories. Although there are several variations of the method, the “dye terminator” approach is by far the most common. The method involves the use of a DNA template, which is often a PCR product, a DNA primer, DNA polymerase, and dNTPs as well as specially modified dNTPs, 2,3 dideoxynucleotide triphosphates, or simply ddNTPs. ddNTPs lack a hydroxyl group at the third carbon position of the deoxyribose molecule. Once incorporated into a strand of DNA, ddNTPs do not allow additional dNTPs or ddNTPs to be added. For this reason, these are often called chain terminators. Each of the four different ddNTPs (ddATP, ddCTP, ddGTP, ddTTP) is labeled with a different fluorescent molecule. Typically ddATP is labeled with green, ddCTP with blue, ddGTP with yellow, and ddTTP with red. The reaction is thermal cycled, similar to PCR, to allow the primer to bind to the DNA template and the DNA polymerase to synthesize a new strand of DNA by incorporating dNTPs or a ddNTP. When a ddNTP is incorporated, synthesis of the new strand is terminated. DNA fragments are produced that incorporate a ddNTP at each different base position of the template DNA. The result is a population of DNA fragments that differ by one base in length and are differentially fluorescently labeled based on the terminal ddNTP. The products are analyzed by capillary electrophoresis, which can separate the fragments and determine the fluorescent label attached to each (Fig. 3.14). A computer algorithm interprets the fluorescent peaks to generate the four-letter sequence. The algorithm can be adjusted to be more sensitive (detect low-level mutations) but at the expense of reduced specificity (more false positives). One should always visually inspect the electropherogram to ensure that the base calls made by the computer algorithm are correct.

The major disadvantages of Sanger sequencing have been cost, analysis speed, and an insufficient lower limit of detection. The high cost of Sanger sequencing results largely from the use of fluorescent molecules to label the sequencing products.



**Fig. 3.14** Sequencing data generated by three different sequencing methods. The *red arrow* identifies a mutation at a relatively low allele frequency. Sanger sequencing (a). Pyrosequencing (b). Next-generation sequencing (c)

The speed of Sanger sequencing is relatively low because only one sequence is generated in each capillary. This problem resulted in the creation of capillary sequencers with 96 or more capillaries, which could analyze sequencing reactions in parallel. These instruments greatly improved the speed of Sanger sequencing, but speed is relative. Using an instrument that could analyze a million bases of sequence per day, sequencing of the entire three billion bases of the human genome would take well over 5 years. Finally, the limit of detection of Sanger sequencing can be insufficient for applications that involve heterogeneous populations of DNA. The limit of detection in most Sanger sequencing applications is approximately 20 % mutant alleles. Thus, at least 40 %

of the cells present in the sample must contain the sequence abnormality in order for it to be detected. There is a significant risk of false-negative results when using Sanger sequencing to evaluate heterogeneous populations such as viral populations and tumor specimens. The limitations of Sanger sequencing have been largely overcome with next-generation sequencing methods (described later).

### 3.6.2 Pyrosequencing

Pyrosequencing, a “sequencing by synthesis” method, was developed in the mid-1990s [33–36]. It is similar to Sanger sequencing in that it

requires a DNA template that is often a PCR amplification product, a DNA primer, and dNTPs. In contrast to Sanger sequencing, pyrosequencing relies on the detection of a light signal that is generated when a dNTP is incorporated into a strand of DNA rather than the use of fluorescently labeled ddNTPs. Because fluorescent molecules, and therefore an instrument capable of fluorescence excitation and detection, are not required for pyrosequencing, it is generally less expensive than Sanger sequencing. Pyrosequencing requires that each dNTP (dATP, dTTP, dCTP, dGTP) is added to the reaction one at a time. The base that is incorporated into the DNA strand is identified by the light signal it produces, while bases that are not incorporated will not generate signal.

Pyrosequencing has a much better limit of detection (approximately 5 %) than Sanger sequencing (approximately 20 %) (Fig. 3.14). This is due in part to the fact that pyrosequencing analyzes each position for each potential base independently, compared to Sanger sequencing which analyzes each position for all four bases simultaneously. Pyrosequencing is also less expensive because sequence is detected by the generation of a light signal rather than by fluorescent molecules.

Small pyrosequencers are used in clinical laboratories to sequence short regions of DNA to detect a number of DNA alterations such as *BRAF* V600E and mutations in the *NRAS* and *KIT* genes (see Chap. 5). Pyrosequencing technology has been incorporated in a next-generation sequencing platform called 454 (see below), which can generate 400 million bases of DNA sequence in about 10 h.

### 3.6.3 Next-Generation Sequencing

As this book goes to print, next-generation sequencing (next-gen sequencing, or NGS) is poised to replace many, if not all, of the methods described above—not just the sequencing methods but essentially all methods.

What is next-gen sequencing? There is no official definition of “next-generation” sequencing, but it loosely refers to high-throughput sequencing methods other than Sanger

sequencing. These methods involve generating very large numbers (thousands to millions) of relatively short sequencing fragments simultaneously, often referred to as “massively parallel.” The sequences are then aligned to a “standard” sequence using a computer algorithm. Areas of the genome are “covered” multiple times, which is referred to as the depth of the sequence. The term “deep sequencing” does not have a specific definition, but generally refers to sequence covered at least 7X. Ultra-deep sequencing involves coverage of a sequence target at  $\geq 100$ -fold. Figure 3.14 depicts multiple short reads generated by next-gen sequencing that have been aligned to identify overlapping regions. In this example, the sequencing depth is eight-fold at the position identified by the arrow. Multiple fold coverage is needed to ensure that the sequences can be aligned correctly, to identify mutations that occur at a low frequency, and to assess for sequencing errors. It also results in two major advantages for next-generation sequencing. Because each sequence is read independently and then aligned, next-gen sequencing has a superior limit of detection (<10 %) compared to Sanger sequencing (approximately 20 %). In addition, the multiple reads allows next-gen sequencing to be quantitative or at least semiquantitative. Algorithms have been generated to “count” the frequency at which specific DNA sequences are generated. This allows next-gen sequencing to be used for copy number changes such as gene amplifications or deletions, alterations which typically cannot be detected by Sanger sequencing.

Table 3.8 gives a brief description of five different next-gen sequencing technologies. The list is not complete. New methods and improvement to old methods are rapidly becoming available. A resource for information on current and upcoming next-gen sequencing methods is available at the AllSeq Sequencing Knowledge Bank, which is a collection of continuously updated next-gen sequencing information, opinions, evaluations, and news ([www.allseq.com/knowledgebank/home#](http://www.allseq.com/knowledgebank/home#)). Each next-gen sequencing method has advantages and disadvantages in terms of time, cost, accuracy, and throughput [37]. The chemistries used in each method are significantly different, but they do have some commonalities.

**Table 3.8** Next-generation sequencing methods

Name	Vendor	Description
454	Roche	Emulsion PCR followed by massively parallel pyrosequencing
SOLiD	Life Technologies/Applied Biosystems	Emulsion PCR followed by sequencing by ligation
HiSeq and MiSeq	Illumina (Solexa)	Sequencing by synthesis. Capture of target sequences by immobilized probes. Extension with reversible dye terminators. Image capture
Ion Torrent	Life Technologies/Ion Torrent Systems	Standard sequencing chemistry with detection of hydrogen ions released during addition of a base to a strand of DNA
Single molecule real time (SMRT)	Pacific Bio	Single molecule real-time sequencing

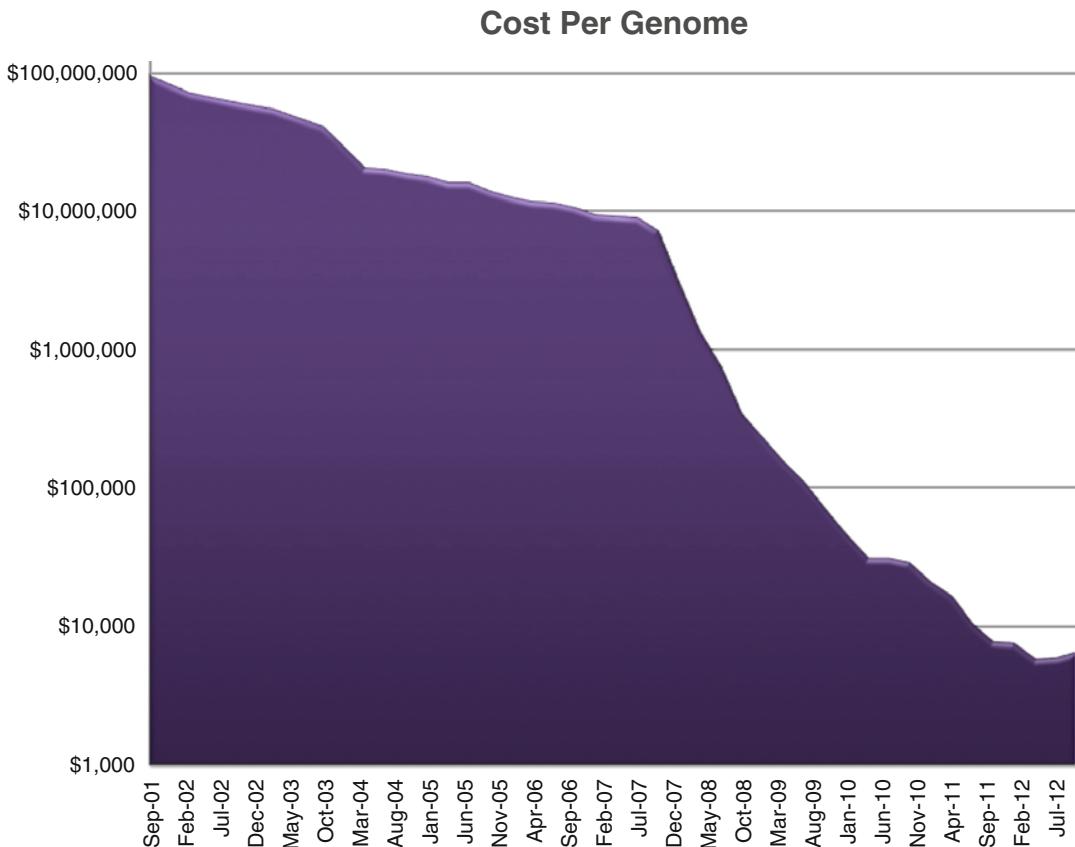
As opposed to Sanger sequencing, some of the next-gen sequencing methods rely on light or pH changes for detection methods rather than fluorescence. This is one of the reasons they are significantly less expensive than Sanger sequencing. In addition, all of the next-gen sequencing methods use very small reaction volumes, which help manage reagent costs. Two of the next-gen sequencing methods use emulsion PCR to generate products for sequence analysis. Emulsion PCR relies on the capture of single DNA molecules within a water-in-oil emulsion “bubble.” Within each emulsion, a single DNA molecule is PCR amplified independently of the others. The amplification products from each can be isolated and analyzed individually. This is essentially equivalent to running thousands of PCR reactions each with a single DNA starting molecule. Advantages of emulsion PCR include the elimination of PCR competition between DNA strands and the ability to detect rare events or low-frequency mutations.

An important point to remember is that all of the data generated from next-gen sequencing methods are “interpreted” by a computer algorithm. Certain types of mutations such as rare occurrence and translocations can be difficult for the algorithm to interpret. Strengths and weaknesses of individual algorithms will vary. As with any test, the algorithm can be adjusted to be more sensitive but less specific (generates false positives) or less sensitive but more specific (generates false negatives). An additional problem is that the sequence generated is aligned to a reference standard that is not absolute.

Differences identified between an individual and the reference sequence must be analyzed to determine if they are likely to be disease causing.

Why is next-gen sequencing so appealing? As a single technology, next-gen sequencing has the potential of detecting virtually all types of mutations and alterations discussed above: point mutations, insertions, deletions, amplifications, etc. It can detect all of these alterations simultaneously and therefore more quickly and with a limit of detection that is equivalent or superior to most of the methods described above. Assays can be designed to sequence the whole genome, the whole coding portion of the genome (sequencing all exons, whole-exome sequencing), or fewer, targeted genome regions of interest. In a single next-gen sequencing reaction, we can detect alterations in *NRAS*, *BRAF*, *KIT*, and many other genes in DNA isolated from melanoma specimens (see Chap. 5). In addition, the development of next-gen sequencing has led to a massive reduction in sequencing cost. Figure 3.15 demonstrates the cost to sequence a human genome over the period from September 2001 until September 2012. Note that the y-axis is a log scale, which is needed to demonstrate the reduction from almost 100 million dollars (\$100,000,000) in September 2001 to the current cost of approximately 7,000 dollars (\$7,000). Also note that the sudden and rapid decline in cost beginning in January 2008 is due to sequencing centers transitioning from Sanger sequencing to next-generation DNA sequencing technologies.

Although whole-genome sequencing (WGS) is beyond the scope of this chapter, it is worthy of



**Fig. 3.15** Cost to sequence a human genome. Data are presented from the period of time covering September 2001 until September 2012. Note that the y-axis is a log scale. The sudden and rapid decline in cost beginning in

January 2008 is due to sequencing centers transitioning from Sanger sequencing to next-generation DNA sequencing technologies

a few comments. Next-gen sequencing has made WGS a reality. While the potentials are huge, the pitfalls are many. One important caveat is that WGS, by any technology, does not truly cover the whole genome. Some areas of the genome, including some gene-coding regions, are covered, but not in a high enough density to be confident of the data, and some regions are not covered at all due to technical limitations. Initial clinical applications will likely focus on whole-exome sequencing because of the reduced cost and reduced number of variants of uncertain significance that it identifies. Less than 5 % of the genome is exons that code for specific proteins. This 5 % is enriched for variations that are likely to be of clinical significance. Looming questions for WGS include: Who is going to interpret the

test results? How will results be interpreted? How will results be reported to clinicians? to patients? While it is important to understand the science and technology involved in next-gen sequencing, perhaps more important is understanding the statistical analysis that is performed on the millions of data points and how that data translates into a biologic setting. A recent commentary noted that interpretation of WGS data required molecular and computation biologists, geneticists, pathologists, genetic counselors, and IT support specialists among others [38]. This led the author to muse that although we may soon be able to perform WGS for \$1,000, the data analysis may cost logarithmically more than that.

What do we do with all the information that is generated? NGS generates large amounts of

sequence information, only some of which will be diagnostic. Secondary, incidental findings will be regularly generated. An example is finding an unanticipated variant likely to cause disease in the future. Should this result be reported, and to whom? The American College of Medical Genetics and Genomics has recently released their reporting recommendations, which will likely evolve over time (*ACMG Recommendations for Reporting of Incidental Findings in Clinical Exome and Genome Sequencing*, [www.acmg.net](http://www.acmg.net)). An additional challenge is that the significance of some of the sequence data generated will not be known for years. We are still learning which alterations are benign genetic variants (polymorphisms), which increase risk for disease, and which are disease causing. And we are in our infancy with understanding which alterations predict response or resistance to the drugs currently in use and the large number of targeted therapies in the pharmaceutical pipelines.

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### 3.7 Practical Considerations

#### 3.7.1 What to Look for in a Laboratory and/or Test Result

There are relatively few FDA-approved molecular tests outside of high-volume infectious disease applications. Many molecular tests are lab developed and lab validated. Therefore, it is important to have confidence in the laboratory you select for analysis of clinical specimens. Molecular diagnostics is changing rapidly, making it difficult to know the most appropriate molecular test(s) to order. Some assays are appropriate for certain diseases or drugs, while others are not. For example, detection of *EGFR* mutations is useful to predict response to the EGFR-targeted small-molecule inhibitors erlotinib (Tarceva) and gefitinib (Iressa) in lung cancer patients, but it does not predict response to the EGFR-targeted monoclonal antibody therapies cetuximab (Erbitux) and panitumumab (Vectibix) in patients with colon cancer. Ensuring that the most appropriate test(s) is ordered is essential for

patient management and cost control. Your laboratory should be a resource for you. Not only should you be able to call the lab with questions about which test(s) to order, but you should expect a call from the lab if the test ordered does not seem appropriate for the specimen type and/or clinical scenario.

As mentioned above in Sample Considerations, when testing for somatic alterations, it is very important to ensure that the specimen is sufficient and appropriate for testing. Review by a pathologist is crucial for accurate analysis. There will be times when a test cannot be performed because the sample is insufficient either due to low tumor density or degradation of nucleic acids. A lab that does not reject some samples is not doing its job. Rejection of a sample is preferred to reporting a result that may not be accurate. Similar to other aspects of pathology, results can, in part, be subjective. It is important that a highly trained and experienced individual interpret molecular test results. Lab personnel should be available for questions regarding test methods, potential interfering substances, risk for false-positive or false-negative results, etc.

Laboratory reports should convey patient results effectively to nonexpert physicians. The College of American Pathologists requires that the final report includes a summary of the methods, the loci or mutations tested, the analytical interpretation, and the clinical interpretation if appropriate. The method summary should clearly describe the test performed and whether the test is lab developed. “Analytical interpretation” means examining the raw data to reach a conclusion about the quality or quantity of the target. Genes, loci, and mutations should be designated according to standard nomenclature. At times this may be confusing, as gene nomenclature has undergone some changes over time. For example, the gene commonly referred to as *HER2* has been designated *ERBB2*. In cases like this where a common name is widely in use in the medical literature, it should also be given in the report to improve clarity and prevent misunderstanding. One approach is to put the common name in the diagnosis section of the report and the standard name in the method or

interpretation section. The “clinical interpretation” involves reaching a conclusion about the implications of the result for the patient. The clinical interpretation may be stated in general terms, or may be specific to an individual patient’s situation, and should include a discussion of the limitations of the findings. For assays performed on histology samples, the interpretation should include correlation with the morphologic findings, if applicable. Knowledgeable laboratory personals should be available for questions regarding test results.

### 3.7.2 Costs and Reimbursement

Cost can be an important consideration when deciding what test methodologies are feasible for a given application. The cost of a test includes instrument and reagent costs, technologist time, and overhead. The costs of molecular tests have significantly decreased over the last few decades primarily due to decreases in instrument and reagent costs and the reduction of technologist time attributed to automation. The most cost-effective tests tend to be high-volume testing, which primarily are infectious disease tests. Oncology and genetic tests are generally much more complex and low volume, limiting their cost-effectiveness. A factor keeping the cost of some molecular tests higher is the intellectual property/patents surrounding a gene sequence, the association of mutation(s) to disease or therapy, and/or the specific methodologies for mutation detection. Fortunately, the Supreme Court ruling in June of 2013 that human genes and the information they encode cannot be patented will likely provide some relief. The justices’ decision in *Association for Molecular Pathology v. Myriad Genetics* reversed decades of federal patents and will likely result in increased availability of genetic tests and reduced pricing.

Potential and realized reimbursement for tests must also be considered. Insurance companies and other payers have been slow to appreciate and value the significance of molecular testing and to understand when reimbursement is appropriate. It is not surprising given the reimbursement

system and environment. For common laboratory tests, the American Medical Association (AMA) assigns current procedural terminology (CPT) codes that are used to determine reimbursement. A CPT code for a glucose test is reimbursed at the same rate regardless of what instrument was used to generate the test result. Until 2012, CPT codes for specific molecular assays did not exist. Instead, CPT codes for different steps of the testing methods were “stacked” together. Thus, one lab performing a *BRAF* test would “stack” the CPT codes for DNA extraction, PCR amplification, and Sanger sequencing. (Note that this code stack may be used for several different molecular tests [*KRAS*, *BRAF*, *PIK3*, etc.].) Another lab performing a *BRAF* test by a different molecular method might stack the CPT codes for DNA extraction, multiplex PCR amplification, and detection by fluorescent probe. Both labs performed and charged for a *BRAF* test but submitted different sets of CPT codes for reimbursement, neither of which identify the test as *BRAF*. No wonder the payers are confused! In 2013, the AMA finally implemented specific CPT codes for some of the most common molecular tests. For molecular tests not included on the list, however, the CPT coding is still vague, and reimbursement will be challenging.

An additional obstacle for reimbursement is the need to maintain current knowledge about what molecular testing is truly needed for patient care. The amount of time from initial discovery of a genetic alteration to the accumulation of sufficient data to demonstrate clinical utility has shortened dramatically. However, with reimbursement somewhat dependent on the recommendations of national or international society guidelines and with some groups updating guidelines only every 2–3 years, authority on appropriate use of proven testing regularly lags. Thus, while significant data demonstrating the utility of a test may be available for years, with the test not incorporated into guidelines, insurance companies and other payers believe they are justified in not reimbursing for the test. On this front, action is needed by laboratories and clinicians to communicate with payers. Laboratories need to provide scientific data

demonstrating the analytical and clinical performance of the test. Clinicians need to provide examples of how the test results affect patient management. Reimbursement will continue to be a problem using the current system, and unfortunately, it may inevitably hamper providing state-of-the art personalized medicine to patients.

### 3.8 Summary and Looking Ahead

Molecular methods are rapidly advancing. Although all are based on a few basic elements, including hybridization and enzymatic reactions, the exact combination of these elements and the conditions under which they are carried out results in assays with specific strengths and weaknesses. Regardless of the method, the composition and quality of the specimen will affect test results. The testing algorithm must include a pathologist with training in molecular diagnostics.

Many of the advancements in molecular testing involve the ability to multiplex and miniaturize testing to allow thousands of results to be generated in a single reaction. The results of these tests are generally interpreted by a computer algorithm. Review of the primary data, if possible, is needed to ensure the accuracy of the results. Clearly there will be an increasing demand for computer programmers, statisticians, and genetic counselors to ensure accurate analytical interpretation of the thousands of data points, accurate clinical interpretation of the data, and clear communication of the results to the physician and patient.

Not surprisingly, improvements in technology have outpaced our understanding of the potential impact(s) of genetic variants for an individual. For example, we have known for well over a decade that tumors have mutations in the *BRAF* gene, yet it was only in the last few years that this became relevant to patients as drugs were developed to target this specific genetic abnormality. We will soon have the ability to sequence an individual's entire germline genetic constitution and/or their entire tumor genetic constitution within a reasonable

cost and time. The data generated will facilitate research studies to determine the clinical significance of molecular alterations including their diagnostic, prognostic, and therapeutic potential.

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# Melanoma. Part I. Risk Assessment, Diagnosis, and Prognosis: Using Molecular Tools to Diagnose Melanoma, Predict Its Behavior, and Evaluate for Inheritable Forms

## Contents

4.1	<b>Introduction.....</b>	64
4.2	<b>The Genetics of Melanoma: Assessing Risk.....</b>	66
4.2.1	Loci Associated with Melanoma Risk .....	67
4.2.2	Testing for Germline Mutations.....	70
4.3	<b>Diagnosis.....</b>	71
4.3.1	Comparative Genomic Hybridization (CGH) .....	75
4.3.2	Fluorescence In Situ Hybridization (FISH) .....	77
4.3.3	Mutational Analysis.....	81
4.3.4	Gene Expression Profiling .....	82
4.4	<b>Prognosis.....</b>	82
4.4.1	Molecular Evaluation of the Sentinel Lymph Node .....	84
4.4.2	Chromosomal Aberrations by FISH .....	84
4.4.3	Ocular Melanoma .....	85
4.4.4	Other Molecular Prognostic Biomarkers .....	85
4.5	<b>Practical Considerations for Ordering and Performing Molecular Tests .....</b>	85
4.5.1	Genetic Testing for Familial Melanoma .....	85
4.5.2	CGH Versus FISH.....	86
4.5.3	Mutational Analysis of Melanoma Signaling Molecules and Gene Expression Profiling...	89
4.5.4	Prognostic Testing.....	89
4.6	<b>Classification of Melanoma: Current and Near-Future Perspectives .....</b>	90
	<b>References.....</b>	92

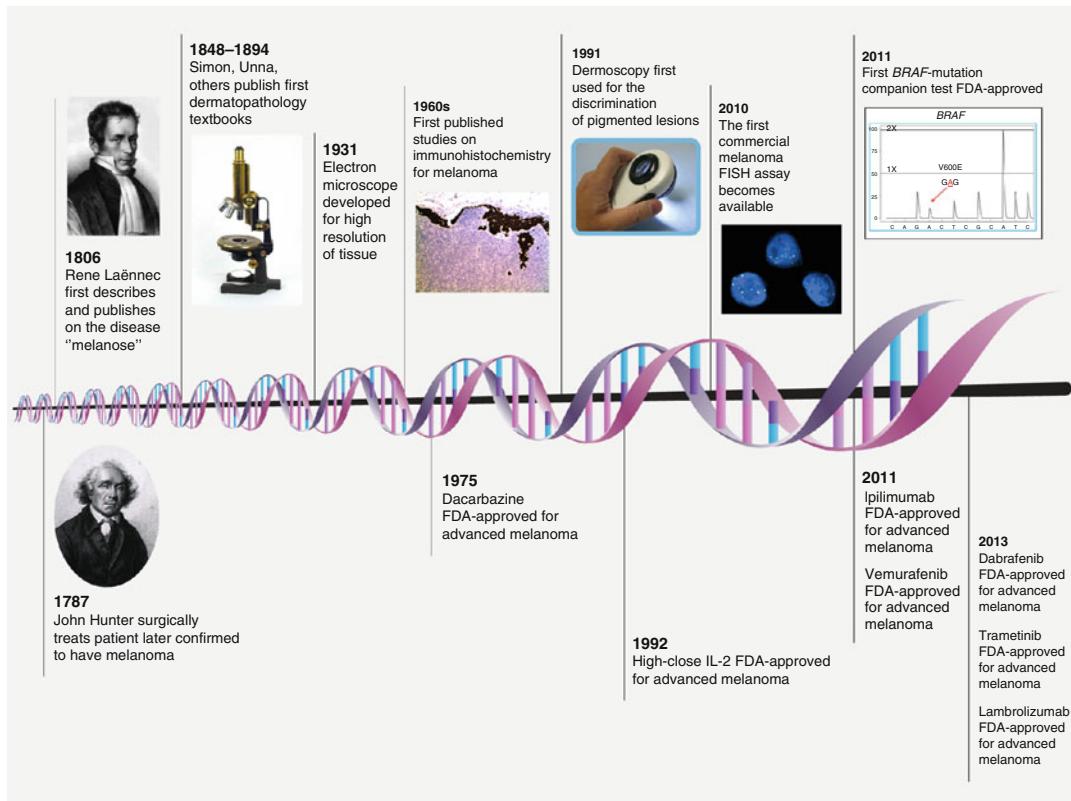
### Key Points

- Melanoma is no longer considered a single entity but a complex, molecularly heterogeneous group of different cancers, similar only in their melanocytic origin.
- Molecular testing is now performed for all stages of management of the melanoma patient: assessment of risk through germline mutational analysis, diagnosis of melanoma by FISH and CGH, assessment of biologic behavior of the tumor and patient prognosis, and determination of eligibility for targeted therapy by tumor mutational analysis.
- Testing for germline (familial) mutations should not be performed on every melanoma patient. Due to assay limitations, testing should be restricted to a select group of patients based on pretest probability (multiple primary melanomas, strong family history, etc.). Testing requires whole blood or buccal swabs.
- FISH and CGH are new available molecular tests for the diagnosis of melanoma. Their use should be restricted to a small subset of histologically ambiguous lesions, and their results should supplement, not trump, conventional histomorphologic interpretation. Testing can be performed from the paraffin block.

- Mutational analysis is useful for determining a patient's eligibility for targeted therapy but has no current role in the diagnosis of melanocytic lesions.
- Molecular evaluation of sentinel lymph nodes, FISH analysis of primary tumors, and gene expression array analysis are all potential applications to better define tumor behavior and patient prognosis, but these are not currently in widespread use for these purposes.
- The molecular heterogeneity of melanoma will undoubtedly shape current and future classification schemes, categorizing tumors based on predicted behavior and response to therapy rather than mere observed histologic variations.

## 4.1 Introduction

When contemplating the evolution of diagnosis and treatment of melanoma, it is hard not to compare it, for example, to walking the geologic timeline at the Smithsonian National Museum of Natural History in Washington, DC. One begins at a standstill at the “Beginning of Time” and then proceeds to slowly stroll from the Precambrian era through the Paleozoic and Mesozoic eras, anxiously anticipating the arrival of one’s own existence on the timeline. Upon arriving, the real estate devoted to the existence of man seems implausibly minuscule. Within this little sliver is an incredible acceleration—a hyperbolic tracing—of change and progress. Changes in the approach to the melanoma patient have similarly accelerated, attributed to recent advances in the understanding of the molecular heterogeneity within melanomas and the development of molecular assays (Fig. 4.1).



**Fig. 4.1** Timeline of the diagnosis and treatment of melanoma. For centuries, there was little change in the diagnosis and treatment of melanoma. Change has accelerated

since the onset of molecular diagnostics. Select events are highlighted

Melanoma, the disease, has existed since at least the fifth century, B.C., with its first archived descriptions by Hippocrates himself [1]. It was first recognized and published in Western scientific literature as a distinct disease, “melanose,” by Dr. Rene Laennec (France, 1806). In this era, the diagnosis of melanoma relied completely upon clinical observations, with published, often colorful, descriptions like “cancerous fungous excrescence” (Dr. John Hunter, UK, 1787), “nutmeg” (Dr. William Norris, UK, 1820), and “purple mark or mole about the size of a mulberry” (Dr. Isaac Parrish, USA, 1837). These descriptions provided a foundation for clinical diagnostic criteria used today. The “soft and black … cancerous fungous excrescence” (retrospectively diagnosed as melanoma) annotated by the Scottish surgeon John Hunter in 1787 was surgically removed from the jaw of a 35-year-old man and provided one of the first documented melanoma treatments. Assessment of risk factors for melanoma can be traced back to the British physician Dr. William Norris. In 1820, he described a family with melanomas and numerous moles, suggesting an inheritable component [2].

Over the next two centuries, the diagnosis of melanoma remained reliant on clinical and microscopic observation, with limited technological advancements. (One of these advancements was the development of a new illumination method for the microscope by August Kohler in the late nineteenth century, roughly coinciding with the first dermatopathology textbooks by Karl Gustav Theodor Simon and Paul Gerson Unna, among others.) Also during this timeframe, there was little change in the sophistication behind risk assessment, and the only effective therapy was surgery. Use of cookbook-like surgical algorithms was the primary treatment modality for localized disease, and with advanced disease, there was no hope for cure. Subdividing melanoma based on anatomic location or minor histologic variations seemed like an academic exercise with little practical importance, prompting the credo that “melanoma is melanoma is melanoma.”

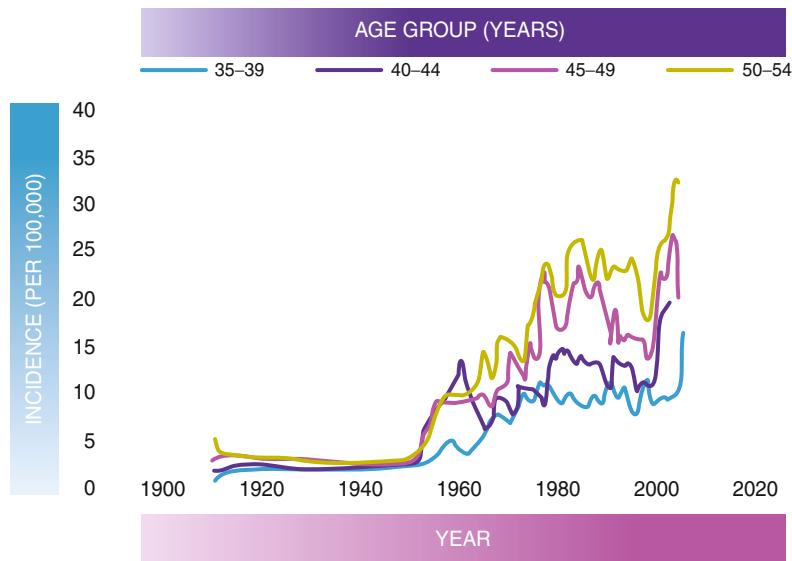
Recent deciphering of melanoma’s genetic underpinnings and signaling pathways, however, is relieving this stagnation and revolutionizing the

complete management of the melanoma patient. Assessment of patient risk is no longer limited to ultraviolet exposure but includes evaluation of the patient’s genome. Diagnosis is no longer limited to clinical and microscopic inspection of the tumor, but includes assessment of the *tumor’s* genome for chromosomal abnormalities and signaling molecule mutations. Prognosis is no longer linked only to tumor size but can be impacted by the amplification of tumor oncogenes and/or the molecular detection of micrometastases. And, finally, treatment is no longer limited to “excise and pray” tactics but can be tailored to the individual, reversing the action of the very mutations that led to melanomagenesis. With these new molecular tools, it has become clear that melanoma is not a single tumor but a complex array of tumors, each with a unique molecular profile, similar only in their genesis within a host melanocyte. Indeed, “melanoma is *not* melanoma is *not* melanoma.”

Today, melanoma remains a pervasive, resilient, and deadly disease. According to the American Cancer Society’s 2013 estimates, there were approximately 76,690 new cases of melanoma diagnosed in the United States with 9,480 deaths. The estimated lifetime risk of developing melanoma is now 1 in 50 [3]. These figures have been on an upward trajectory for the last 60 years (Fig. 4.2). And while over 90 % of patients will achieve a cure, these cures remain almost exclusively surgical and in patients with low-stage tumors, and the survival of the advanced melanoma patient remains unacceptably low. The decryption of the molecular infrastructure of melanoma and the development of novel molecular assays and therapeutic strategies are necessary to improve upon these statistics and have shown initial promise, but this journey remains in its infancy.

In this chapter, the discussion on melanoma begins with the contribution of molecular events in the risk of developing melanoma (i.e., hereditary melanoma). This is followed by a review of new molecular assays used in the diagnosis of melanoma, the use of molecular tools to assess a patient’s prognosis, and potential classification schemes using molecular information. Keep in mind, the molecular diagnostic world is vast.

**Fig. 4.2** Incidence of melanoma. The incidence of melanoma has steadily increased over the past century, for all age groups. In particular, the incidence among women under age 40 has jumped since 1970 (data shown not separated by gender)



There are numerous exciting, and sometimes clever, applications for molecular testing in the context of melanoma, but many of these are currently in the investigative stage. The focus here remains on *practical*, or *current*, applications.

## 4.2 The Genetics of Melanoma: Assessing Risk

Genetic and environmental factors arguably play a role in the epidemiology of any disease, including melanoma. This fact was observed as early as the beginning of the nineteenth century when Dr. William Norris, a general practitioner in the United Kingdom, described a family with numerous moles and melanoma and subsequently described eight melanoma patients, some with light hair, pale complexion, and possible industrial pollution exposure [1, 2]. Since then, a large body of literature weighing risk factors for melanoma has been compiled. With the exception of large numbers of atypical or common nevi and certain germline mutations, which have a fairly high relative risk, most of these factors have a relative risk in the 1.5–2.5 range (Table 4.1) [4, 5].

Approximately 10 % of melanomas are familial, or hereditary, meaning there is a strong family history and/or a documented melanoma-related germline mutation. The majority of

familial melanomas are inherited in an autosomal dominant fashion, meaning the susceptibility is passed directly from one parent to the child, irrespective of gender. Family history is variably defined but typically includes a single first-degree family member or multiple more distant family members affected on the same side of the family. In an individual with a positive family history, the risk varies greatly depending on the circumstances. On average, a positive family history equates to about a twofold risk in developing melanoma. However, patients with a documented *CDKN2A* mutation, with a family history, and who are living in Australia have a 91 % cumulative risk of developing melanoma by age 80 [6]. Identification of individuals who harbor the mutation in the high-risk family has importance because they may develop melanoma at an earlier age, may have a higher risk of developing a second primary melanoma, may have a higher risk for other visceral malignancies such as pancreatic carcinoma, and may pass on their susceptibility gene to their offspring. All of these reasons introduce potential points of intervention, by increased surveillance.

Melanoma susceptibility genes can be broken into high-, moderate-, and low-risk genes, and excellent reviews are available on this topic (see Table 4.1) [7]. Of the high-risk genes, mutations in *CDKN2A* and *CDK4* are the most common. In

**Table 4.1** Risk factors for melanoma

Clinical risk factor	Approximate relative risk <sup>a</sup>
>5 dysplastic nevi	10 RR
Previous melanoma	9 RR
>100 total nevi	7 RR
Red hair	4 RR
Family history	1.5–2.5 RR
1 dysplastic nevus	
16–40 total nevi	
Light skin	
Light eyes	
Light hair	
Freckling	
Poor tanning ability	
Tanning bed use	
History of sunburns	
Genetic risk factor	Risk
<i>High risk</i>	
<i>CDKN2A</i> mutation in high-risk families	0.91 (Australia) <sup>b</sup> 0.76 (United States) <sup>b</sup> 0.58 (Europe) <sup>b</sup>
<i>CDKN2A</i> mutation (unselected)	0.28 <sup>b</sup>
<i>CDK4</i>	(Same as <i>CDKN2A</i> )
<i>RB1</i>	4–80 RR
<i>XP (A-G)</i>	600–8,000 RR
<i>Medium risk</i>	
<i>MC1R</i>	2.7 RR (one allele)
<i>MITF</i>	2.2 RR (p.E318K variant)
<i>Low or unknown risk</i>	
<i>BAP1</i> , <i>TERT</i> , <i>XRCC3</i> , <i>ASIP</i> , <i>TYR</i> , <i>TYRP1</i> , others	<sup>c</sup>

<sup>a</sup>Relative risk (RR) is defined as the risk of developing melanoma if the risk factor is present versus absent. A relative risk of 1.0 means there is no increase in risk. A relative risk of 2.0 means there is double the risk.

<sup>b</sup>Penetrance by age 80. A value of 1.00 means the patient has a 100 % chance (cumulative risk) of developing melanoma by age 80.

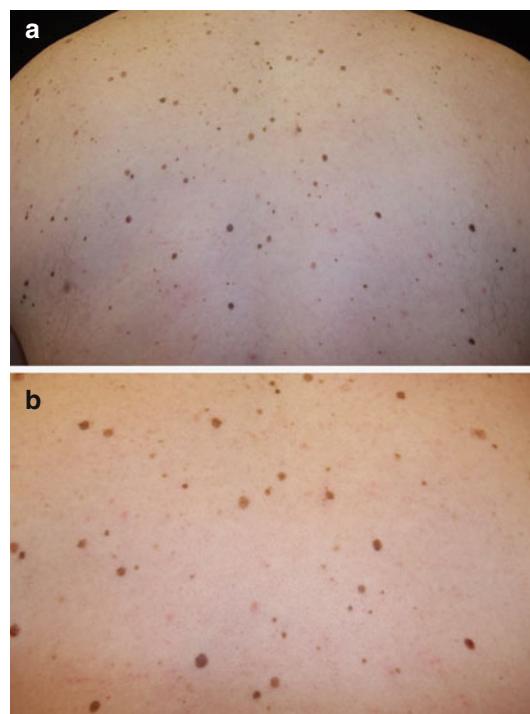
<sup>c</sup>Sufficient data not available

a recent study by the melanoma research consortium GenoMEL, of the 466 melanoma families studied, 41 % had mutations in *CDKN2A* (40 %) or *CDK4* (1 %) [8]. These data support *CDKN2A* and *CDK4* as attractive targets for studying the biology of hereditary melanoma and developing genetic tests but also highlight the fact that more than half of melanoma families have different or yet-to-be-determined molecular defects.

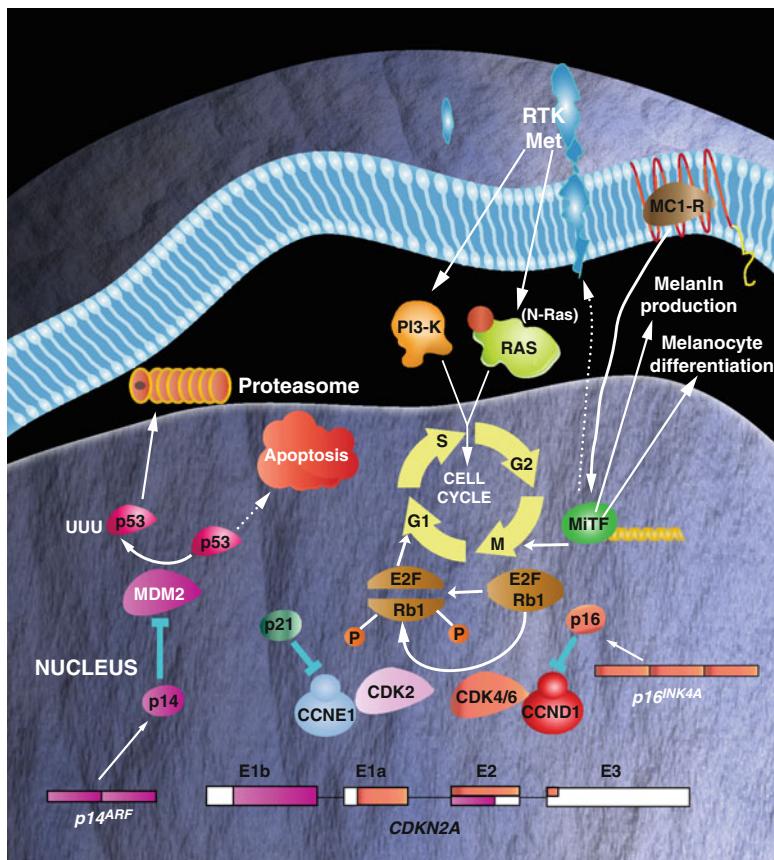
## 4.2.1 Loci Associated with Melanoma Risk

### 4.2.1.1 *CDKN2A*

Germline alterations in *CDKN2A* (cyclin-dependent kinase N2A) are the most common genetic defects found in melanoma families. This gene, located on chromosome 9p21, was identified using linkage analysis on large melanoma families and is the primary locus involved in the well-characterized B-K mole and familial atypical mole melanoma (FAMM) syndromes [9, 10]. Patients with these syndromes have an increased number of clinically atypical moles (Fig. 4.3), although it remains controversial whether these lesions are precursors to melanoma or merely connote higher risk for the development of melanoma elsewhere on the body. Families with *CDKN2A* mutations have a higher risk for



**Fig. 4.3** Multiple atypical nevi in patients with mutations in *CDKN2A* or *CDK4*. Atypical nevi may be numerous in patients with *CDKN2A* or *CDK4* mutations (a). Atypical nevi can have overlapping clinical features with melanoma, including variable degrees of asymmetry, irregular borders, and pigment heterogeneity, to name a few (b) (Images courtesy of Dr. Stephen Weis, Ft. Worth, TX)



**Fig. 4.4** Molecular pathways in heritable melanoma. *CDKN2A* has three exons (E1a/b, E2, E3), encoding two proteins, p16<sup>INK4A</sup> and p14<sup>ARF</sup>, by an alternative splicing mechanism. p14 inhibits the activity of MDM2, which is involved in the ubiquitination and degradation of p53. A loss of (or defective) p14 leads to increased degradation of p53 and steering of the cell away from programmed cell death and toward cell proliferation and survival. p16 inhibits CCND1, which is involved in the phosphorylation

developing melanomas and pancreatic cancer, with variable penetrance.

*CDKN2A* is an interesting gene, encoding for two distinct proteins, p16<sup>INK4a</sup> and p14<sup>ARF</sup>, by alternative splicing of exon 1 (Fig. 4.4). p16 is a well-studied tumor suppressor. It acts by inhibiting the kinase activity of CDK4/6, which normally acts to release E2F from Rb by phosphorylation of Rb. This allows the transcription factor E2F to transition the cell from G1→S phase and initiate the cell cycle leading to cell division. A mutated or absent p16 cannot block CDK4 and thus cannot put the brakes on cell cycling and

of Rb and the downstream transition of the cell into the cell cycle. A loss of (or defective) p16 fails to block this signaling pathway, leading to uninhibited cell cycling. Other proteins that may be defective or lost in heritable melanoma, such as CDK4, MC1R, and MiTF, are also shown. Dotted lines correspond to indirect and/or less understood interactions (MiTF may transcriptionally activate Met)

proliferation. p14<sup>ARF</sup> is also a well-known tumor suppressor that prevents MDM2 (mouse double minute 2) from allowing the ubiquitination and thus degradation of p53. Since p53 is a tumor suppressor and a major player in the apoptosis, or programmed cell death, pathway, a defective or absent p14, which causes the rapid degradation of p53, steers the cell away from apoptosis and cell death and toward survival.

There are many reported mutations in *CDKN2A*, primarily involving and spanning exons 1-α and 2, and thus primarily involving p16<sup>INK4A</sup>. A few common mutations stand out,

including 225–243del19 (aka “Leiden”), M53I (aka “Scottish”), and G101W. Each of these three mutations has been observed in over 15 extended families worldwide. Of the known familial *CDKN2A* mutations in melanoma families, approximately 96 % involve p16<sup>INK4A</sup>, with the remaining 4 % involving p14<sup>ARF</sup> [8].

Environmental factors, specifically ultraviolet radiation, appear to play a significant role in the development of familial melanoma. In a study looking at the risk of developing melanoma among carriers of *CDKN2A* defects from different geographies, by age 80, 58 % of Europeans, 76 % of Americans, and 91 % of Australians developed melanoma [6]. Additionally, mutations or variants in other melanoma risk genes (*MC1R*, for example; see below) may have an additive or synergistic effect on an individual’s risk. In a separate study from Australia, individuals with both a *CDKN2A* mutation and an *MC1R* variant had an 84 % cumulative lifetime risk of melanoma versus 50 % risk with the *CDKN2A* mutation alone [11]. Of note, while defects in *CDKN2A* are more commonly associated with familial melanoma, somatic mutations and somatic loss of 9p21 (the locus for *CDKN2A*) in sporadic melanomas are also quite common, supporting the direct role of *CDKN2A* in oncogenesis [5, 12].

#### 4.2.1.2 *CDK4*

*CDK4* (cyclin-dependent kinase 4) is located on chromosome 12q14 and encodes for the CDK4 protein. CDK4 is part of a network of cyclins and cyclin-dependent kinases involved in cell cycling, specifically the G1→S transition, leading to cell division. CDK4 is an intermediary between p16 and Rb. It is a kinase and activates Rb by phosphorylation. Mutations in *CDK4* account for approximately 1 % of familial melanomas [8]. Defects in *CDK4* interfere with p16 binding, preventing inhibition by p16, and thus allowing uninhibited activation of Rb. Just as p16 mutations prevent the normal brake-applying function of p16, mutations in *CDK4* that prevent binding of p16 have the same downstream effect. These mutations in *CDK4* are rarer than *CDKN2A* but, when present, are also considered equally “high risk” and have a similar clinical presentation (see

Fig. 4.3) [13]. The most common mutations, R24C and R24H, are found at the p16 binding region of the protein [7].

#### 4.2.1.3 *MC1R*

*MC1R* (melanocortin-1 receptor) is located on chromosome 16q24 and encodes for a 7-transmembrane protein belonging to the family of G-protein-coupled receptors involved in melanin synthesis. Its ligand is α-MSH (melanocyte-stimulating hormone). Upon binding to MSH, *MC1R* activates CREB (cAMP response element binding protein), which upregulates MiTF (microphthalmia transcription factor), leading to the preferential generation of eumelanin (dark pigment) over pheomelanin (red pigment). This process is mediated by tyrosinase and tyrosinase-related protein 1 (Tyrp-1) among other melanin-synthesizing enzymes. Eumelanin protects the skin from damage induced by UV exposure better than pheomelanin.

Certain variants of *MC1R* are associated with “moderate” risk for developing melanoma, increasing risk by 2.7-fold. *MC1R* variants can impact the relative balance of eumelanin and pheomelanin production. The moderate-risk variants are present in most patients with red hair, increasing their risk for melanoma, while other variants have increased risk without the red hair phenotype, suggesting a more complex role for *MC1R* [14]. The variants associated with the highest risk include D84E, R142H, R151C, I155T (I155T is not the red hair phenotype), R160W, and D294H. These are designated as “R” or “RHC” alleles. There are other variants, “r” or “rhc” alleles, which also increase risk, but to a lower degree. These latter variants include V60L, V92M, and R163Q. All the *MC1R* germline variants associated with an increased risk of melanoma are associated with somatically *BRAF*-mutated tumors (see Chap. 5) [15].

#### 4.2.1.4 Others

Other genes implicated in hereditary melanoma are altered at a much lower frequency than those described above and thus are considered minor players. Some, like the *XP* (xeroderma pigmentosum) family of genes, when mutated, lead to

an astonishingly high risk for developing melanoma (600–8,000 relative risk) but are overall extremely rare. Other mutated genes in this “minor” category include *BAP1* (primarily uveal melanomas), *TERT* (telomerase reverse transcriptase), *RB1* (retinoblastoma), *MITF* (specifically the p.E318K variant), *BRCA1* (breast cancer), and others. The corresponding protein products of some of these genes participate in the same molecular signaling pathways (see Fig. 4.4). In addition to these aforementioned players, many genes identified through genome-wide association studies (GWAS) are being studied to assess their role in the biology of melanoma and their association with melanoma risk [7]. These are not discussed further.

#### 4.2.2 Testing for Germline Mutations

The role of genetic testing is controversial, but what is not controversial is the importance of genetic counseling in order for the individual to have a clear understanding about the true meaning of positive and negative test results [12]. Clearly, having the information can have some benefits to individuals, their family, and their physicians. A positive result can potentially identify individuals and family members at high risk for melanoma, allowing consideration for more rigorous or frequent dermatologic screening. With a higher risk for pancreatic cancer in some populations (mainly Europeans and North Americans), these individuals may also benefit from rigorous screening for visceral disease, if such methods become more effective and available (i.e., ultrasound or CA19.9 blood levels) [16, 17]. Testing can identify a family “signature,” or “fingerprint,” mutation, which can be followed among subsequent offspring. Individuals who test positive for a family signature mutation will have a better understanding of their likelihood for the development of melanoma, as this should follow published penetrance data. Additionally, those negative for their family signature mutation can be reassured that their risk is much lower (but not zero given other

potential genetic and environmental factors), thus alleviating potential psychological stress.

Of course, testing is not without its pitfalls. The false-negative rate is high, as less than half of individuals from melanoma families will have a detectable mutation. Moreover, because most commercially available tests only screen the *p16<sup>INK4a</sup>* regions of *CDKN2A*, individuals with negative results may have other known but undetected melanoma-associated germline mutations (e.g., *CDKN2A* exon 1β for *p14<sup>ARF</sup>*, *CDKN2A* intron defects, and *CDK4* mutations). Negative results may also have unintended consequences such as increasing the risk for melanoma with somatic mutations as overly optimistic individuals abandon practices to limit UV exposure. Because *CDKN2A* mutations occur with relative frequency in certain populations and are not uniformly drivers of melanoma (silent mutations, e.g., not associated with melanoma), without a known family signature mutation, these results must be interpreted with caution as there could be a different melanoma-causing gene. There are also the usual concerns with genetic testing, including but not limited to cost, the willingness for insurance companies and other payers to bear the cost, genetic discrimination, and the effect on family dynamics.

In an unscreened population, only 1 % of individuals will test positive for a *CDKN2A* mutation, and this rate varies widely depending on geography [18]. This raises the typical concerns regarding tests with a low pretest probability, and this approach is not recommended. There may be an argument to test certain subgroups, however. The likelihood (and thus the positive predictive value) a patient from a melanoma family has a *CDKN2A* mutation increases with the number of primary melanomas, the number of family members affected, and the presence of pancreatic cancer in the family. This is enhanced even more if the patient is from a geographical region with a *low* incidence of melanoma, since areas with a high incidence are significantly diluted by nonfamilial cases. A family history of melanoma is the strongest predictor of a *CDKN2A* mutation, with approximately 40 % of families harboring a detectable

**Table 4.2** Proposed criteria for hereditary melanoma testing

Incidence of melanoma in patient's geographical region	Criteria
Low	Two primary melanomas in an individual <i>or</i>
	Two cases of melanoma among first- or second-degree relatives <i>or</i>
	One case of melanoma and one case of pancreatic cancer in first- or second-degree relatives
High	Three primary melanomas in an individual <i>or</i>
	Three cases of melanoma in first- or second-degree relatives <i>or</i>
	Two cases of melanoma and one pancreatic cancer in first- or second-degree relatives <i>or</i>
	One case of melanoma and two of pancreatic cancer in first- or second-degree relatives

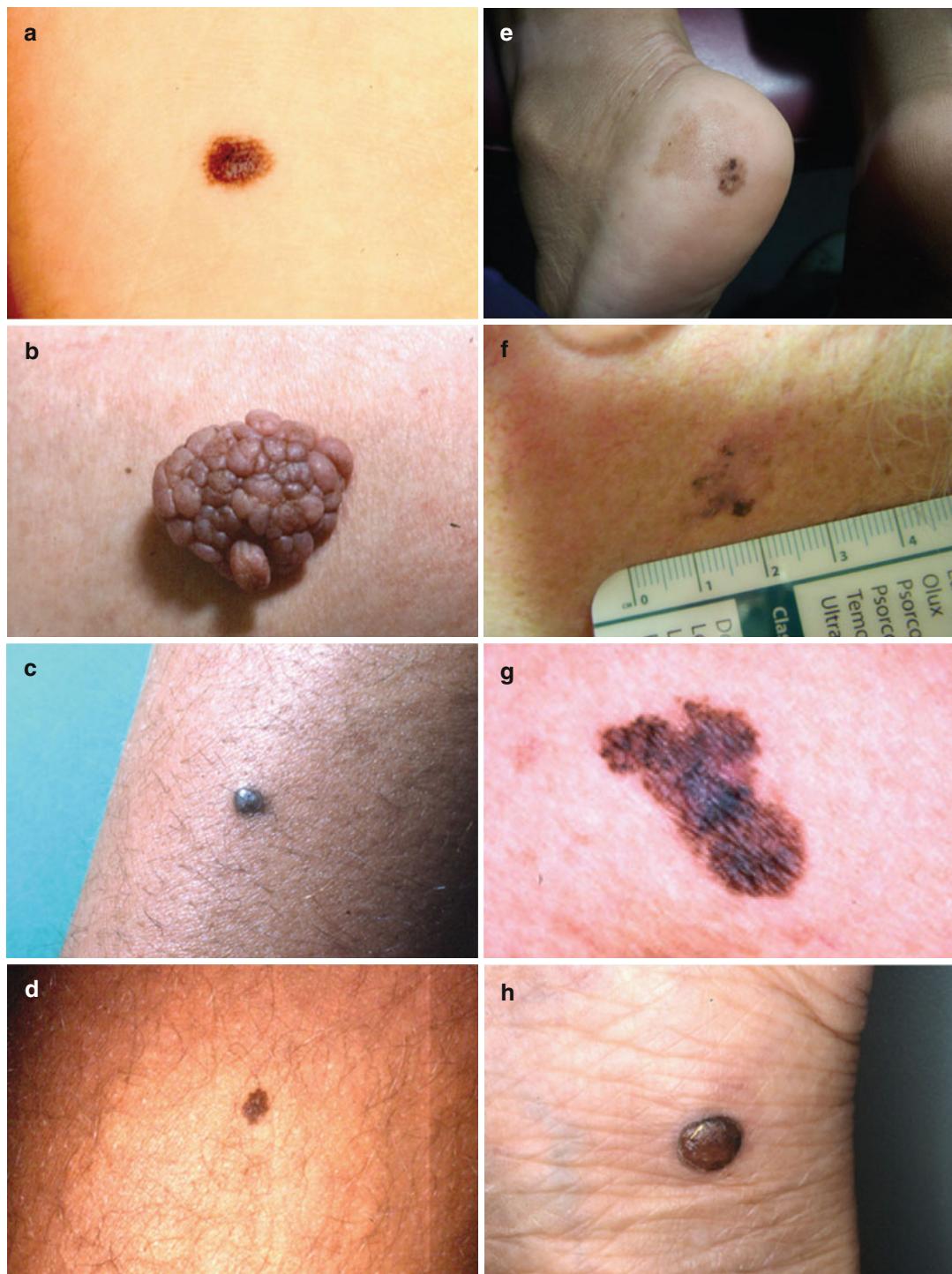
mutation [19]. In patients with multiple primary melanomas, 2.9 % have detectable germline mutations [18]. In order to increase the positive predictive value of the test, algorithms have been proposed based on these aforementioned factors. One proposed method of screening is to test the following: individuals with three or more cutaneous melanomas *or* patients with a strong family history including at least one invasive melanoma *and* two or more diagnoses of melanoma and/or pancreatic carcinoma among first- or second-degree relatives [12]. Other similar algorithms are available, and the stringency of the criteria for testing may be loosened if the patient is from a low-risk geographical zone (Table 4.2) [20].

### 4.3 Diagnosis

Molecular testing is a recent addition to the diagnostic armamentarium for melanoma. The current gold standard for the diagnosis of melanoma remains the clinical evaluation and biopsy of suspicious lesions. For centuries, clinicians have recognized most melanomas by their appearance, and more recently, these observa-

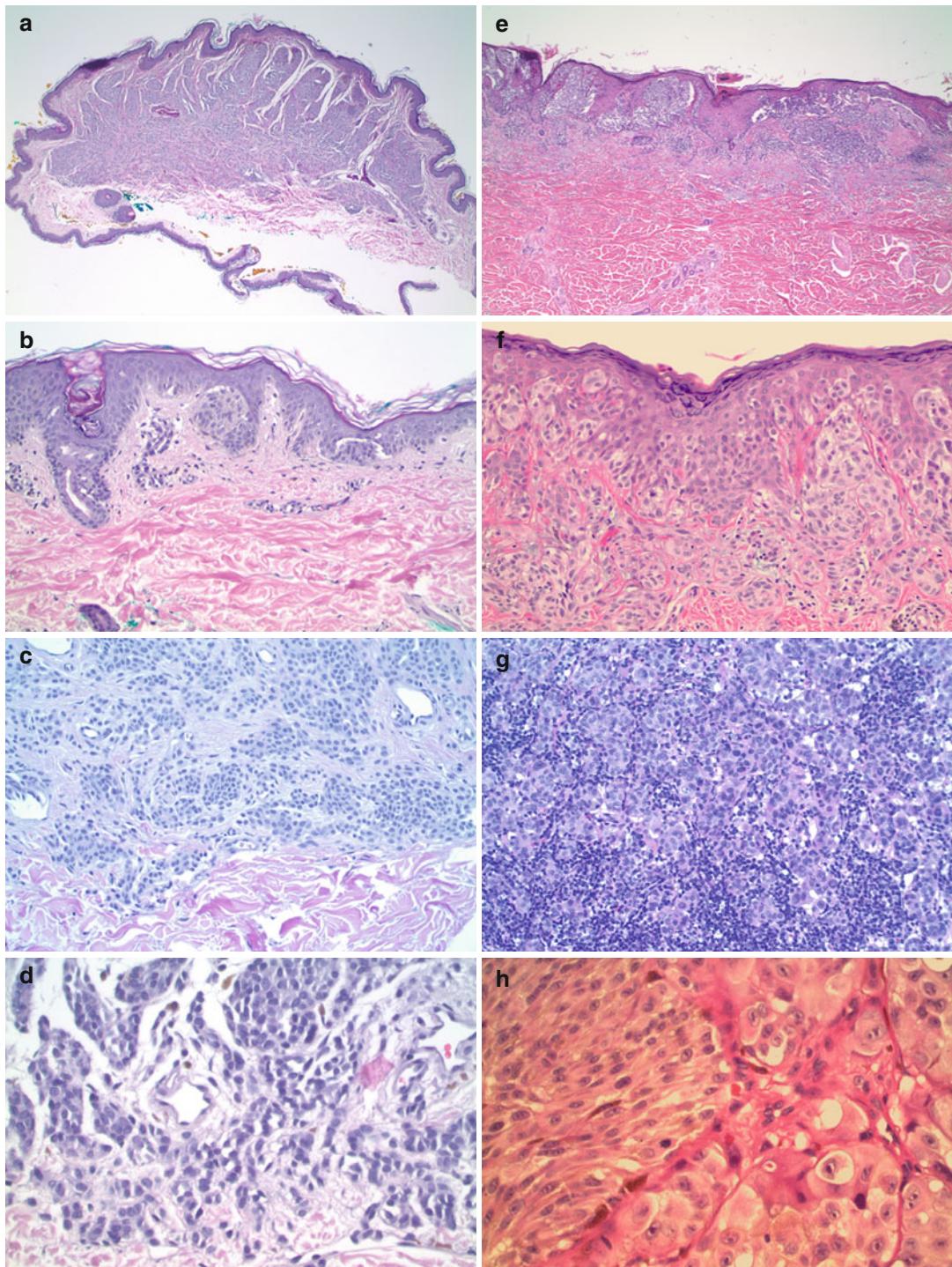
tions have been cataloged into the ABCDEs of melanoma. This refers to the *asymmetry*, *irregular borders*, *color (or pigment) heterogeneity*, *large diameter*, and *evolution* typical of melanomas (Fig. 4.5). The diagnosis is confirmed by microscopic histomorphologic analysis, with the pathologist evaluating the lesion for the presence or absence of features that have been well described (Fig. 4.6) [21].

It is convenient to think that the histologic evaluation of the melanocytic lesion is definitive and final, like a “+” or “-” sign appearing in the microscope, but anyone who has attempted this knows life is just not so simple. There are a variety of lesions, including nevi with architectural disorder (or so-called dysplastic nevi), spindled and/or epithelioid cell (or Spitz) nevi, special site nevi, and atypical nevi NOS that share some but not all features with melanoma [22]. Moreover, the interobserver variability and inability to obtain a clear consensus on ambiguous lesions, even among experts, are well documented [23, 24]. At times, the distinction is inconsequential as the biopsy itself is often curative. But many times, as with Spitzoid lesions, for example, the lesions measure several millimeters in depth, and therefore, the histologic differential diagnosis includes both a benign Spitz nevus and a high-Breslow (and therefore high-stage) melanoma. Immunohistochemical analysis has been available to pathologists for several decades, to help with this problem, but its diagnostic utility in this setting has remained limited (Fig. 4.7 and Table 4.3) [25]. The role of immunohistochemistry has largely been relegated to confirming the lesion’s melanocytic origin, as markers to distinguish benign from malignant have remained elusive. HMB-45 (gp100) and MIB-1 (Ki-67) are probably the most used for this purpose, although limitations exist, preventing their widespread use. It is exactly this necessity to better discriminate between nevi and melanoma that has been the driver of invention of new ancillary techniques, including comparative genomic hybridization, FISH, and other molecular assays, to ultimately improve our diagnostic accuracy. An overview of these techniques is provided in Chap. 3.



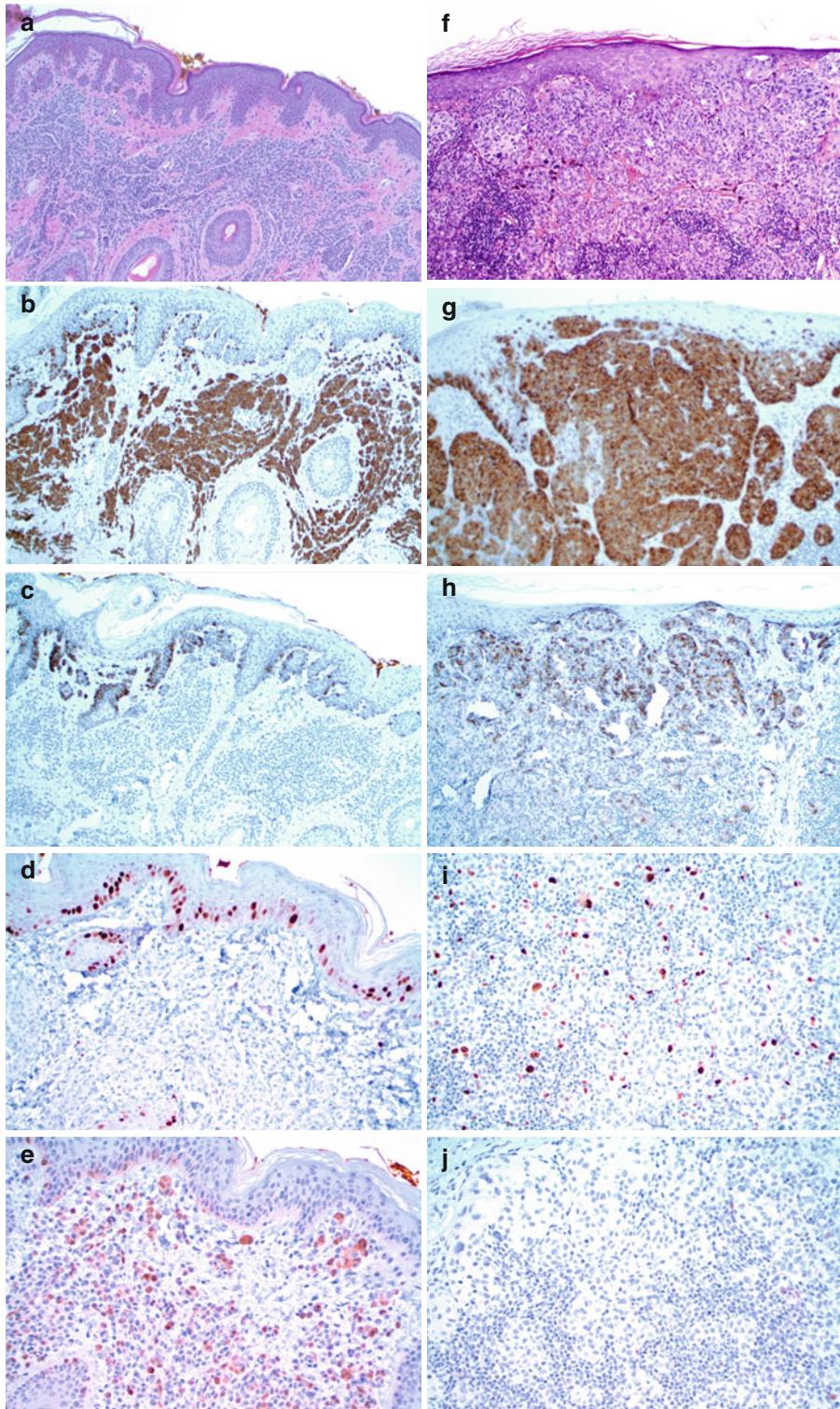
**Fig. 4.5** Clinical features of nevi and melanoma. Melanocytic nevi are clinically heterogeneous. They may have a fried-egg (**a**), polypoid (**b**), blue (**c**), or flat (**d**) appearance. They may be pigmented or amelanotic. With some exceptions, they are usually small and symmetric with smooth borders and regular pigmentation. The clinical features of melanoma are defined by the ABCDE

criteria—namely *asymmetry*, *irregular borders*, *color (or pigment) heterogeneity*, *large diameter*, and *evolution*. Shown are examples of acral lentiginous melanoma (**e**), lentigo maligna melanoma (**f**), superficial spreading melanoma (**g**), and nodular melanoma (**h**). (Several images courtesy of Dr. Stephen Weis, Ft. Worth, TX, and Dr. Alan Menter, Dallas, TX)



**Fig. 4.6** Histomorphologic features of nevi and melanoma. The histomorphologic features of nevi and melanoma have been well described. Briefly, nevi may be raised (**a**, H&E, 40 $\times$  original magnification) or flat (**b**, 200 $\times$ ) and are typically symmetric. Within the epidermis, melanocytes are mostly nested and reside along the epidermal-dermal junction. Within the dermis, there is evidence of melanocytic maturation (cells get smaller deeper within the lesion) (**c**, 100 $\times$ ). The cells are cytologically bland, without nuclear irregularities or

mitotic figures (**d**, 400 $\times$ ). There is minimal stromal reaction or inflammation. Melanomas, in contrast, are more often asymmetric (**e**, 40 $\times$ ). The melanocytes ascend into the epidermis with pagetoid growth (**f**, 200 $\times$ ) and may track along adnexal units. In the dermis, the tumor cells fail to mature, and an inflamed or desmoplastic stroma is commonly observed (**g**, 100 $\times$ ). Melanoma cells are usually cytologically atypical, with variable degrees of pleomorphism, irregular nuclear borders, pigment heterogeneity, and mitotic activity (**h**, 400 $\times$ ).



### 4.3.1 Comparative Genomic Hybridization (CGH)

It is well recognized that chromosomal instability is a frequent event in tumorigenesis. With chromosomal instability comes the creation of genetic diversity and selection of tumor subpopulations

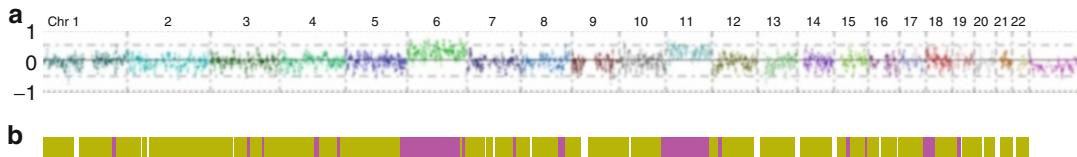
with a growth and survival advantage [26, 27]. Amplifications of oncogenes and deletions of tumor suppressor genes are just two simplified examples of how this mechanism may lead to malignancy. Comparative genomic hybridization, or CGH, is a very powerful technique that can detect and localize genome-wide variations

**Table 4.3** Antibodies for the immunohistochemical analysis of melanoma

Antibody	Antigen	Staining localization	Sensitivity for melanocytic lesions	Comments
Anti-S100	S100	Nuclear and cytoplasmic	>95 %	Positive in melanoma, nevi, neural tumors, fat, cartilage, dendritic cells
Anti-SOX10	SOX10	Nuclear	>95 %	Positive in melanoma, nevi, peripheral nerve sheath tumors
HMB-45	gp100	Cytoplasmic	>95 % for nevi, less so for melanoma	Very specific for melanocytes Stratified staining in nevi Diffuse staining in blue nevi and related lesions Patchy staining in melanoma Positive in angiomyolipomas, PEComas
anti-melan-A anti-MART-1 A-103	Melanoma antigen recognized by T cells 1 (MART-1)	Cytoplasmic	>95 % Does not stain spindle cell lesions	Positive in melanoma, nevi, melanin-laden keratinocytes, angiomyolipomas, PEComas
Anti-MiTf	Microphthalmia transcription factor (MiTF)	Nuclear	>95 %	Positive in melanoma, nevi, hematopoietic cells, fibroblasts, Schwann cells Useful in sun-exposed skin as replacement for MART-1
Anti-tyrosinase	Tyrosinase	Cytoplasmic	Similar to HMB-45	Similar to HMB-45
anti-Ki67 MIB-1	Ki-67	Nuclear	N/A	Proliferation marker Should be low (<1 %) and stratify with nevi High (>5 %) and no stratification with melanoma
Anti-neuropilin-2	Neuropilin-2	Cytoplasmic	>95 % for melanoma Less for Spitz nevi	May be useful for differentiating Spitz nevi from melanoma

**Fig. 4.7** Immunohistochemistry in nevi and melanoma. Immunohistochemistry has a limited role in the distinction between nevi and melanoma. Shown are H&E examples of a benign nevus (**a**, 100× original magnification) and a nodular melanoma (**f**, 100×) with corresponding select immunohistochemical markers. MART-1 is a cytoplasmic stain, diffusely highlighting all melanocytes in the nevus (**b**, 100×) and melanoma (**g**, 100×). Focal pagetoid growth is observed in the melanoma. HMB-45 is cyto-

plasmic and will stratify from superficial to deep in nevi (**c**, 100×) but will be patchy and irregular, including focal deep staining, in melanoma (**h**, 100×). Ki-67 is a nuclear stain, a proliferation marker that should highlight few superficial melanocytes, if any, in nevi (**d**, 200×) but will be increased with some marking of deep melanocytes in melanoma (**i**, 200×). p16 is also a nuclear stain that is positive in most melanocytes within the nevus (**e**, 200×) and is often lost in melanoma (**j**, 200×)



**Fig. 4.8** Comparative genomic hybridization. CGH measures copy number changes. Different platforms vary by targeted regions (whole genome, one chromosome, etc.) and by resolution (ability to discriminate two separate genetic data points), among others. In an example of full-genome CGH, copy number changes are displayed as tracings above the line (net gains) or below the line (net

losses). This example has abnormal gains of the entire chromosome 11 and chromosome 6 (a). These data can also be displayed in a Hidden Markov Model (b), with *yellow* representing two copies of the DNA region (normal) and *pink* representing three copies (*other colors* are used for losses, not shown)

in (whole or partial) chromosomal copy number. Detection of these aberrations can be used to further understand the biology of the tumor by localizing the macrodefects to known sites of oncogenes or tumor suppressor genes as well as for prognostic or diagnostic purposes.

The general principle behind CGH is to compare the tumor genome with a background normal genome and identify differences, such as gains and losses of genetic material, between tumor and normal cells (Fig. 4.8). There are many available methods. Traditional CGH entails labeling tumor and reference DNA with differently colored fluorochromes. The labeled material is hybridized to immobilized metaphase spreads, and then the fluorescent signals are measured along the long axes of the chromosomes, looking for relative gains and losses. Microarray technology, or array CGH (aCGH), is now more widely used. The detection resolution for conventional metaphase CGH is defects of at least 20 Mb [28], and for newer aCGH platforms, the resolution can be as high as 200 bp, depending on the technology and design [29]. For this reason and others, aCGH has virtually replaced the older conventional method.

Melanomas, as with most malignancies, have a higher number of chromosomal aberrations than their nonmalignant counterparts [30, 31]. Not only are there more aberrations, but these are fairly consistent and reproducible. Using CGH analysis on 132 melanomas and 54 nevi, Bastion et al. showed 96 % of melanomas had aberrations (Table 4.4). The most common of these were losses in 9p (64 %), 9q (36 %), and 10q (36 %) and gains in 6p (37 %), 1q (33 %), 7p

**Table 4.4** Chromosomal aberrations present in nevi and melanoma

Chromosomal copy number change	Frequency (%)
<i>Nevi</i>	
11p gains	Up to 20 (for Spitz nevi)
<i>Melanoma</i>	
9p losses	64
9q losses	36
10q losses	36
10p losses	29
6q losses	26
11q losses	21
6p gains	37
1q gains	33
7p gains	32
7q gains	32
8q gains	25
17q gains	24
20q gains	22

(32 %), and 7q (32 %) [31]. Only seven benign nevi, which were all Spitz nevi, had abnormalities. All seven of these lesions had gains in 11p, the entire short arm of chromosome 11 [31]. This and subsequent analyses have estimated 10–20 % of Spitz nevi have gains in 11p, with a minor subset having gains in 7q. These 11p+ lesions are typically morphologically large with a sclerotic base and also carry *HRAS* mutations (see Chap. 5) [32–34]. Melanomas may also have gains in 11p, but this occurs in the context of multiple other chromosomal abnormalities—gains in 6p and 17q and losses in 1p and 15p—including partial chromosomal gains and losses [34]. Partial

gains and losses appear much more commonly in melanomas than with nevi, possibly reflecting different mechanisms behind the development of chromosomal aberrations.

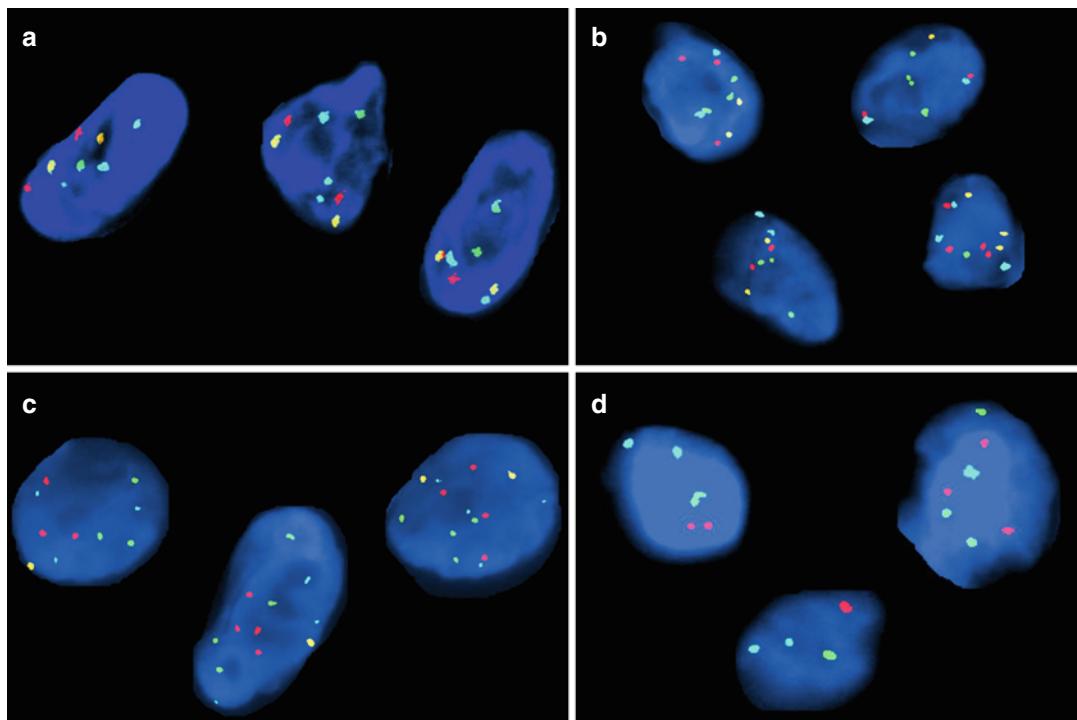
The ability of CGH to distinguish melanoma from nevi based on differences in chromosomal copy numbers provided a potential platform for using CGH as a diagnostic assay. Indeed, CGH has been shown to help distinguish Spitz nevi from melanoma, blue nevi from blue-nevi-like malignancies [35], and melanoma arising in a congenital nevus from benign proliferative nodules [36], among other applications. While the potential power of CGH is clear, it has some limitations preventing its widespread use as a melanoma diagnostic assay.

One such limitation is interpretation of the data. CGH can evaluate the entire genome, and while this is one of its main attractions, this has shifted the focus from data collection to interpretation. The massive amounts of data collected require a bioinformatic solution to sift through all the “noise” and determine what data are meaningful. Other limitations for CGH include its technical restrictions on the type of sample that can be analyzed, its limited availability, and the high level of expertise required (which contribute to its availability, slow turnaround time, and potentially elevated costs). Due to the nature of the assay, there must be relatively high tumor purity in the sample, and the chromosomal aberration must be present in at least 30–50 % of the cells to establish a positive result. Because of this restriction, there is the benefit of an inherent high specificity incorporated into the assay (false positives are less likely because the defect is present in a lot of cells, not just a few), but obviously, this prohibits its use on certain samples. CGH cannot be performed on biopsies with only small foci of tumor, superficially invasive tumor, heavily inflamed tumor, or, unknown at the time of performing the test, genetically heterogeneous tumors. Additionally, the interpreter of CGH does not have the luxury of being able to know if the molecular aberration is present in the atypical melanocytic population of interest, short of performing delicate microdissection or laser capture, and balanced translocations and

inversions are not detectable. Melanoma CGH is only offered at a few centers around the world for clinical samples. CGH is a high complexity test, both to perform and, more so, to interpret. Isolation of the tumor DNA, running the assay, and interpreting the data, in conjunction with the test’s limited availability, all contribute to the slow turnaround time of several weeks to months. This may not be an acceptable option for an anxious patient, parent of patient, or physician. These same factors, along with test reagents, potentially increase the cost of the assay, which is not FDA approved and often not covered by insurance. Of course the issue of cost (for CGH or any test) is always complex as the test’s cost must always be weighed against the potential cost of under- or overtreatment. The development of microarray platforms, or aCGH, has increased optimism for more widespread use of CGH by alleviating, but not eliminating, some of the above concerns.

### 4.3.2 Fluorescence In Situ Hybridization (FISH)

Many of the limitations imposed by CGH, outlined above, provided the impetus to pursue a fluorescence in situ hybridization (FISH) assay for melanoma. FISH uses fluorescently labeled segments of DNA which are designed to hybridize to targets in the genome. As normal human cells are diploid, a normal cell will have two and only two signals per target. This method readily identifies deviations in target, or gene, copy number, and its relative simplicity and ease in interpretation make it attractive as a surrogate for CGH. An example of a FISH-positive and FISH-negative cell during the evaluation for melanoma is provided in Fig. 4.9. It should be noted that while there are many contributors to the field, the lions’ share of the credit for developing the melanoma FISH assay should go to Dr. Pedram Gerami, Dr. Boris Bastian, and their colleagues at the University of California San Francisco (San Francisco, CA), Northwestern University (Chicago, IL), and Abbott Molecular (Abbott Park, IL), among others [37]. The development of



**Fig. 4.9** FISH in the evaluation of melanoma. Paraffin-embedded tissue FISH (PET-FISH) was performed on histologically ambiguous lesions using two separate probe sets. Probe set one includes probes to *RREB1* (red), *MYB* (gold), *CCND1* (green), and *D6Z1* (centromere 6) (aqua). Two signals for each probe are observed in normal cells (a). Melanoma often has increased copies of some probes, specifically *RREB1* and *CCND1*, as in this case from the trunk of a 59-year-old male (b, bottom right cell and top

right cell, respectively). Loss of *MYB* may also be observed (not shown). A second-generation probe set uses probes to *RREB1* (red), *CCND1* (green), *MYC* (aqua), and *CDKN2A/p16* (gold). In this case from the calf of a 69-year-old female, increases in signals for *RREB1* and *CCND1* suggest a diagnosis of melanoma (c). A separate lesion from the back of a 32-year-old male shows absence of *CDKN2A/p16* signals (homozygous loss), also suggestive of melanoma (d)

the melanoma FISH assay by these investigators is worth summarizing due to its historical significance and as it has set a new standard for assay development in this field [38, 39].

In their 2009 publication in The American Journal of Surgical Pathology, Gerami et al. used combinatorial analysis of CGH data for the development of a melanoma FISH diagnostic assay [37]. The CGH data pointed to 13 areas on the genome that had copy number variations that could potentially segregate melanomas from benign nevi. FISH probes were targeted toward known oncogenes (if there was an addition of genetic material) and tumor suppressor genes (with deletions) within these macrogenomic areas, and a fourteenth FISH probe to *KIT* was added due to potential therapeutic implications.

The probes were analyzed in combinations of four-probe sets (as this is the maximum number of probes that can be used in a single assay due to limitations placed by wavelengths of fluorochromes). In an elegant study using 497 pigmented lesions in four separate cohorts, an optimal probe set was systematically selected and then validated. The first cohort of lesions (97 unequivocal melanomas, 93 unequivocal nevi) was used to evaluate the probe sets for their ability to discriminate benign from malignant lesions. The best performing set was selected and included FISH probes targeting the following: *RREB1*, *MYB*, *CCND1*, and *CEP6* (Table 4.5). This panel was then applied to a second cohort (58 unequivocal melanomas, 51 unequivocal nevi) to establish cutoff values for positive and

**Table 4.5** Published FISH probe panels for the diagnosis of melanoma

Probe set 1				
Region	Gene	Function	Criteria	Cutoffs <sup>a</sup> (%)
Centromere 6	( <i>CEP6</i> )	Mitosis	<i>RREB1</i> > <i>CEP6</i>	>53
6p25	<i>RREB1</i>	Zinc finger protein	>2 signals	>16
6q23	<i>MYB</i>	Transcription factor	<i>MYB</i> < <i>CEP6</i>	>42
11q13	<i>CCND1</i>	Cyclin D1 (bcl-1), cell cycle regulator	>2 signals	>19
Probe set 2 <sup>b</sup>				
Region	Gene	Function	Criteria	Cutoffs <sup>c</sup> (%)
9p21	<i>CDKN2A</i>	Locus for p16/p14 tumor suppressors	Homozygous deletion	>29
6p25	<i>RREB1</i>	Zinc finger protein	>2 signals	>29
11q13	<i>CCND1</i>	Cyclin D1 (bcl-1), cell cycle regulator	>2 signals	>29
8q24	<i>MYC</i>	Transcription factor	>2 signals	>29

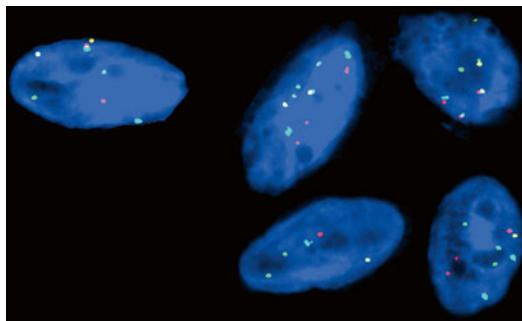
<sup>a</sup>Probe set 1 cutoffs from NeoGenomics 1st-generation FISH assay values

<sup>b</sup>Commercial version of assay uses the addition of centromere 9

<sup>c</sup>Cutoffs from Gerami et al. [39]

negative results. The results were interpreted as positive (likely to be melanoma) if any of the following criteria were met: >38 % of scored cells have more than two signals for *CCND1* (*CCND1*>2), >29 % of scored cells have more than two signals for *RREB1* (*RREB1*>2), >55 % of scored cells have more signals for *RREB1* than centromere 6 (*RREB1*>*CEP6*), and >40 % of scored cells have fewer signals for *MYB* than centromere 6 (*MYB*<*CEP6*). A third cohort of pigmented lesions (83 unequivocal melanomas, 86 unequivocal nevi) was used to establish the performance characteristics of the assay. Using receiver operating characteristic (ROC) curve analysis, the assay's sensitivity and specificity parameters were determined and fixed at 86.7 and 95.4 %, respectively. Lastly, a fourth cohort of histologically ambiguous lesions (*n*=27) was evaluated by the probe set to determine if the assay could predict patient outcome. Patients were either disease free at 5 years or had recurrence, defined as bulky lymphadenopathy, distant metastases, or death. All patients (6/6) with recurrences were FISH positive, while only 6/21 of the patients without recurrence had a positive result, demonstrating the power of this test as a diagnostic assay. Other studies with outcome data have shown variable results but an overall similar utility [40, 41].

This first-generation melanoma FISH assay has its limitations. There are recognized examples of false positives, including Spitz nevi and dysplastic nevi [37, 42], as well as false negatives [38, 43]. Additionally, the sensitivity of the assay appears to vary based on melanoma subtype, ranging from almost 100 % with acral melanomas to 81 % for superficial spreading melanoma down to 50 % for desmoplastic melanoma [44, 45], suggesting the utility of the assay may be limited to certain histologic variants of melanoma. The main source for false-positive results is tetraploidy. Up to 10 % of Spitz nevi exhibit tetraploidy, enough to exceed the assay's cutoff values [46], and it has been estimated that up to 27 % of false-positive results can be attributed to tetraploidy [47]. Tetraploidy refers to a form of polyploidy, where the entire genome is duplicated, resulting in four copies of every gene and chromosome, instead of the normal two. As mentioned above, this phenomenon is likely due to macrochromosomal replication due to errors occurring with the centromere during mitosis and not genomic instability seen with malignancies. An example of a tetraploid cell by FISH is shown in Fig. 4.10. Even if the technician and pathologist are astute enough to recognize tetraploidy, the interpretation of this finding is complicated by the fact that tetraploidy may also



**Fig. 4.10** Tetraploidy observed by FISH in a Spitz nevus. Paraffin-embedded tissue FISH (PET-FISH) was performed on an atypical Spitzoid lesion from the cheek of a 1-year-old female using probes to *RREB1* (red), *MYB* (yellow), *CCND1* (green), and D6Z1 (centromere 6) (aqua). The number of signals for each probe is increased. Tetraploidy may result in four signals for each probe, as is the case here, but exactly four signals for each probe are not required for an interpretation of tetraploidy in PET-FISH, as cells may overlap or exit the visualized plane

occur in melanomas. And regarding the false-negative problem, the sensitivity of the assay for Spitzoid melanomas is only around 70 % [43]. Interestingly, the majority of FISH-negative melanomas appear to have aberrant loss of 9p, the location of *CDKN2A/p16* and the most common aberration in melanoma overall, not represented in this original four-probe set and thus not detectable by this assay [38].

A second-generation FISH assay has been developed to address some of these issues [39]. Several parameters were changed in this new version. Following methodical steps for probe selection similar to the original assay development, a new four-probe set was created. It should be noted that for FISH, the number of probes is ideally limited to four as using more will introduce technical challenges with overlapping fluorochrome wavelengths, and therefore, five or more probes would require at least two separate setups/assays. The new panel added 9p21 (*CDKN2A*) and 8q24 (*MYC*) to two members of the original panel, *RREB1* and *CCND1* (see Table 4.5). These four targets reside on four different chromosome arms, alleviating some of the technical challenges of identifying tetraploidy. New cutoffs were calculated with a requirement that at least eight cells have any particular defect to be called positive, and *CDKN2A* must have *homozygous* loss to be considered positive. Heterozygous deletion of 9p

occurs in benign nevi, including nevi with architectural disorder, and this likely explains why it was unable to effectively discriminate between melanoma and benign nevi in the original assay [48, 49]. Two new scoring rules were adopted to help standardize the assay: cells with three or four signals of *RREB1*, *MYC*, and *CCND1* and at least one signal for *CDKN2A* were counted and scored as negative (attributed to tetraploidy), and cells with no signals in three or more probes were excluded from the count (attributed to being technically invalid). With these revisions, the new second-generation melanoma FISH assay has improved performance characteristics with a reported sensitivity of 94 % and specificity of 98 % in controlled laboratories. NeoSITE Melanoma (NeoGenomics Laboratories, Irvine, CA) is a commercial version of this assay that uses the above four probes along with a centromere 9 internal control probe. This second-generation assay is becoming implemented worldwide and has virtually replaced the original version in the United States.

Since its inception, the melanoma FISH assay has been used in a variety of diagnostic settings, mirroring CGH studies. It has been used for distinguishing conjunctival nevi from melanoma [50], epithelioid blue nevi from blue-nevi-like malignancies [51], mitotically active benign nevi from nevoid melanoma [52], and dysplastic nevi from superficial spreading melanoma [53], among others [40, 42, 54, 55]. Some chromosomal abnormalities are now becoming more clearly associated or disassociated with specific histologic subtypes (e.g., 8q24 gains associated with amelanotic and nevoid melanoma and not with Spitzoid lesions) [56]. And some chromosomal abnormalities are emerging as more important in certain diagnostic settings (e.g., homozygous loss of 9p21 more meaningful than a 6q23 deletion in Spitzoid tumors, and 11q13 gains are very significant in any lesion). These interpretative nuances have added another layer of sophistication to FISH as a diagnostic assay.

In addition to its diagnostic applications, FISH has also been used to guide surgical management by assessing surgical margins for histologically undetectable tumor and field cells [57], in a form of staging by distinguishing capsular or intranodal nevi from lymph node metastases

[58], and as a potential prognostic marker since *CCND1*, *MYC*, and topoisomerase I amplifications all have been associated with poor prognosis [59–61]. While the prognostic and surgical management applications have potential for more widespread use, the primary role for FISH is in the context of the multi-probe melanoma FISH assay for the diagnosis of histologically ambiguous melanocytic lesions. It will be interesting, going forward, to see if there is an increase in the reported incidence of melanoma in low-risk populations, like pediatric patients. FISH/CGH data may provide courage to the otherwise wavering pathologist for these challenging lesions previously uniformly designated “atypical.”

### 4.3.3 Mutational Analysis

There has been a recent explosion in the understanding of signaling pathways in melanoma [62]. Decoding the events that lead to melanoma genesis offers promise for new assays designed to diagnose melanoma, to predict its behavior, and to provide therapeutic strategies for the advanced melanoma patient. *To date, however, mutational analysis is not used for diagnostic purposes in the context of melanoma.* The primary reasons for this include the following: the same mutations are observed in both melanomas and nevi, the mutation is observed in malignancies other than melanoma, and/or the molecular information does not add anything to standard clinical, morphologic, and immunohistochemical diagnostic criteria. This is not to say, however, that emerging narrow applications will not exist. Potential candidates are briefly addressed, *but the bulk of this exciting developing story is focused on theranostics, and detailed descriptions of the signaling pathways and testing are provided in the next chapter (see Chap. 5).*

#### 4.3.3.1 BRAF

*BRAF* is a good example of a gene mutated in melanoma but not used for diagnosis. *BRAF* is mutated in 50–60 % of melanomas. These mutations are primarily observed in melanomas from intermittently sun-damaged skin (59 %), which typically have nodular and superficial spreading

histology. The mutation is fairly consistent, a T→A mutation at the second position in codon 600, resulting in p.V600E (or Val600Glu) and an increase in B-Raf activity [62]. Developing an assay for diagnostic purposes has been hampered by several observations. This same mutation has been observed in many other malignancies, including tumors of the thyroid, lung, gastrointestinal tract, genitourinary system, and hematopoietic system, among others [63]. Additionally, subsequent studies identified the same mutation in up to 82 % of benign nevi, including common acquired nevi (87.5 %), nevi with architectural disorder (dysplastic nevi) (52–62 %), small (88 %-)- and medium (30 %-)-sized congenital nevi and congenital-like nevi, and, to a lesser extent, blue nevi (up to 12 %) [64, 65]. Because of its apparent inability to distinguish malignant from benign or melanoma from other malignancies, the *BRAF* V600E mutation assay’s diagnostic utility has yet to be realized. With that said, however, it has a clear role in determining patient eligibility for B-Raf V600E inhibitor therapy [66] and may have a more prominent future role in the reclassification of melanomas (see below) [67].

#### 4.3.3.2 NRAS and HRAS

Mutations in members of the *RAS* family—*NRAS*, *KRAS*, and *HRAS*—are observed in a variety of settings [68], limiting the utility of *RAS* mutation-detection assays for the purpose of melanoma diagnosis. This is true despite the presence of oncogenic-driving mutational hotspots (Q61, G12, G13) very amenable for detection and present in all *RAS* family members. *NRAS* is the primary player in this family for melanoma. It is mutated in 15–20 % of all melanomas and more commonly in those from intermittently sun-damaged skin (22 %) and, slightly less so, chronically sun-damaged skin (15 %) [67]. Like with *BRAF*, *NRAS* mutations are observed in nevi, but at less and variable frequencies [69]. They are observed in 81 % of giant congenital nevi [70]. *HRAS* mutations and/or amplifications are seen in 10–29 % of Spitz nevi, but are not typical for melanoma [33]. This fact offers promise for a diagnostic application, but because *HRAS*-mutated Spitz nevi have reproducible morphologic characteristics that do not necessarily pose a diagnostic dilemma

and because histologically ambiguous Spitzoid lesions have not been fully studied regarding *HRAS* mutational status, *HRAS*-mutation diagnostic assays have not yet gained traction. It is possible that *HRAS* copy number changes (11p) may become implemented into future FISH panels (and CGH algorithms) for this purpose.

#### 4.3.3.3 *GNAQ* and *GNA11*

*GNAQ* and *GNA11* mutations are fairly common in a narrow range of lesions, including up to 80 % of uveal melanomas and most blue nevi (either *GNAQ* or *GNA11* but not both) [71]. These genes also have mutational hotspots, including Q209 and R183 [72]. This makes *GNAQ/11* a potential target for a diagnostic assay, but a firm diagnostic application has not yet been established, and currently, more focus has been placed on its role in prognosis and as an oncogenic driver.

#### 4.3.3.4 *BAP1*

*BAP1* is a tumor suppressor gene that is inactivated in a subset of uveal melanomas and a smaller subset of cutaneous melanomas (as well as meningiomas and mesotheliomas) [73, 74]. *BAP1* mutations have been reported in families susceptible to melanoma and in up to 5 % of sporadic cutaneous melanomas. *BAP1* mutations in uveal melanomas are associated with a poor prognosis. The *BAP1*-mutated cutaneous melanomas have been histologically described as Spitz-like and epithelioid, and most contain the *BRAF* V600E mutation. Like any tumor suppressor gene, *BAP1* can become inactivated by many different genetic events, including but not limited to deletions and point mutations, making the development of a molecular diagnostic assay a challenge. Immunohistochemistry for *BAP1* appears to correlate well with functional inactivation of the *BAP1* gene and is currently preferred over a molecular approach.

### 4.3.4 Gene Expression Profiling

Analyzing tumor RNA transcripts from an array of different genes (gene expression profiling) can potentially be used for diagnostic and prognostic purposes. Very recently, commercially available assays became available to aid in differentiating

benign from malignant melanocytic lesions. These assays may be pre-biopsy, in the form of tape-stripping directly on skin (DermTech, La Jolla, CA), or post-biopsy from paraffin-embedded material (myPath, Myriad Genetics, Inc., Salt lake City, UT). Little data are currently available. The true utility of these assays will likely be realized over the next few years. Gene expression profiling for prognostic purposes is discussed below.

## 4.4 Prognosis

The prognosis of the melanoma patient is currently determined by their clinical and pathologic stages. These stages have been defined by an international effort based on survival data and published in their most recent form in the 2011 7th edition of *The American Joint Committee on Cancer (AJCC) Cancer Staging Manual* [75, 76] (Table 4.6). (Websites, including [www.melanomaprognosis.org](http://www.melanomaprognosis.org) and [www.melanomacalculator.com](http://www.melanomacalculator.com), have been created as educational tools for patients and caregivers, translating the staging data into a meaningful form for the individual.) The clinical stage is dependent on microstaging of the primary tumor along with clinical, radiographic, and laboratory evidence for metastatic disease. The pathologic stage is determined by microstaging of the primary tumor and (if applicable) pathologic evaluation of a sentinel lymph node and/or completion regional lymphadenectomy.

As has held constant over the AJCC editions, the pathologic stage (in node-negative disease), and therefore prognosis, is largely determined by the depth of tumor invasion, specifically the Breslow depth. The Clark level, which uses anatomic barriers instead of raw measurements for tumor depth, is often reported but no longer required for staging. Ulceration continues to be a strong negative prognostic parameter as it upstages the pathologic stage a→b (1a→1b, 2a→2b, etc.). This effectively upstages the clinical stage (IA→IB, IB→IIA, etc.) as the presence of ulceration shifts the survival curve to basically overlay the curve corresponding to the next highest Breslow category without ulceration (e.g., T1b=T2a). New to the AJCC 7th edition is the addition of mitoses as a prognostic parameter. For thin melanomas (T1, <1.0 mm), the

**Table 4.6** Melanoma staging using tumor-node-metastasis (TNM) criteria

TNM classification for melanoma	
<i>Primary tumor (T)</i>	
TX	Primary tumor cannot be assessed
T1	No evidence of primary tumor
Tis	Melanoma in situ
T1	Melanomas ≤1.0 mm in thickness
T1a	Without ulceration and mitosis <1/mm <sup>2</sup>
T1b	With ulceration or mitoses ≥1/mm <sup>2</sup>
T2	Melanomas 1.01–2.0 mm
T2a	Without ulceration
T2b	With ulceration
T3	Melanomas 2.01–4.0 mm
T3a	Without ulceration
T3b	With ulceration
T4	Melanomas >4.0 mm
T4a	Without ulceration
T4b	With ulceration
<i>Regional lymph nodes (N)</i>	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	1 node
N1a	Micrometastasis <sup>a</sup>
N1b	Macrometastasis <sup>b</sup>
N2	2–3 nodes
N2a	Micrometastasis <sup>a</sup>
N2b	Macrometastasis <sup>b</sup>
N2c	In-transit met(s)/satellite(s) <i>without</i> metastatic nodes
N3	Clinical, 1 node with transit met(s)/satellite(s); pathologic, four or more metastatic nodes, or matted nodes, or in-transit met(s)/satellite(s) <i>with</i> metastatic node(s)
<i>Distant metastasis (M)</i>	
M0	No distant metastasis (no pathologic M0; use clinical M to complete stage group)
M1	Metastases present
M1a	Metastases to skin, subcutaneous tissues, or distant lymph nodes
M1b	Metastases to lung
M1c	Metastases to all other visceral sites or distant metastases to any site combined with an elevated serum LDH

<sup>a</sup>Micrometastases are diagnosed after sentinel lymph node biopsy and completion lymphadenectomy (if performed)<sup>b</sup>Macrometastases are defined as clinically detectable nodal metastases confirmed by therapeutic lymphadenectomy or when nodal metastasis exhibits gross extracapsular extension

presence of mitoses bumps the stage, T1a→T1b. All of these elements are now included in standard pathology synoptic reports.

Outside of patients with distant metastases (M1 patients), the single most important predictor of survival is the regional lymph node status. Melanoma first metastasizes to the sentinel lymph node, and therefore, evaluation for tumor in this lymph node is an effective mechanism for determining prognosis. A single positive lymph node (N1), however defined, is stage III, with approximately 60 % 5-year survival. A lymph node is considered positive if there are satellite or in-transit metastases, or pathologic evidence of melanoma in the lymph node itself, as determined by routine H&E sections *or* immunohistochemistry, with melanocyte-specific antibodies such as Melan-A or HMB-45. There is no tumor burden cutoff defining a positive lymph node in melanoma, unlike in breast cancer, where a micrometastatic tumor burden of <0.2 mm is considered as “isolated tumor cells” and NOT considered a positive node. Of note, regarding the evaluation of a sentinel lymph node, there is no standard for the number of levels needed to be viewed or the immunohistochemical markers or sequence of markers to use. In our practice, we bisect all material submitted as “sentinel node” along the long axis, regardless of the number of lymph nodes, and generate one H&E slide. In negative or suspicious cases, immunohistochemistry for MART-1, SOX10, and gp100 (HMB-45) is performed. Other laboratories will cut one or multiple H&E sections with interval unstained slides. The unstained slides will be used for immunohistochemistry (some combination of S100, MART-1, HMB-45, or a cocktail) if the original H&E slide is suspicious or negative. Other variants on this theme are available in the literature [77, 78].

Notably absent in the 7th edition *AJCC Cancer Staging Manual* is the inclusion of molecular criteria. There can be many reasons for this, including lack of sufficient data to show a clear independent impact on patient survival, insufficient numbers of patients for statistical significance, the molecular test is cost prohibitive or not considered standard of care for the evaluation of the tumor, etc. The drive to incorporate molecular data into prognostic considerations continues as our knowledge of the biology of melanoma increases and new more robust molecular assays are developed.

#### 4.4.1 Molecular Evaluation of the Sentinel Lymph Node

Logic tells us that if sentinel lymph node status is the most important predictor of survival in the absence of distant metastases, finding any tumor burden in the node, however small and by whatever method, would predict a worse prognosis. Moreover, given the fact that some patients with negative sentinel lymph nodes (as determined by standard methods) do progress to metastatic disease, it is logical to assume that at least some of these nodes were falsely negative due to sampling (alternatively, these patients' tumors could be "pure hematogenous" spreaders). Our current process for evaluating lymph nodes is not infallible. Overdiagnosis of metastatic deposits can lead to unnecessary aggressive treatment with associated morbidity, and underdiagnosis can misclassify patients according to prognosis and, in some cases, may prevent a complete lymph node dissection and potentially impact outcome. There are histologic and immunophenotypic features that help the pathologist diagnose metastatic melanoma, but interpretation can be impacted by sampling (grossing and/or microtome cutting), un-evaluatable small deposits, artifacts introduced by collection and/or processing, and benign nodal nevi, among others.

There is a whole body of literature devoted to PCR and RT-PCR evaluation of sentinel lymph nodes [79–83]. In general, as expected, using these molecular approaches has increased the sensitivity for detecting a positive sentinel lymph node, and the more markers used, the higher the sensitivity. One of the main barriers to their widespread use, however, is the lack of a good melanoma-specific marker. The majority of studies use RT-PCR to look for transcripts of tyrosinase, Melan-A, MAGE-A3, gp100, or some combination of these or with others. In breast cancer, sentinel lymph node evaluation by molecular means is facilitated by lack of keratin-positive cells in the normal lymph node. With melanoma, however, finding nodal nevi during routine evaluation of sentinel lymph nodes is a common occurrence, in fact more common in melanoma patients than in breast cancer patients, and therefore melanocyte-specific testing will lead to false-positive results and impact specificity [84].

In addition to the presence of transcripts from benign nevi, there are other sources of false-positive results. Contamination (or carryover) is a potential issue with all amplification procedures. Additionally, the assay may be "too sensitive," meaning it is detecting melanocyte-specific transcripts with no biologic consequence. This may occur with detection of transcripts from dead or dying cells, within macrophages ingesting cells, or perhaps live but biologically inert or dormant tumor cells [85]. False-negative results may occur with contaminated, degraded, or inhibitor-laden samples. Additionally, the tumor may no longer express the target or may be absent in the sample due to sampling artifact. There are other complicating factors that have prevented widespread implementation of molecular assays for the evaluation of the sentinel lymph node. Among them are the lack of standardized methodology including choice of targets, questions about specificity given the conflicting published results when compared with immunohistochemistry, and logistical concerns including tissue procurement (where and how much within node), storage, validation on frozen samples, and personnel, among others.

Nonetheless, the increased sensitivity and the potential short turnaround time and low cost remain the driving forces behind the development of these molecular tests. Additionally, new potential melanoma-specific markers are continually being discovered and evaluated, offering hope to bolster specificity [86]. Other directions have been explored too, including looking for a signature chromosomal aberration in the lymph node to distinguish metastases from nodal nevi [58] and looking for signature mutations to do the same, although the latter is complicated as *BRAF* V600E has been shown to be present in nodal nevi [87], forcing investigators to look for alternative options.

#### 4.4.2 Chromosomal Aberrations by FISH

Standard prognostic tests have been developed in other organ systems, one example being the detection of copies of *ERBB2* (*HER2/NEU*) for

breast cancer. Recent studies have identified several potential prognostic markers based on FISH-detectable copy number changes in primary melanoma samples. In a study of 97 primary melanomas with at least 5 years of follow-up data, Gerami et al. evaluated eight FISH probes directed to chromosomes that are commonly gained or lost in melanoma to assess the prognostic power of these chromosomal aberrations. Gains in 11q13 (*CCND1*) or 8q24 (*MYC*) were linked to poor prognosis and were second only to ulceration in their ability to predict metastasis using multivariate logistic regression analysis [61]. Amplification of topoisomerase I is another potential marker for poor prognosis, warranting further study [60]. Currently, FISH analysis is not routinely performed for the purposes of risk stratification.

#### 4.4.3 Ocular Melanoma

Uveal melanoma is the most common intraocular malignancy. There are chromosomal aberrations and mutations in signaling molecules that are highly associated with uveal melanoma. For example, aberrations in chromosomes 1, 3, 6, and 8 are commonly found in uveal but not cutaneous melanomas. *GNAQ/GNA11* mutations are present in uveal melanomas and blue nevi, but not cutaneous melanoma. Loss of the tumor suppressor gene *BAP1* (BRCA1-associated protein 1, located on 3p21.1) is also observed in uveal melanomas. To date, however, identification of these defects has been important in understanding the biology of the tumor, but there is a limited role for using molecular detection assays for the purpose of diagnosis or guiding therapy. Current applications focus more on prognosis. The main negative prognostic features for uveal melanoma include posterior location (ciliary body, choroid), larger tumor size, extrascleral invasion, epithelioid morphology, increased mitoses, monosomy 3 (and loss of heterozygosity of *BAP1*), 8q gain, and a “class 2” gene expression profile [88–90]. Monosomy 3 in primary uveal melanomas is highly predictive of metastases. Testing for class 1/2 gene expression profiles is now commercially available as DecisionDx-UM

(Castle Biosciences, Inc.) [91]. Testing for chromosomal copy number alterations, by FISH, for example, is not routinely performed.

#### 4.4.4 Other Molecular Prognostic Biomarkers

This is an area of intense investigation, beyond the scope of this text, and reviews on the topic are available [92–95]. Molecular tests for prognosis are largely restricted to the research setting, but a few commercially available tests have emerged. One example is DecisionDx-MELANOMA (Castle Biosciences, Inc.), which is a quantitative RT-PCR-based melanoma gene expression profile analysis of 31 genes [96]. By quantitatively analyzing the expression levels of these genes in the primary melanoma, this assay effectively stratifies node-negative stage I and II melanoma patients into low-risk (class 1) and high-risk (class 2) groups. Class 1 individuals virtually have a 100 % 5-year metastasis-free survival compared to 38 % for class 2 individuals, providing valuable data to the patients and their physicians. While there is no current data to suggest that altering the management of class 2 (poor prognosis) patients can impact their survival, this direction is being investigated.

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### 4.5 Practical Considerations for Ordering and Performing Molecular Tests

#### 4.5.1 Genetic Testing for Familial Melanoma

Not all melanoma patients should be tested for a germline mutation for the reasons discussed (see Assessing Risk section). Example guidelines for screening individuals are presented in Table 4.2. If an individual meets these criteria, a discussion with him or her and referral to a genetic counselor should occur before testing. Testing is achieved with a blood draw, or buccal swab/saliva, and not from biopsy material. If a family signature mutation has not been

established, the specimen is ideally from the patient with the melanoma, in order to increase the likelihood that any detected mutation is the oncogenic driver of the melanoma and not a spurious/incidental finding. Without a signature mutation, a negative result has little meaning.

Most laboratories perform complete sequencing of the *p16<sup>INK4A</sup>* region. The cost of sequencing ranges from \$700 to \$1,000 and, in many cases, is covered by insurance. Some laboratories offer “discounts” if the mutation is already known, allowing for more focused testing. In patients with a very high pretest probability of a positive result but a negative test, sequencing of other areas such as *CDKN2A* exon 1β (*p14<sup>ARF</sup>*), *CDKN2A* intronic regions, and *CDK4* can be performed, with an additional cost. Testing for *BAP1* and *TERT* mutations can also be considered, but familial mutations of these genes are rare and testing is not widely available. Testing for *MCIR* variants is not routinely performed. A list of laboratories that offer genetic testing for *CDKN2A*, *CDK4*, and others is available at [www.genetests.org](http://www.genetests.org).

Any laboratory interested in performing genetic testing needs to develop a relationship with a genetic counselor and offer their services to clinicians and their patients. As stated above, most laboratories sequence only the *p16<sup>INK4A</sup>* exons within *CDKN2A*, as this will cover >90 % of known mutations, with reflexing to testing of other known mutational regions if the original testing is negative. Testing can be achieved by standard Sanger sequencing, but most laboratories are moving to high-throughput (next-gen) platforms. Next-gen sequencing may obviate the use of reflex testing as all mutation candidates can be assayed in parallel.

#### <sup>1</sup>CPT coding

81404 is the current code for sequencing all *CDKN2A* exons. This code applies to analy-

sis of 2–5 exons. This would cover the entire *CDKN2A* gene, but many laboratories choose only to analyze the p16 region (exons 1α, 2, and part of 3). *CDK4*, *MCIR*, and other familial melanoma genes are currently under 81479, which is the unlisted molecular procedure code.

#### 4.5.2 CGH Versus FISH

From a practical standpoint, the decision to use FISH or CGH to help with diagnosis largely resides with the dermatopathologist. That is not to say, however, that the dermatologist or other clinician should not be just as informed of these tools. In fact, it is not unreasonable for the clinician to engage in a conversation over performing one of these tests whenever they receive a report suggesting an “indeterminate” biology of a lesion or a report tagged with a “238.2” ICD (International Statistical Classification of Diseases and Related Health Problems) code. Because both tests are performed on paraffin-embedded tissue, these tests can be added on at any point, without requiring collection of new tissue.

If ancillary testing is desired, there may be confusion on whether CGH or FISH is most appropriate. Because these assays are gathering different types of information, there is the possibility that performing both tests on the same lesion would be appropriate, in certain settings, but with the caveat that they could yield discrepant results [40]. Therefore, it is important to understand their limitations in order to select the most appropriate assay.

In these assays’ current forms, for most lesions, FISH is likely the better choice and is currently more widely used. This could change, however. The advantages of FISH include its applicability to a more broad set of lesions, its shorter turnaround time, and its current possible cost edge (with insurance coverage) (Table 4.7). For example, because a relatively large and pure tumor load is required for CGH, this assay performs poorly with superficial tumors and tumors with mixed populations [53]. Some laboratories require a minimum of 0.4–0.5 mm of invasion to

<sup>1</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [97]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

**Table 4.7** Comparison between FISH and CGH

	FISH	CGH
Availability	Becoming more widely available	Currently not widely available
Turnaround time	Several days–week	Weeks–month
Cost	Expensive (\$500–\$1,500)	Currently more expensive (approx. \$1,500–\$2,500) but decreasing
Insurance coverage	Covered by most insurance plans	Insurance coverage variable
Extent of evaluation	Evaluates only probed loci	Evaluates entire genome (potentially)
Scope of detection	Detects amplifications, deletions, potentially balanced translocations, inversions	Detects amplifications, deletions, <b>not</b> balanced translocations, inversions
Sensitivity and Specificity	Fairly high sensitivity and specificity	Lower sensitivity, higher specificity
Tissue requirements	Tumor can be smaller in size, needs minimum of 25–50 dermal cells to score	Needs abundant and pure DNA and may require microdissection. Requires $\geq 0.5$ mm of invasion
Localization of cells with defects	Can be focused on morphologically atypical cells	Abnormalities from normal and atypical cells are mixed and cannot be distinguished

run CGH. FISH has the important advantage of being able to evaluate the chromosomal makeup of cells that are *histologically* atypical. Using 4',6-diamidino-2-phenylindole (DAPI) to counterstain the nucleus, fluorescent signals can be evaluated while the overall morphology of the cell remains visible and intact, allowing analysis of small or mixed areas with focus on the atypical cells. This allows FISH to have a potentially (and paradoxically) higher sensitivity than CGH even though only four regions of the genome are being targeted, and this may partially explain reports of discrepant results between FISH and CGH when evaluating the same lesion [40].

While FISH clearly has advantages over CGH, newer generations of array CGH (aCGH) have narrowed the gaps on some of these points, and there appears to be a resurgence of aCGH use. Because aCGH evaluates the entire genome compared to only four targeted areas for FISH, it is not unreasonable to predict that there will be cases of melanoma that are false negative by FISH but positive by CGH. On this point, other specific loci (e.g., 11p gains in Spitz nevi) can be assessed. There will also be cases with borderline or ambiguous FISH results that may become more clearly characterized by aCGH analysis. And because aCGH requires such a high percentage of cells containing an abnormality to be considered positive, the test has an attractive inherent

high specificity. As the cost and user-friendliness of aCGH continues to improve, it may become more widely implemented, and the next major hurdle to overcome will be interpretation of the immense data it generates.

Although the temptation to reflexively use the melanoma FISH assay (and/or CGH) is strong due to the repercussions of under- and overdiagnosis of melanoma, the use of these assays should be restricted to a very small subset of pigmented lesions. An example of one laboratory's utilization data of ancillary studies, specifically immunohistochemistry and FISH, for the diagnosis of pigmented lesions is shown in Table 4.8 (for reference only, not a guide). For routine biopsies of pigmented lesions from the community, immunohistochemistry was used in approximately 2.4 % of cases, and FISH in 0.03 %, or roughly 1 in 100 of the atypical lesions that required immunohistochemistry. The utilization of FISH increased to 0.9 % of pigmented lesions submitted for consultation. Other authors have reported using FISH in 1 % of lesions biopsied to rule out melanoma. With the cost of these assays (which can range between several hundred and a thousand dollars) and limitations on sensitivity/specificity, these assays have the highest impact on histologically ambiguous lesions which extend into the deep dermis. These lesions have such divergent possible biologic behaviors that

**Table 4.8** Utilization of immunohistochemistry and FISH for the evaluation of 50,000 pigmented lesions at one laboratory

	Routine biopsies	Consultation biopsies	Total
Immunohistochemistry	1,168 (2.4 %)	86 (8.2 %)	1,254 (2.5 %)
FISH	15 (0.03 %)	9 (0.9 %)	24 (0.05 %)
Total	49,522	1,049	50,571

Biopsies were evaluated at ProPath in Dallas, TX. A set time period in 2013 was evaluated to include approximately 50,000 histologically diagnosed nevi, atypical nevi, and melanomas. These were included in the calculation, regardless of clinical impression

the importance of diagnostic accuracy alone justifies the test. Management ranges from watching or conservative re-excision to wide excision with sentinel lymph node procedure and potential adjuvant therapy. And the importance of diagnostic accuracy is magnified in these lesions with the emergence of effective targeted therapy and immunotherapy [66].

One should not lose sight of the fact that FISH and CGH are ancillary tests. Not unlike immunohistochemistry for melanoma, the performance characteristics cannot justify these as stand-alone tests (see Fig. 1.2 for elaboration). The FISH and CGH data must be incorporated into the entire picture, including the clinical scenario, morphology, and immunohistochemical profile. When considering the test, it is important to gauge the pretest probability for melanoma and have an idea of how a positive or negative result will impact the final diagnosis. In our practice, cases that are very concerning for melanoma (by clinical, morphologic, and/or immunohistochemical features) and have a positive FISH result are called melanoma. Cases with low suspicion (a diagnosis of an atypical nevus, Spitz, or otherwise) and a negative FISH are signed out as a type or variant of nevus. When the FISH result is not in complete concordance with the other data and our level of suspicion, we will often (and admittedly unsatisfactorily) release these cases as lesions with “indeterminate biology,” leaving management up to a risk/benefit conversation between the clinician and patient. A similar approach to using FISH has been suggested by others

[41, 47]. CGH is used similarly, but due to its lower sensitivity and inherent high specificity, more weight is given to a positive result. While FISH and CGH have improved our diagnostic accuracy of melanoma and reduced the pool of ambiguous melanocytic lesions, a population of melanocytic lesions that cannot be clearly classified will continue to exist.

For dermatopathologists considering offering FISH or aCGH, there are some points to consider. With FISH, there are several permutations. The most common scenario is to send a paraffin-embedded tissue block and/or cut slides (typically 5 µm sections) to a third party that will perform and interpret the test. A third party may also offer to perform the test, score the cells, and then return the data to the dermatopathologist to interpret and incorporate into his/her report (a so-called technical component/professional component split). Alternatively, more laboratories worldwide are bringing up a version of the melanoma FISH test in-house, and kits are available internationally.

As a note of caution for developing an in-house melanoma FISH test, FISH requires a high level of expertise, both on the parts of the cytogenetics/FISH technologist to process tissue and score the cells appropriately as well as the dermatopathologist to interpret the results and navigate through false positives and negatives. The melanoma FISH assay is performed on paraffin and thus is an example of paraffin-embedded tissue FISH, or PET-FISH. While FISH on single-cell spreads, cytologic smears, and aspirates allows for the analyzed cells to align in a

single plane, with PET-FISH, cells are embedded within wax, with a third dimension, introducing some technical challenges. There may be overlapping nuclei or portions of nuclei, which can cause the probes to misrepresent the true number of copies within a single cell. For this reason, the cutoffs for positive results in PET-FISH can be uncomfortably high (see Table 4.5) and are routinely much higher than for non-PET-FISH. Newer generations of the melanoma FISH assay have addressed some of the technical challenges, such as interpreting tetraploidy [39], but new issues will surface, and so the expertise requirement should not be taken lightly. And as with any new test, internal validation is required to establish one's own performance characteristics, including cutoff values, analysis of receiver operating characteristic (ROC) curves, sensitivity, specificity, etc.

#### **<sup>2</sup>CPT coding**

FISH probe codes include 88365 or 88367 (quantitative with computer assistance) or 88368 (quantitative, manual) (all are per probe) or the combination of 88274 (per probe) and 88291, depending on how the test is performed and interpreted. CGH has no code yet and therefore falls under 81479, which is the unlisted molecular procedure code.

### **4.5.3 Mutational Analysis of Melanoma Signaling Molecules and Gene Expression Profiling**

To date, molecular testing for mutations in signaling molecules (*BRAF*, *NRAS*, *KIT*, etc.) has no role in the diagnosis of melanoma and should

<sup>2</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [97]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

not be performed for that purpose. These assays are becoming very important for therapeutic selection and therefore are covered in the next chapter (Chap. 5). Gene expression profiling has only recently become commercially available (myPath, Myriad Genetics, Inc. Salt Lake City, UT). This approach has potential for generating important data for differentiating nevi from melanoma, but little is currently known about its true utility as an ancillary test.

#### **<sup>3</sup>CPT coding**

All testing performed in this section currently falls under 81479, which is the unlisted molecular procedure code.

### **4.5.4 Prognostic Testing**

For the most part, molecular testing for prognosis is not routinely performed outside the investigative arena, with the few exceptions (like DecisionDx-MELANOMA) outlined above. Prognostic assays are potentially important for providing patients and their physicians with additional data for future planning. Before ordering or designing a prognostic test, however, we should ask ourselves what we will do with the information. Is the information “clinically actionable”? In the future, patients with more aggressive tumors may opt for more aggressive therapy up front, potentially impacting their survival, but currently there is no data to support this correlation.

#### **<sup>3</sup>CPT coding**

All testing performed in this section currently falls under 81479, which is the unlisted molecular procedure code.

<sup>3</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [97]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

## 4.6 Classification of Melanoma: Current and Near-Future Perspectives

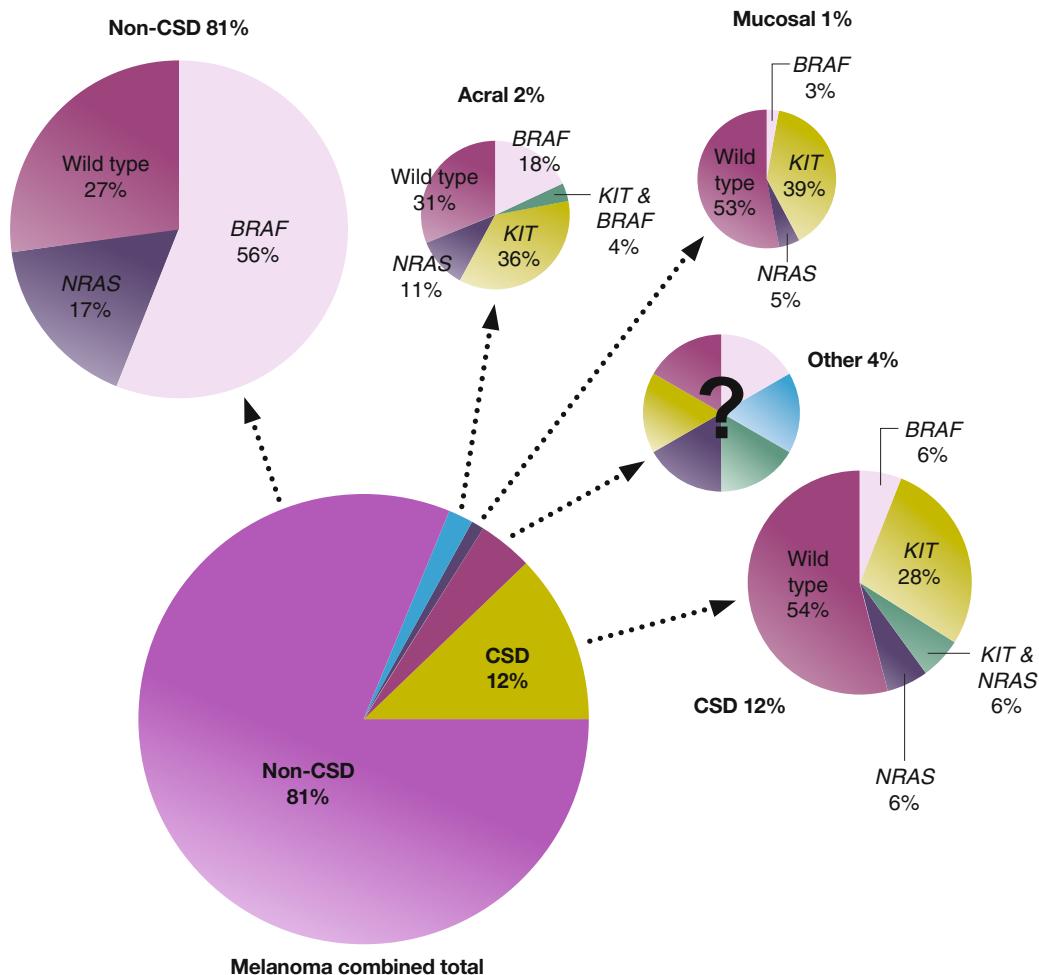
It is crystal clear that melanoma is not one disease, but a complex array of different tumor types, with, in many cases, the only apparent common trait being the cell of origin. Conventionally, melanoma is divided into subgroups based on histomorphologic and, to some extent, clinical features—superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, and acral lentiginous melanoma [98]. Desmoplastic melanoma is also recognized as a distinct subtype [99]. This has been convenient as it allows carving of the melanoma pie into smaller sections to provide some understanding of the biology of the tumor and prognosis of the patient. Until now, however, treatment strategies have been largely surgical and virtually independent of melanoma subtype. As the molecular signaling pathways are uncovered, melanoma will likely be better classified based on its mutational profile and predicted response to therapy. The melanoma pie may need to be re-carved, perhaps into more or resized slices.

It is likely that melanoma classification will undergo this facelift, not unlike other fields (e.g., hematopathology). With leukemias and lymphomas, classification schemes now integrate morphology, immunophenotype, and molecular data. Some tumor types, like promyelocytic leukemia, were previously lumped with others but are now separated based on a molecular event that defines them and, in some cases, an event that drives therapeutic decision making.

Proposals to reclassify melanoma based on molecular profile and degree of UV exposure have already begun. Using aCGH, Curtin et al. looked at DNA copy alterations and mutational status of *BRAF* and *NRAS* on 126 melanomas divided into four categories based on UV exposure: tumors from chronically sun-damaged skin,

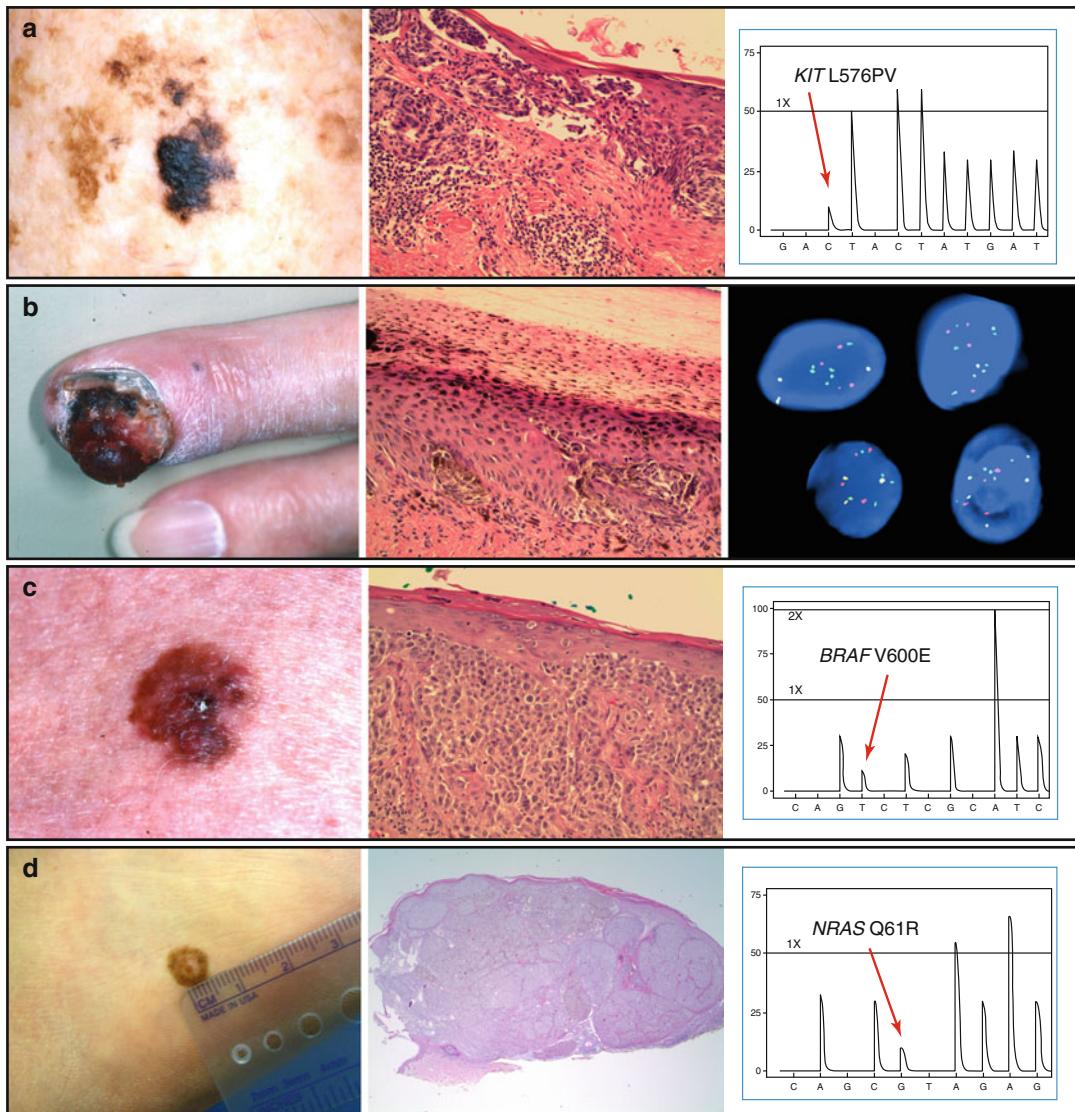
skin without chronic sun damage, acral sites, and mucosal sites [67]. In this study, the mutational status of *BRAF* and the DNA copy number of certain loci (specifically *CDK4* and *CCND1*) could accurately classify the tumor into one of the four categories. Furthermore, 81 % of tumors from skin without chronic sun damage had mutations in *NRAS* or *BRAF*, whereas this was uncommon in the other categories. A follow-up study analyzed 102 melanomas by aCGH to specifically look for DNA copy number changes and candidate oncogenes in the tumors with wild-type *BRAF* and *NRAS* [100]. In this study, *KIT* mutations and/or copy number changes were identified in 39 % mucosal melanomas, 36 % acral melanomas, 28 % melanomas on chronically sun-damaged skin, and 0 % in skin without sun damage (Fig. 4.11) [67, 100, 101]. Subsequent studies have expanded on this idea with the following conclusions: *BRAF* mutations are frequent in superficial spreading melanoma (and melanomas from non-chronically sun-damaged skin), while *NRAS* mutations are frequent in nodular melanomas (and melanomas from chronically sun-damaged skin) in a large meta-analysis [102], *BRAF* mutations are not seen in desmoplastic melanoma [103], *BRAF* mutations but not *NRAS* or *KIT* mutations are present in spindle cell melanomas [104], and others.

Based on these studies and more, it does not require a big leap to imagine new classification schemes for melanoma. With conventional classification relying on histomorphology and anatomic site, newer approaches may classify melanomas based on UV exposure and degree of sun damage, the mutational status of *BRAF*, *NRAS*, and *KIT*, or likely, a combination of all of the above (Fig. 4.12). Detailed descriptions of the melanoma signaling pathways and therapeutic strategies for shutting down oncogenic drivers within these pathways are provided in Chap. 5.



**Fig. 4.11** Mutational frequency of signaling molecules in melanomas with different UV exposure. Mutations in *BRAF*, *NRAS*, and *KIT* occur with variable frequencies depending on the type of melanoma. Melanomas can be categorized based on anatomic site and sun exposure (largest pie). The size of the other pies varies to reflect different contributions to the overall melanoma pie (not to scale). The “other” category includes melanomas not localized to specific anatomic sites and a mixture of

poorly characterized and poorly reported tumors, such as desmoplastic, Spitzoid, and spindle cell melanomas, among others. Few tumors contain more than one activating mutation (between *BRAF*, *NRAS*, and *KIT*). Wild type refers to lack of mutations in only *BRAF*, *NRAS*, and *KIT* (Data are compiled from several references [67, 100, 101]). CSD chronically sun-damaged skin, non-CSD non-chronically (or intermittently) sun-damaged skin



**Fig. 4.12** Evolution of the classification of melanoma. Conventional clinical and histologic melanoma classifications of lentigo maligna melanoma (**a**), acral lentiginous melanoma (**b**), superficial spreading melanoma (**c**), and nodular melanoma (**d**) may ultimately be replaced by classifications incorporating clinical (*left panels*),

histologic (*middle panels*, H&E), and molecular (*right panels*, sequencing and FISH) data. These tumors could be potentially renamed as *KIT*-mutated melanoma (**a**), *CCND1*-amplified melanoma (**b**), *BRAF*-mutated inhibitor-responsive melanoma (**c**), and *NRAS*-mutated melanoma (**d**), just as examples

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## Melanoma. Part II. Personalized Medicine: Using Molecular Tools to Guide Targeted Therapy

### Contents

5.1	<b>Introduction.....</b>	98
5.2	<b>Melanoma Tumor Progression.....</b>	99
5.3	<b>Melanoma Signaling Pathways and the Biology of Melanoma.....</b>	100
5.3.1	MAP Kinase Pathway .....	102
5.3.2	KIT .....	104
5.3.3	PI3K/AKT/mTOR Pathway.....	105
5.3.4	Others.....	106
5.4	<b>Clinical Trials and Therapeutic Strategies.....</b>	106
5.4.1	Signaling Molecule and Pathway Inhibition.....	107
5.4.2	Immunotherapy .....	113
5.4.3	Resistance to Therapy and Clinical Relapse.....	114
5.4.4	Combination Therapy and Emerging Therapeutic Strategies.....	115
5.5	<b>Practical Considerations for Ordering and Performing Molecular Tests .....</b>	117
5.5.1	Targeted Mutation-Specific Molecular Assays .....	117
5.5.2	Immunohistochemistry .....	124
5.5.3	Companion Testing: The New Reality?.....	125
5.6	<b>Summary.....</b>	126
	<b>References.....</b>	127

### Key Points

- The treatment for advanced melanoma has evolved from ineffective cytotoxic chemotherapy to approaches tailored to an individual's tumor. Tying treatment selection to mutational analysis (or other molecular diagnostic assays) has emerged into a new medical discipline termed theranostics.
- The MAP kinase and PI3K/Akt/mTOR signaling pathways are commonly activated in melanoma, providing attractive candidates for therapeutic targets.
- The *B-Raf<sup>V600E</sup>* mutation is the most common mutation in melanoma, occurring most frequently in melanomas from intermittently sun-damaged skin.
- Mutations in *NRAS* occur in a similar subset of melanomas as mutations in *BRAF*, but at a lower frequency. N-Ras may play a role in resistance mechanisms to B-Raf inhibitor therapy.
- KIT* mutations occur with relatively high frequency in acral melanomas, primary mucosal melanomas, and melanomas from chronically sun-damaged skin.
- The FDA has recently approved three new therapies directed at inhibiting signaling pathway molecules in advanced melanoma patients: vemurafenib (*B-Raf<sup>V600E</sup>* inhibitor), dabrafenib (*B-Raf<sup>V600E</sup>* inhibitor), and trametinib

(MEK inhibitor). Many more agents, including those directed toward mutated Kit, are in clinical trials as monotherapies or in combination therapies and have shown promising early results.

- Because signaling molecule mutation status predicts response to these therapies, effective diagnostic tests are required. Activating mutations in the *BRAF* and *NRAS* genes are easily detected by molecular assays as they are fairly consistent and localized. *KIT* mutations are spread across multiple exons, requiring more complex testing.

diminishing quality of life during that final year. A common thread between these treatment strategies, and contributing to their minimal success, is lack of tumor specificity. These agents act by targeting rapidly dividing cells or modulating the immune system, but are not specific to the individual melanoma. With the onset of melanoma theranostics, these statistics will undoubtedly improve.

*Theranostics* refers to a new discipline in the era of personalized medicine, combining therapy with diagnostics. Specifically, therapeutic decisions are based on diagnostic tests for an individual. In the context of oncology, theranostic success usually hinges upon the presence of a detectable defect within the tumor, a defect that is specifically responsible for driving the cell to malignancy (“oncogenic driver”). Additionally, that defect must result in an activated protein that can be blocked, or inhibited, by a designed targeted therapy. There have been several success stories outside of dermatology. Chronic myelogenous leukemia (CML) has consistent breakpoints in the tumor genome leading to the t(9;22) translocation, or Philadelphia chromosome. This results in the *BCR-ABL* fusion gene, which encodes for a constitutively active tyrosine kinase. The translocation can be detected by FISH or RT-PCR methods. Small-molecule inhibitors, imatinib mesylate (Gleevec), and later-generation molecules, dasatinib and nilotinib, were developed to dampen the activity of the fusion gene and effectively treat the disease in this population [3]. Another example is with breast cancer. A subpopulation of breast cancer patients has amplification of the gene *ERBB2* (*HER2/NEU*), leading to oncogenesis. This can

## 5.1 Introduction

For centuries, palliative surgery was the only treatment option for the advanced melanoma patient (refer back to Fig. 4.1, for timeline). More recently, standard therapy for advanced disease includes cytotoxic chemotherapeutic agents, specifically dacarbazine (DTIC) and temozolomide, interferon- $\alpha$ , and high-dose interleukin-2 (IL-2). Prior to 2011, only DTIC and high-dose IL-2 were FDA approved for this purpose [1, 2] (Table 5.1). While these regimens will initially shrink the total tumor burden, there is minimal impact on overall survival. The median survival for stage IV disease remains a dismal 1 year, and as few as 10 % of patients are long-term survivors. Moreover, these treatment regimens are hampered by a high toxicity profile, severely

**Table 5.1** FDA-approved treatments for advanced-stage melanoma

Therapy	Brand name	Mechanism	Year approved
Dacarbazine	DTIC-Dome	Cytotoxic chemotherapy	1975
Aldesleukin	Poleukin	High-dose IL-2	1992
Ipilimumab	Yervoy	Anti-CTLA4 immunotherapy	2011
Vemurafenib	Zelboraf	B-Raf <sup>V600</sup> inhibitor	2011
Dabrafenib	Tafinlar	B-Raf <sup>V600</sup> inhibitor	2013
Trametinib	Mekinist	MEK inhibitor	2013
Lambrolizumab	–	Anti-PD-1 immunotherapy	2013

As of January 2014, with more in progress

be detected by FISH. An immunotherapy, trastuzumab (Herceptin), effectively treats this subpopulation [4].

Melanoma has been a little late to the theranostic party, largely due to the incomplete understanding of the molecular events leading to melanomagenesis. In the past, melanoma was considered a single disease, and because of this, the quest for the therapeutic magic bullet had gone unfulfilled. In the last few decades, however, great strides have been made with the understanding of signaling pathways within the melanocyte and corresponding mutations leading to malignant transformation. Examples include activating mutations in *BRAF*, *NRAS*, and *KIT*, among others, which encode key cellular signaling pathway molecules. These discoveries have helped fill some molecular gaps within our tumor progression models, allowing them to begin to emulate models in other organ systems, like colon cancer [5]. These strides in basic research have also enlightened us to the molecular heterogeneity of melanoma. We now recognize that not just anatomic site, UV exposure, and histologic features *but also specific molecular events* impact tumor biology. These exact molecular events now provide a rationale for therapeutic innovation, and “designer drugs” have now become a reality.

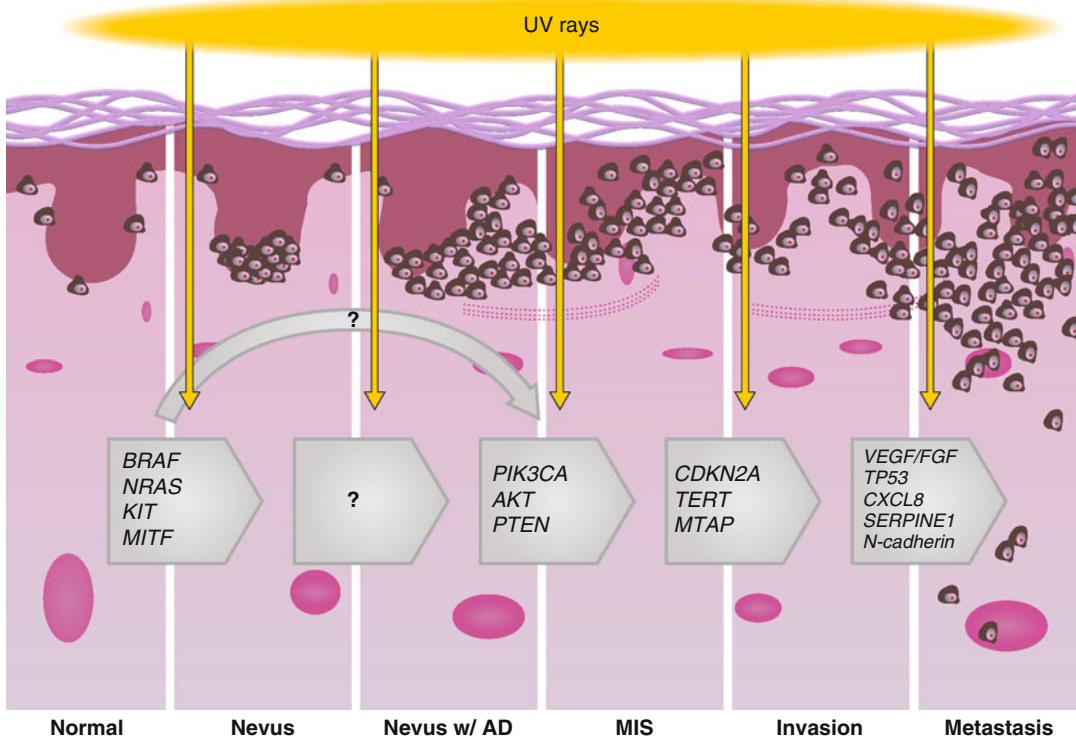
Chapter 4 began the discussion on melanoma by reviewing the use of molecular assays to assess patient risk for melanoma, to diagnose and reclassify melanomas, and to predict tumor behavior and prognosis. This chapter continues the discussion, highlighting, in sequence, the main molecular signaling pathways in melanoma, current therapeutic strategies based on the tumor’s mutational status, and applicable mutation-detection assays. The year 2011 marked the beginning of the melanoma theranostic era, with the FDA approval of vemurafenib, a small-molecule inhibitor to mutant B-Raf<sup>V600E</sup>, detectable in approximately half of all melanomas. The ability to detect this molecular defect combined with the ability to effectively use therapy targeted to the mutant protein defines melanoma theranostics. Two more inhibitor therapies for mutant-B-Raf<sup>V600</sup> melanoma—dabrafenib and trametinib—are now also FDA approved, and

many more examples are in the pipeline. Indeed, personalized medicine for the advanced melanoma patient is now a reality, leaving behind the era of cytotoxic chemotherapy.

## 5.2 Melanoma Tumor Progression

Tumor progression models are well characterized in other tumors, such as colorectal cancer, with a defined number of molecular “hits” required for malignant transformation [5]. An average of six “hits” has been proposed to complete the journey from a normal cell to cancer. Similar models have been proposed with melanoma, with progression from *melanocyte*→*nevus*→*atypical nevus*→*melanoma in situ*→*invasive melanoma*→*metastasis*, following a series of somatic mutations and epigenetic events (Fig. 5.1) [6, 7]. Melanoma progression models remain controversial for a variety of reasons, including an abundance of “molecular gaps” with disagreement on the number of required “hits” for transformation, clear examples of cases skipping steps, and poorly histologically defined steps (atypical/dysplastic nevi), among others. Nonetheless, development of tumor progression models remains important for understanding the molecular players in the growth/survival↔arrest/death balance of the cell, molecular events leading to uninhibited growth, and points of potential therapeutic intervention. The formal introduction of these molecular players and their roles in cellular signaling, in melanomagenesis, and as therapeutic targets are in the sections that follow (see Fig. 5.2 for an overview schematic of signaling molecules).

Within the melanoma tumor progression models, the *BRAF* mutation is considered to be an early event [8]. This is supported by the finding that the identical V600E mutation in melanoma is also observed in up to 82 % of nevi, and there is molecular evidence for clonality in nevi [9–11]. Not all nevi become melanoma, however. Only one-fourth of melanomas are estimated to arise from a nevus [12]. B-Raf<sup>V600E</sup> results in constitutive activation of the MAP kinase pathway, but it appears that, at least in some scenarios, isolated *BRAF* mutations induce senescence, possibly after a finite number of cell



**Fig. 5.1** A tumor progression model for melanoma. In concert with UV exposure, somatic mutations and epigenetic events may lead the normal melanocyte through a progression to malignancy and metastasis. *BRAF* mutations, for example, are thought to be early molecular events, as they are observed in most nevi. Not all melan-

omas progress through all stages. The role of nevi with architectural disorder (nevi w/AD) in the progression to melanoma remains controversial. Listed mutations are theoretical. They do not always occur in sequence and are not present in all melanomas. *AD* architectural disorder, *MIS* melanoma in situ

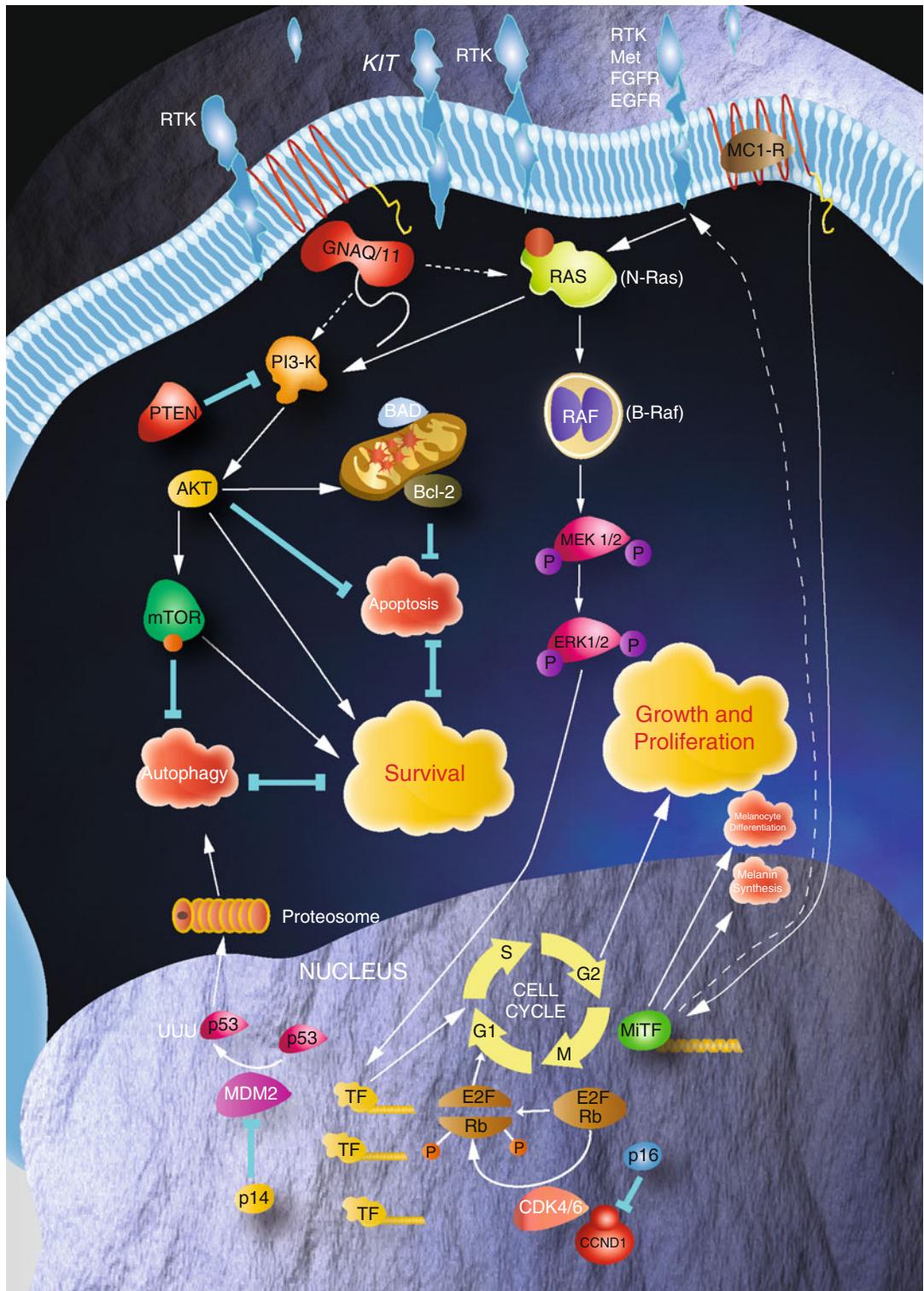
divisions, to create a nevus and not melanoma [13]. This concept has been termed oncogene-induced senescence [14, 15]. *BRAF* mutations may require another molecular ‘hit’ in an alternative signaling pathway, such as the PI3K/Akt/mTOR pathway, to transform. Otherwise, the cell undergoes oncogene-induced senescence [16, 17]. While *BRAF* and *NRAS* mutations are common in melanoma (40 % of *in situ* tumors and 81 % of invasive melanomas), both are not required for malignancy as their mutations are largely mutually exclusive of one another. Mutual exclusivity is typical for proto-oncogenes in the same signaling pathway. *NRAS* mutations may be more powerful oncogenic drivers than *BRAF* mutations since N-Ras is upstream and can activate both MAP kinase and PI3K/AKT/mTOR pathways, effectively causing a “two-for-one hit.”

While a stepwise progression predicted by the model may occur in some tumors, it is clear that melanocytes can find many different paths to

melanoma. Furthermore, melanoma has a fairly unique and stubborn ability to arrest its progression in its later stages. In melanoma patients disease-free at 10 years post diagnosis (of invasive melanoma), approximately 7 % will have metastases at 15 years and 11 % at 20 years [18]. The exact roles of these signaling molecules and their mutational or epigenetic events in the progression from normal melanocyte to metastatic melanoma continue to be investigated.

### 5.3 Melanoma Signaling Pathways and the Biology of Melanoma

In order to understand the strategies behind melanoma therapeutics, it is necessary to delve into the signaling pathways of the melanocyte. Figure 5.2 depicts a detailed, albeit still oversimplified,



**Fig. 5.2** Signaling pathways in melanoma. Depicted simplified cellular signaling pathways are commonly activated in melanoma, leading to an imbalance of autophagy, apoptosis, proliferation, and survival signals, resulting in

uninhibited growth. Activating mutations of some components—Ras (N-Ras), Raf (B-Raf), Kit, and GNAQ/11—frequently occur in melanoma. RTK receptor tyrosine kinase, TF generic transcription factors

schematic of key signaling pathways for melanoma. *B-Raf*, *N-Ras*, and *Kit* are spotlighted, as activating mutations in these molecules occur with relatively high frequency in melanoma, and therefore they are attractive theranostic targets. There are many other integral molecules important in orchestrating melanomagenesis, but mutations in these molecules do not appear to be frequent events and thus are not highlighted here.

The signaling pathways can be separated into two groups: those with germline mutations (covered in Chap. 4) and those with somatic mutations. Somatic mutations account for the vast majority of molecular defects in melanoma, as is the case in most tumor types. Because these defects are not present in the germline, they are not heritable. Many of the somatic mutations are oncogenic drivers, i.e., their presence is directly related to progression to malignancy. Activation of these molecules and their pathways, by direct stimulation, mutation, loss of inhibition, or other means, allows the cell to hijack its own regulatory machinery, leading to uninhibited growth. These pathways and corresponding mutations are not all specific for melanoma as many are global players in oncogenesis.<sup>1</sup>

### 5.3.1 MAP Kinase Pathway

The MAP (mitogen-activated protein) kinase pathway is one of the main conduits for transmitting signals from the cell surface, usually through ligand binding to surface receptors, to the nucleus. Signaling through this pathway typically leads to activation of the cell, resulting in stimulation of other signaling pathways, progression through the cell cycle, and regulating apoptosis and survival mechanisms. Cellular activation begins by upstream molecules sequentially activating more

downstream molecules, and, in most cases, through phosphorylation (i.e., phosphorylation cascade), in the direction of Ras→Raf→MEK→ERK [21]. Over 90 % of melanomas have an activated MAP kinase pathway. Of course, the linearity of this pathway suggested by the diagrams is an oversimplification. There are at least three Ras, three Raf, two MEK, and two ERK molecules. There are also elaborate checkpoints, compensatory pathways, and feedback mechanisms. Key components of this pathway are discussed.

#### 5.3.1.1 RAS

Ras (*Rat* sarcoma) is a superfamily of G proteins, specifically small GTPases [22]. There are three main gene members of its human subfamily, *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue) located on 12p12.1 encoding GTPase KRas (K-Ras), *NRAS* (neuroblastoma rat sarcoma viral oncogene homologue) located on 1p13.2 encoding GTPase NRas (N-Ras), and *HRAS* (v-Ha-ras Harvey rat sarcoma viral oncogene homologue) located on 11p15.5 encoding GTPase HRas (H-Ras). These Ras proteins are localized to the plasma membrane and function to transmit signals from a cell surface receptor, such as a receptor tyrosine kinase like Kit, to downstream signaling pathways, such as the MAP kinase and PI3K/Akt/mTOR pathways. Ras proteins are activated by binding guanosine triphosphate, or GTP. Activated Ras protein dimerizes and activates Raf in the MAP kinase pathway. This is mediated by a process called farnesylation (posttranslational addition of a prenyl group), which is also important because farnesylation is a potential therapeutic target [23]. The net effect of activated Ras is cell growth, division, and survival.

The *RAS* superfamily of genes contains the most commonly mutated oncogenes in human cancer, found in up to one-third of human malignancies [22]. Germline *RAS* mutations do exist, present in cases of Noonan syndrome (*KRAS*, *NRAS*), Costello syndrome (*HRAS*), cardiofaciocutaneous syndrome (*KRAS*), and autoimmune lymphoproliferative syndrome 4 (*NRAS*), but are not associated with melanoma (see Chap. 10, Genodermatoses, for tables containing germline

<sup>1</sup>Note: In the following discussion, currently recommended gene names and chromosomal locations are used. Short abbreviations of protein names, as defined in the text, are used for clarity. As the names of genes, their locations, and names of proteins continually evolve, the reader is directed to several websites, [www.genenames.org](http://www.genenames.org) and [www.uniprot.org](http://www.uniprot.org), for the most current data and recommendations [19, 20].

mutations in *RAS* and other signaling genes covered in this section).

In melanoma, *NRAS* is a bigger player than *KRAS* and *HRAS*. Somatic *NRAS* mutations are observed in 15–20 % of tumors, compared with a combined 3 % harboring mutations in *KRAS* or *HRAS* [15, 22, 24, 25]. The mutations in *NRAS* most commonly affect residues Q61, G12, and, less so, G13, preventing GTP hydrolysis and leading to a constitutively activated N-Ras [26]. *NRAS* is also mutated in up to 40 % of melanomas *in situ*, a subset of dysplastic nevi, and up to 81 % of congenital nevi, demonstrating its complex role in melanomagenesis [27–31].

*NRAS* mutations are more commonly observed in certain subsets of melanoma, specifically those on intermittently sun-damaged skin [32]. These tumors more closely correlate with superficial spreading and nodular histologic variants, similar to *BRAF*-mutated melanomas. *NRAS* mutations are not common in acral and primary mucosal melanomas. There is a suggestion that *NRAS* mutations predict a more aggressive biology, with reports of *NRAS*-mutated melanoma having a deeper tumor depth and higher mitotic rate at diagnosis. However, *NRAS*-mutant melanomas are not uniformly bad actors, and some may even have a better response to high-dose IL-2 [33]. Overall, the relationship between *NRAS* mutational status, morphology, and overall survival remains unclear at this time [34–36].

The other *RAS* gene family members, *KRAS* and *HRAS*, are mutated less frequently in melanoma, observed in 2 and 1 % of tumors, respectively [15]. *KRAS* is the most commonly mutated *RAS* family member in human malignancies overall, but less so in melanoma, and is a primary focus for targeted therapy for tumors of the gastrointestinal tract. *HRAS* mutations are more often associated with Spitz nevi (*NRAS* and *BRAF* mutations are not typical) [29]. *HRAS* mutations and/or copy number changes of 11p (the site of the *HRAS* gene) are observed in 10–29 % of Spitz nevi, and these lesions tend to have distinct morphologic features such as a large size and a sclerotic base [37, 38].

### 5.3.1.2 RAF

Raf (rapidly accelerated fibrosarcoma) is a family of serine/threonine-protein kinases. There are three family member genes—*ARAF*, *BRAF*, and *CRAF*—with *BRAF* (v-raf murine sarcoma viral oncogene homologue B1) located on chromosome 7q34 garnering the most attention in melanoma [39]. *BRAF* encodes serine/threonine-protein kinase B-Raf (B-Raf). These B-Raf molecules reside in the cytoplasm. B-Raf becomes dimerized, as a homodimer (B-Raf/B-Raf) or with one of its isoforms as a heterodimer (B-Raf/C-Raf). B-Raf is recruited to the plasma membrane, and activated by Ras. In turn, B-Raf phosphorylates MEK, continuing the MAP kinase cascade to cell proliferation and survival.

The *BRAF* gene is mutated in many cancers, including papillary thyroid carcinoma, colorectal carcinoma, ovarian carcinoma, lung carcinoma, hairy cell leukemia, and melanoma [40, 41]. Germline *BRAF* mutations do occur but result in cardiofaciocutaneous syndrome, Noonan syndrome, and LEOPARD syndrome, and not melanoma. These germline mutations are not the classic V600E mutations observed in cancer [42, 43]. Somatic *ARAF* and *CRAF* mutations do occur but at a low rate and are apparent minor players in tumor development.

*BRAF*, however, is the most commonly somatically mutated proto-oncogene in melanoma, with over 65 different mutations identified [44–46]. Approximately 50–60 % of melanomas have some activating *BRAF* mutation. Other non-activating mutations exist, but have no known functional relevance [44, 47, 48]. In one of the first studies to screen melanoma patient samples ( $n=15$ ) and melanoma cell lines ( $n=34$ ), it was shown that the majority of activating *BRAF* mutations (over 95 %) occur on exon 15 at residue V600, with 95 % of those resulting in a valine to glutamic acid (p.V→E) substitution within the kinase domain [44]. In some earlier studies, this was erroneously reported as V599E. Many subsequent studies have validated the high representation of B-Raf<sup>V600E</sup> in melanoma, as well as reported other activating mutations at V600 and at other loci within the *BRAF* gene [46, 49]. The V600E mutation causes a conformational change

to the catalytic domain, leading to constitutive activation with a several-log increase in activity of B-Raf and thus downstream activation of the MAP kinase pathway [21, 50]. Interestingly, the identical V600E mutation is seen in up to 82 % of nevi (including common acquired, dysplastic, and small and medium congenital and congenital-like nevi), raising questions regarding the role of V600E in tumor progression and its role in a diagnostic assay [9, 10].

The mutations observed in *BRAF* for melanoma are not typical for UV-induced mutations, but there is an association between *BRAF*-mutated melanomas and sun exposure. It appears that intermittent, not chronic, UV exposure is predictive of *BRAF*-mutated melanoma, with the mutation observed in 59 % of melanomas from intermittently sun-exposed sites, while it is present in only 23 % of acral melanomas, 11 % of primary mucosal melanomas, and 11 % of melanomas from chronically sun-damaged sites [32]. As with *NRAS*, this may explain the observation of *BRAF* mutations in melanomas of younger individuals (chronic sun damage is more common with older individuals). Other than the widely accepted association between *BRAF* mutations and histologic subtypes of melanomas (nodular and superficial spreading), there are no reproducible specific histologic findings associated with *BRAF* mutations [16, 51, 52]. Although there are some reports suggesting melanomas with *BRAF* mutations have more aggressive biology [48], there is no clear association between *BRAF* mutational status and clinical outcome [25, 51–53]. Any inferred negative prognostic weight for a *BRAF* mutation would be mitigated by the current availability of B-Raf-mutant inhibitor therapy.

### 5.3.1.3 *MAP2K (MEK)* and *MAPK (ERK)*

These other members of the MAP kinase pathway have multiple designations, cluttering the literature. *MAP2K1* is located on 15q22.1-q22.33 and *MAP2K2* is located on 19p13.3, encoding for two tyrosine-threonine kinases. These proteins are currently designated as dual-specificity mitogen-activated protein kinase kinase 1 and 2 (Map2k1/2 or MEK1/2, referred to as MEK going forward). *MAPK1* is located on 22q11.2

and *MAPK3* is located on 16p11.2, encoding for two serine/threonine kinases further downstream. These proteins are currently designated mitogen-activated protein kinase 1 and 3 (Mapk1/3, or ERK1/2, referred to as ERK going forward). When MEK is phosphorylated by Raf, its catalytic activity is increased, and, in turn, it phosphorylates ERK. ERK is the only known substrate of MEK. ERK is the most downstream of the phosphorylation proteins in this signaling cascade. When phosphorylated, it can directly activate transcription factors, like Myc, for example, which can increase transcription of cell growth and survival genes within the nucleus.

*MAP2K (MEK)* germline defects have been described in the cardiofaciocutaneous syndrome, but not melanoma. Somatic mutations in *MAP2K (MEK)* were reported in 8 % of 127 metastatic melanoma samples in one study [54]. *MAPK (ERK)* mutations do not appear to be common events. The prevalence and significance of primary activating mutations in MEK and ERK proteins for melanomagenesis remain unclear. However, because their activation is very clearly important for tumor progression and survival, and possibly resistance, and they reside downstream of Ras and Raf, these molecules are potential therapeutic targets.

### 5.3.2 *KIT*

*KIT* (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue) is located on 4q11-q12 and encodes mast/stem cell growth factor receptor Kit (also known as CD117, or Kit). Kit is a member of a large family of tyrosine kinases (RTKs), which also includes platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (HGFR, or MET), and many more. Specifically, Kit is a type III transmembrane receptor tyrosine kinase. Kit is not a true member of the MAP kinase pathway, but feeds into it. When it binds its ligand, stem cell factor (SCF), it dimerizes and activates downstream molecules, such as Ras, and by association can activate the MAP kinase and PI3K/Akt/mTOR signaling pathways

as well as MiTF and an alternative signaling pathway—JAK/STAT. Kit plays a yet-to-be-fully characterized role in normal melanocyte migration and development [55]. Amplifications and activating mutations have been described in leukemia, gastrointestinal stromal tumors, seminomas, and melanomas, among others.

Kit overexpression is observed in approximately 3 % of all melanomas. While the contribution of *KIT* mutations appears to pale in comparison to *NRAS* and *BRAF*, the importance of *KIT* is highlighted within subsets of melanoma. Acral melanomas and primary mucosal melanomas account for only 5 and 2 % of total melanomas in Caucasians, respectively. If melanomas are separated into subtypes, *KIT* amplifications and mutations are observed in 39 % of primary mucosal melanomas, 36 % of acral lentiginous melanomas, and 28 % of melanomas on chronically sun-damaged skin [56]. These percentages are slightly lower when accounting for mutations only (10–21, 11–23, 17 %, respectively) [56–58]. *KIT* mutations are uncommon in melanomas from intermittently sun-damaged skin.

Similar to GISTs, the majority of *KIT* mutations in melanoma occur in the juxtamembrane domain (exon 11). This region normally acts to prevent activation of the kinase when Kit is not bound to its ligand. Mutations lead to constitutive activation. Mutations in other components of the molecule can also lead to its activation (see Testing for *KIT* below) [21, 57, 59].

### 5.3.3 PI3K/AKT/mTOR Pathway

The PI3K/Akt/mTOR pathway can be considered an activation pathway running parallel to the MAP kinase pathway (see Fig. 5.2). It is also an enzymatic cascade, regulating autophagy and leading to cellular growth, proliferation, and survival. The signal propagation is in the following direction: PI3K→Akt→mTOR, although this is greatly simplified as there are many other molecules that feed into this pathway, one being PTEN, an inhibitor of this pathway at the point of PI3K. Most cancers, including up to 60 % of melanomas, have alterations within this pathway [60].

#### 5.3.3.1 PI3K

*PIK3CA* is located on 3q26.3 and encodes phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit  $\alpha$  isoform (PI3K). PI3K is activated by Ras, by a receptor tyrosine kinase, or by other mechanisms, including G-protein-coupled receptors. Activated PI3K can subsequently activate Akt. Germline *PIK3CA* mutations can (rarely) result in Cowden syndrome and segmental overgrowth (in the context of mosaicism) [61], but not melanoma. Mutations and copy number changes of *PIK3CA* are common in many types of cancers, specifically breast and colon cancer. In melanoma, while activation of the PI3K/Akt/mTOR pathway appears important and a common event, the genetic alterations of *PIK3CA*, specifically, appear relatively rare, observed in less than 3 % of melanomas [56, 60]. PI3k/Akt/mTOR pathway activation is more commonly mediated by either Akt activation or functional loss of PTEN [62].

#### 5.3.3.2 AKT and mTOR

Akt (v-akt murine thymoma viral oncogene homologue) is a family of serine/threonine kinases. Akt has three main family member genes—*AKT1* located on 14q32.32-q32.33, *AKT2* located on 19q13.1-q13.2, and *AKT3* located on 1q44. These encode RAC- $\alpha$  serine/threonine-protein kinase, RAC- $\beta$  serine/threonine-protein kinase, and RAC- $\gamma$  serine/threonine-protein kinase, respectively (collectively referred to as Akt). Akt is activated by PI3K, among others, and can activate a variety of substrates, including mTOR, bcl-2 family members, and NF- $\kappa$ B, for example. Germline *AKT1* mutations can (rarely) occur with Cowden syndrome, but not melanoma. Akt is activated in many human cancers, including melanoma, as well as Proteus syndrome [60, 63]. Approximately 1 and 3 % of melanomas harbor a mutation at E17 in *AKT1* and *AKT3*, respectively [64].

*MTOR* (mechanistic target of rapamycin) is located on 1p36 and encodes the serine/threonine-protein kinase mTOR (mTOR). mTOR interacts with other molecules forming complexes (mTORC1/mTORC2), which can feed back into the PI3K/Akt/mTOR pathway and activate more downstream molecules such

as S6K1 and 4EBP1, which are mRNA translation regulators. The precise role of mTOR is unknown, but it appears to sense the nutritional status and oxidative stress level of the cell and control protein synthesis. Molecules like insulin and growth factors can regulate mTOR via PI3K and Akt. mTOR is activated with many cancers including melanoma, and therefore is a potential therapeutic target. Driving mutations in *MTOR*, however, are not widely found or well characterized.

### 5.3.3.3 PTEN

*PTEN* (phosphatase and tensin homologue) is located on 10q23.31. It acts as a tumor suppressor gene, encoding phosphatidylinositol 3,4,5-triphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN (referred to as PTEN for clarity). PTEN inhibits cell division and promotes apoptosis. It is a lipid phosphatase, dephosphorylating and blocking PI3K, and thus negatively regulating the PI3K/Akt/mTOR pathway. Germline defects in *PTEN* are observed in the autosomal dominantly inherited *PTEN* hamartomatous syndrome (Cowden, Bannayan-Riley-Ruvalcaba, Lhermitte-Duclos) and Proteus-like syndrome, leading to cancers and hamartomas. *PTEN* is genetically inactivated or has reduced expression in many human cancers, including in 10–30 % of melanomas [62, 65]. In melanoma pathogenesis, *PTEN* mutations are thought to be important when present in conjunction with activating mutations in other signaling pathways, such as *BRAF* in the MAP kinase pathway. In contrast, *NRAS* and *PTEN* mutations are mutually exclusive, supporting their existence along the same signaling pathway (see Fig. 5.2) [66]. Loss of PTEN protein expression is observed in 8 % of nevi, suggesting a role for PTEN in tumor progression [67]. As a tumor suppressor gene, *PTEN* inactivation leads to activation of the PI3K/Akt/mTOR signaling pathway and thus cell growth and survival. Inactivation of *PTEN* can be achieved by a variety of mechanisms: missense mutations, frameshifts, deletions, insertions, loss of heterozygosity, and epigenetic silencing, to name a few [62, 65].

### 5.3.4 Others

*GNAQ*, located on 9q21, and its family member *GNA11*, located on 19p13.3, encode for the  $\alpha$  subunit of the heterotrimeric G-protein complex. These proteins are designated guanine nucleotide-binding protein G(q) subunit  $\alpha$  and guanine nucleotide-binding protein subunit  $\alpha$ -11 (referred to as GNAQ and GNA11, respectively). This G-protein complex is required to transmit signals from transmembrane G-protein-coupled receptors to internal signaling pathways, such as the MAP kinase and PI3K/Akt/mTOR pathways, mediated by activation of phospholipase C. Mutations in Q209 (for both) in exon 5, and, less frequently, R183 in exon 4, result in constitutive activation of the G-protein complex and subsequent downstream signaling. Mutations in *GNAQ* and *GNA11* occur in uveal melanoma and blue nevi, but not cutaneous melanoma [68]. In one study, *GNAQ* mutations were present in 45 % of uveal melanomas and 55 % of blue nevi, while *GNA11* mutations were present in 32 % of uveal melanomas and 7 % of blue nevi [69]. The *GNAQ* and *GNA11* mutations are mutually exclusive in uveal melanoma, with one of the genes (but not both) mutated in up to 80 % of cases, suggesting that these activating mutations contribute to its pathogenesis.

Obviously, there are many other molecules that reside in these pathways, directly or indirectly feed into these pathways, or are in completely separate pathways that potentially contribute to melanomagenesis. Wide-scale screening endeavors have identified many potential candidates, but as they are in early investigative stages, the interested reader is directed to excellent reviews on the subject [15, 70, 71].

## 5.4 Clinical Trials and Therapeutic Strategies

The number of clinical trials for advanced melanoma has grown exponentially in the wake of the landmark vemurafenib study discussed below. In general, targeting aberrantly activated oncproteins is infinitely more achievable than

reconstituting the function of absent or dysfunctional tumor suppressor proteins, and this is reflected in the lists of drugs under investigation (Fig. 5.3). Current strategies include next-generation wild-type and mutant-specific inhibitors, combination therapies, and rescue therapies, among others. This is a rapidly evolving list that is best researched at its current state at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) [72].

### 5.4.1 Signaling Molecule and Pathway Inhibition

#### 5.4.1.1 B-Raf Inhibition

Because of the recognized prevalence of *BRAF* mutations in melanoma, this has long been on investigators' radars as a potential therapeutic target [73]. Early clinical trials of nonselective B-Raf inhibitors, such as sorafenib tosylate, which has primary activity for C-Raf but also some activity against wild-type B-Raf, mutant B-Raf, and receptor tyrosine kinases such as VEGFR, had been disappointing [74]. Newer generations of inhibitors have much more specificity and activity toward mutated protein.

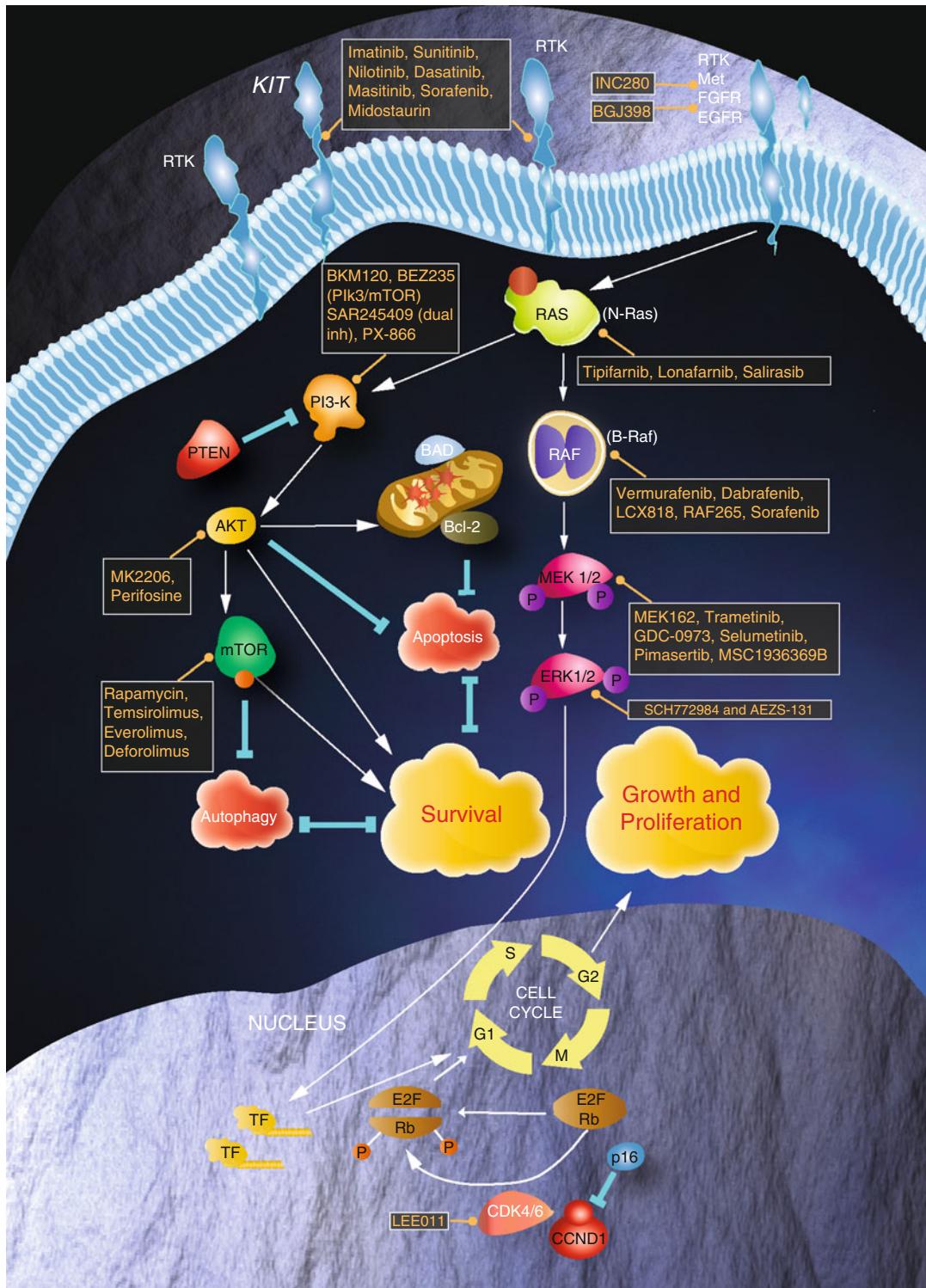
Recently, a series of preclinical studies and clinical trials culminated in the FDA approval of vemurafenib (RG7204/PLX4032, RO5185426, Zelboraf) for treatment of advanced-stage melanoma [75, 76]. The phase II and phase III BRAF-in-melanoma (BRIM-2 and BRIM-3) trial results energized the dermatology and oncology communities. Vemurafenib is an ATP-competitive Raf inhibitor specific for the B-Raf<sup>V600E</sup>-mutant protein. In the phase III randomized clinical trial comparing vemurafenib (960 mg twice daily) with dacarbazine (1,000 mg/m<sup>2</sup> IV q3weeks), 675 patients with untreated stage IIIC or stage IV melanoma harboring the *BRAF* V600E mutation had better overall and progression-free survival rates when treated with the B-Raf inhibitor. At 6 months, the overall survival was 84 % in the treatment arm compared to 64 % in the dacarbazine arm. The median progression-free survival was 5.3 months in the vemurafenib group compared to 1.6 months in the dacarbazine group. The main reported adverse events include

arthralgias, fatigue, rash, alopecia, and cutaneous tumors including squamous cell carcinoma and keratoacanthomas, to name a few. Dose modification was required in 38 % of subjects. Overall, the side effects of this therapy have been transient and manageable.

While vemurafenib captured the early headlines, there are other emerging B-Raf inhibitors. Dabrafenib (GSK2118436, Tafinlar) is now the second FDA-approved B-Raf<sup>V600E</sup>-mutant inhibitor for advanced or surgically unresectable melanoma. This approval followed results of a recent open-label phase III trial of 250 stage IV untreated B-Raf<sup>V600E</sup>-mutated melanoma patients randomized to dabrafenib or dacarbazine [77]. The median progression-free survival was 5.1 months for the dabrafenib group compared to 2.7 months for dacarbazine. The investigators of this study reported fewer cutaneous adverse effects compared with vemurafenib treatment. Other potential benefits of dabrafenib therapy include activity and an acceptable safety profile in patients with brain metastases [78] and apparent activity against B-Raf<sup>V600K</sup>- and B-Raf<sup>V600R</sup>-mutant forms, in addition to B-Raf<sup>V600E</sup> [79].

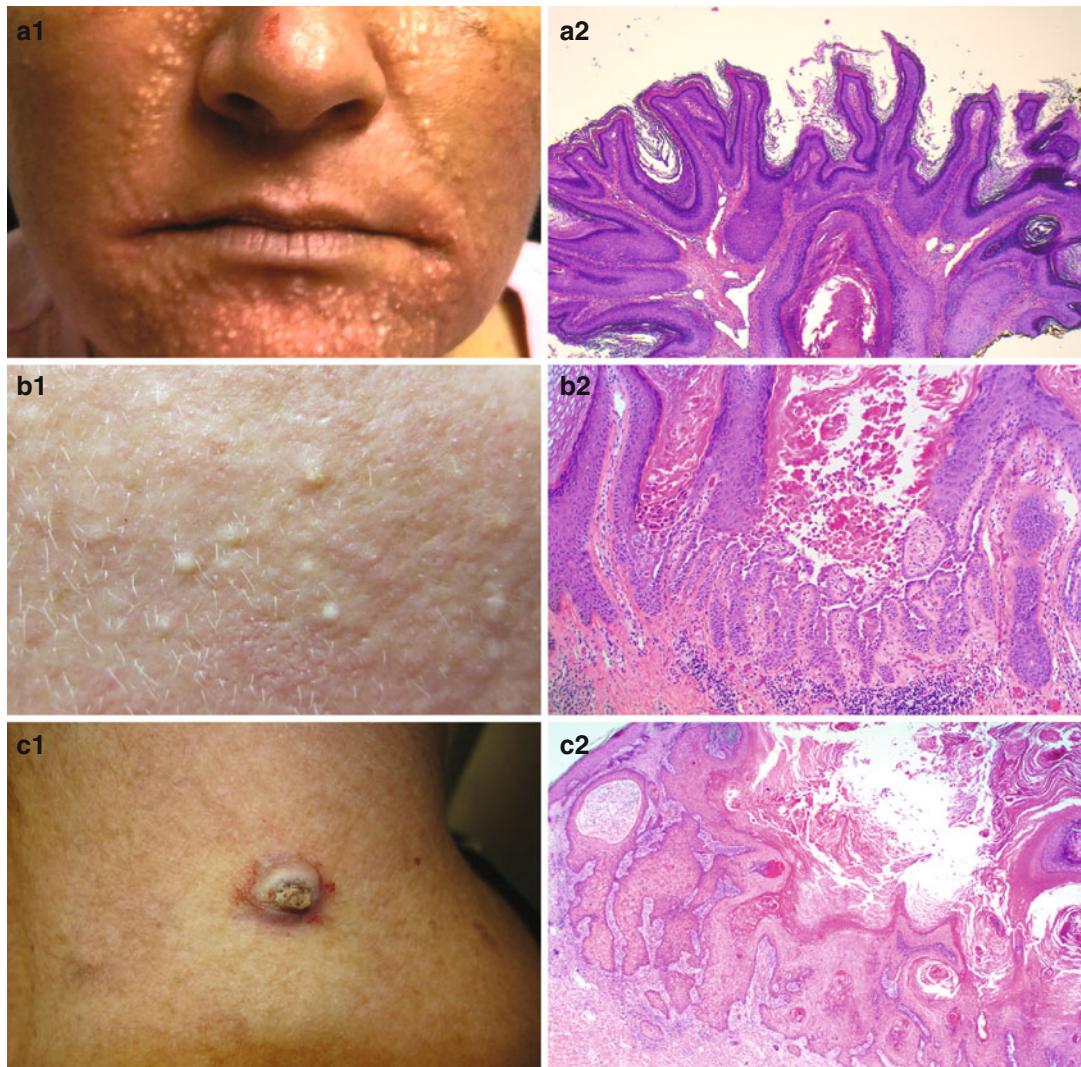
RAF-265 (CHIR-265) is another B-Raf inhibitor, currently in phase I/II, that also has cross-reactivity against vascular endothelial growth factor receptor-2 (VEGFR-2) [80]. This dual inhibition is intriguing as it may act like a combination therapy, attacking two separate mechanisms of oncogenesis (of note, the dose escalation phase of this trial was subsequently canceled and intermediate doses are being explored). LGX818 is another selective inhibitor of B-Raf currently in early clinical trials.

*Cutaneous side effects appear to be relatively common in patients on B-Raf inhibitor therapy*, and this point is worth further discussion. In addition to rashes and alopecia, many of these patients develop cutaneous proliferative lesions, both benign and malignant (Fig. 5.4) [81]. Initial studies on dabrafenib had claimed these side effects occurred with less frequency than with vemurafenib, but recent data suggest this is still a problem [77, 82]. Cutaneous proliferations still occur in over half of dabrafenib-treated patients, with 20 % reporting malignancies, specifically



**Fig. 5.3** Therapeutic agents directed toward melanoma signaling pathway molecules. Numerous therapeutic agents are being investigated for the treatment of advanced

melanoma. Select examples directed toward specific components in the signaling pathways are listed

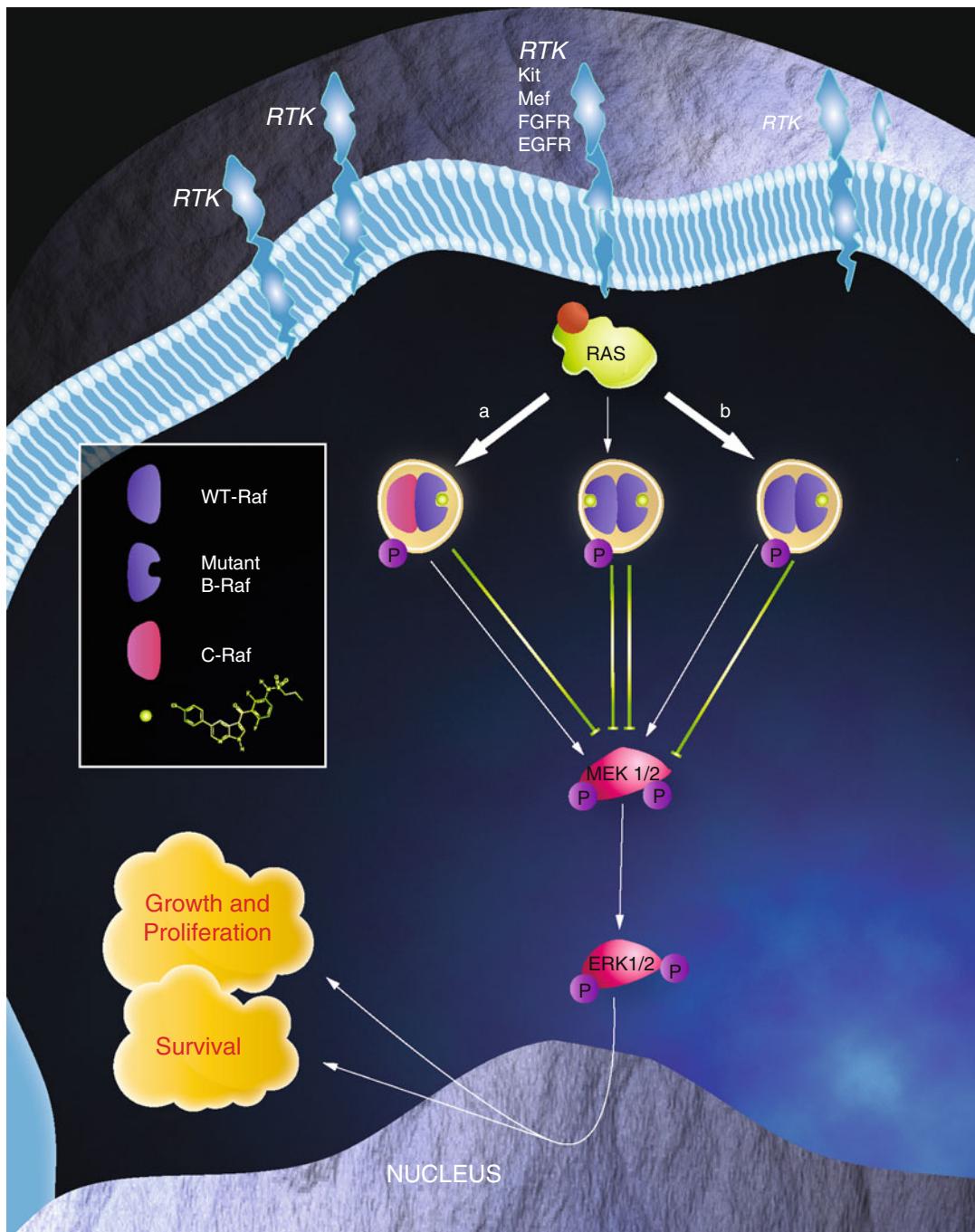


**Fig. 5.4** Cutaneous side effects of B-Raf inhibition. Patients on B-Raf inhibitor therapy commonly develop cutaneous proliferative lesions. These lesions range from benign to malignant. This 41-year-old woman developed numerous matted keratotic papules on her face after 1 month of vemurafenib therapy for metastatic melanoma (**a1, b1**). Biopsies revealed both benign verrucous keratoses (**a2**, H&E, 40 $\times$  original magnification) and acantho-

lytic dyskeratotic acanthomas (**b2**, H&E, 100 $\times$ ). A biopsy-confirmed keratoacanthoma erupted on a different patient, a 39-year-old woman, being treated with a similar therapeutic regimen for metastatic melanoma (**c1, c2**, H&E, 20 $\times$ ). Squamous cell carcinomas are also commonly reported (not shown) (Several images courtesy of Dr. Elaine Miller, Granbury, TX, and Dr. Ern Loh, Dallas, TX)

squamous cell carcinomas [83]. One explanation for cutaneous proliferations is the observation that B-Raf inhibitors are capable of paradoxically activating the MAP kinase pathway (Fig. 5.5). As described above, Ras, an upstream regulator of the MAP kinase signaling pathway, when activated by cell surface molecules or by mutation, dimerizes

and phosphorylates Raf, ultimately leading to cell growth and survival. For Raf to become active, it dimerizes as homodimers (B-Raf/B-Raf) or heterodimers (B-Raf/C-Raf). Raf inhibitors bind and block Raf, but this may have the effect of transactivating its dimerized partner that is unbound by the drug (because the drug either is not specific



**Fig. 5.5** Paradoxical activation of MAP kinase pathway with mutant-B-Raf inhibition. Therapeutic inhibitors to mutant B-Raf can cause paradoxical activation of downstream MAP kinase molecules, such as MEK and ERK. This is postulated to occur in the context of cells with an activated Ras. Ras activation can occur by mutation or by stimulation from upstream molecules such as receptor tyrosine kinases (RTKs). B-Raf<sup>V600E</sup>-specific inhibitors, such as dabrafenib and vemurafenib (green), block signal-

ing by mutant B-Raf (green lines). However, because B-Raf dimerizes with another Raf molecule in the context of an activated Ras, the dimerized (and unbound by drug) partner may be sufficiently activated to propagate signals from Ras to MEK and ERK (white arrows). The dimerized partner, such as C-Raf, may be directly activated by Ras (a) or the dimerized partner (wild-type B-Raf, unbound mutant B-Raf, C-Raf, etc.) may be transactivated by the drug-bound mutant B-Raf (b).

for the partner or is at too low of a concentration). The transactivated partner can then propagate the Ras signal downstream. Alternatively, a selective mutant-specific B-Raf inhibitor may block mutant B-Raf, but activated Ras may directly and preferentially activate the heterodimerized C-Raf [84, 85]. The net effect from these models is the activation of the MAP kinase signaling pathway in cells (including keratinocytes) with an activated Ras (either by mutation or by upstream activation by receptor tyrosine kinases) that are exposed to B-Raf inhibitors. In fact, squamous cell carcinomas have been recently shown to harbor *RAS* (primarily *HRAS*) mutations in patients on vemurafenib and other B-Raf inhibitors [86, 87]. There does appear to be some variability among B-Raf inhibitors in their paradoxical ability to activate the MAP kinase pathway, presumably due to differences in cross-reactivity with wild-type B-Raf or C-Raf. Inhibitors that have limited cross-reactivity have been coined “paradox breakers” for this reason and are currently being investigated in clinical trials. These models may help explain the adverse cutaneous reactions and may lead to new therapeutic strategies to prevent them, such as by using paradox breakers or targeting downstream molecules like MEK. Moreover, paradoxical MAP kinase activation in the context of an activated Ras could potentially play a role in stimulating benign or malignant melanocytes and therefore underlines a potential importance of testing patients for *NRAS* mutations.

#### 5.4.1.2 N-Ras Inhibition

To date, there are no effective Ras or mutant-specific Ras inhibitors, but not for lack of trying. Inactivating the mutated N-Ras protein (reconstituting the GTP hydrolysis function) has proven pharmacologically difficult. Other strategies are being explored, including the use of small interfering ribonucleic acids (siRNA), antisense oligonucleotides, and disrupting post-translational modifications such as geronylation and farnesylation, among others. Blocking farnesyltransferase indirectly inhibits Ras activity by preventing translocation of Ras to the plasma membrane and thus the dimerization of Raf. Agents directed toward disrupting Ras

mobility include tipifarnib, lonafarnib, and salirasib. These and other modalities for indirectly blocking Ras activity have had minimal clinical efficacy to date as monotherapy, but their utility in combination with other agents, as well as focusing on targeting downstream molecules, is being explored [88, 89].

#### 5.4.1.3 MAP2K (MEK) and MAPK (ERK) Inhibition

MEK and/or ERK inhibition seems like a logical strategy to shut down activated melanoma cells as they lie downstream in the MAP kinase signaling pathway. Almost all melanomas have an activated MAP kinase pathway, culminating in activated MEK and ERK, whether by mutated B-Raf, N-Ras, GNAQ/GNA11, or other molecules. Attempts at MEK inhibition predate B-Raf inhibition. Volumes of preclinical work looking at the efficacy of inhibitors to MEK, and to a lesser extent ERK, have yielded promising results. Inhibitors proved effective in inducing cell cycle arrest and apoptosis in melanoma cells. Clinical trials using MEK inhibitor monotherapy had been disappointing, until recently [90].

Trametinib (GSK1120212, Mekinist) was recently FDA approved for the treatment of advanced or surgically unresectable melanoma. Interestingly, trametinib is a MEK inhibitor but has been shown to be moderately effective for treating B-Raf<sup>V600E</sup>- or B-Raf<sup>V600K</sup>-mutant melanomas, and was FDA approved for this purpose. This drug was co-approved with dabrafenib (as monotherapies) in May 2013. Trametinib is an allosteric MEK1/2 inhibitor. In the phase I trial for trametinib, Falchook et al. reported a 33 % response rate in previously untreated patients harboring a *BRAF*-mutated melanoma [91]. The phase II trial for trametinib showed significant clinical activity in patients that had not previously received mutant-B-Raf inhibitor therapy (2 % complete response, 23 % partial response, 51 % stable disease,  $n=57$ ) [92]. Interestingly, patients that had been previously treated with mutant-B-Raf inhibitor therapy did not fare as well, suggesting MEK inhibition monotherapy may not be effective in this resistant or relapsed patient group.

MEK162 is another MEK inhibitor advancing through clinical trials. A phase II trial treating advanced melanoma patients with *NRAS*- or *BRAF*-mutated tumors showed a similar partial response rate (20 %,  $n=71$ ) between the two groups [93]. Although no complete responses were observed, this is one of the first trials to demonstrate clinical efficacy against *NRAS*-mutated tumors. GDC-0973, selumetinib, and pimasertib are other examples of MEK inhibitors currently under investigation [94, 95].

SCH772984 and AEZS-131 are examples of ERK inhibitors under development, with little data currently available. Early preclinical work on ERK inhibitors suggests a possible role in B-Raf and/or MEK inhibitor-resistant tumors [96]. There is no current FDA-approved therapy for ERK inhibition. There are numerous clinical trials exploring inhibitors to these downstream molecules, with current emphasis on MEK. Even with the promising results and FDA approval of trametinib monotherapy, the real value in MEK and ERK inhibition may be realized in other settings: as rescue therapy for B-Raf inhibitor-resistant tumors (despite the recent trametinib data), as a treatment for *NRAS*-mutated melanomas (as an effective direct inhibitor of N-Ras is not yet available), and as combination therapy with drugs directed elsewhere in the signaling pathways or with entirely different mechanisms of action.

#### 5.4.1.4 Kit Inhibition

Inhibitors to Kit, such as imatinib mesylate, have been around for over a decade, due to their efficacy against chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (GISTs) [97, 98]. Subsequent preclinical data and the observation that *KIT*-mutated melanomas share the same activating mutations as imatinib-responsive GISTs led to clinical trials to assess the efficacy of this Kit inhibitor therapy on advanced melanoma patients. Initial trials for melanoma showed minimal or no benefit [99, 100]. While this was disappointing, there remained the possibility that the efficacy of Kit inhibition could be unmasked with selected patient populations. There was a precedent for this in breast cancer

treatment. In the initial clinical trials for trastuzumab (Herceptin) on a large breast cancer population, no clinical benefit was detected. The efficacy of the drug was only appreciated once the patients with amplified *ERBB2* (*HER2/NEU*) were parsed out and analyzed, emphasizing the importance of patient selection when using targeted therapy. More recent phase II open-label clinical trials explored the efficacy of Kit inhibitor therapy on patients with advanced melanoma with known aberrations to *KIT*, not just immunohistochemical expression levels, and had more promising results [59, 101–103].

The phase II trial by Guo et al. studied 43 evaluable patients with metastatic melanoma harboring aberrations in *KIT* [102]. These patients were treated with a continuous dose of imatinib mesylate 400 mg/day. With a median follow-up time of 1 year, 53.3 % of the patients had either a partial response (10 patients) or achieved stable disease (13 patients), with an overall response rate of 23.3 %. Interestingly, 9 of 10 patients with mutations in exons 11 or 13 achieved a partial response. In a study by Carvajal et al. 51 patients were studied, with 28 (25 evaluable) receiving imatinib mesylate 400 mg twice daily [59]. In their treatment group, two patients achieved complete responses (over 94 weeks, one patient over 3 years as of January 2013), two had durable partial responses (over 53 weeks), and two had transient partial responses (12 and 18 weeks), with an overall durable response rate of 16 % and a median overall survival of 46.3 weeks.

Interestingly, the efficacy of Kit inhibitor therapy appears linked to the type of *KIT* aberration [59, 102, 103]. In the Carvajal study, all six responders had mutations in either exon 11 (L576P) or exon 13 (K642E). Guo et al. also reported that only patients with mutations in exons 11 or 13 (and one patient with *KIT* amplification but no mutation) responded to therapy. A third phase II study by Hodi et al. looked at the efficacy of imatinib mesylate in specific subtypes of melanomas with high frequencies of *KIT* aberrancies (acral, mucosal, chronically sun-damaged skin melanomas) [103]. These investigators also observed better responses with *KIT*-mutated

tumors rather than *KIT* amplified tumors. Patients with functional mutations appear to respond best to the inhibitor therapy. Those with only amplifications or nonfunctional mutations respond less well. Additionally, Kit expression levels, as determined by immunohistochemistry, do not correlate with response to therapy. This is in contrast to the observation that imatinib mesylate has efficacy against GISTs with elevated Kit expression, even in the absence of *KIT* or *PDGFR* mutations.

There are newer generation Kit inhibitors currently in early clinical trials [72]. Nilotinib (Tasigna) is currently used worldwide in the treatment of Philadelphia chromosome-positive CML. It has activity against the receptor tyrosine kinases Kit, platelet-derived growth factor receptor (PDGFR), and the Bcr-Abl fusion protein. Currently, nilotinib is in a single-arm phase II clinical trial to explore its efficacy in patients with *KIT*-mutated metastatic melanoma with early promising results, including partial clinical responses in two of three patients with exon 11 *KIT* mutations and a favorable toxicity profile [104]. Other examples of investigative Kit inhibitors include sunitinib malate (Sutent), dasatinib (Sprycel), sorafenib tosylate (Nexavar), masitinib mesylate, and midostaurin.

#### 5.4.1.5 PI3K/Akt/mTOR Pathway Inhibition

Targeting the PI3K/Akt/mTOR pathway is an attractive strategy as activation of this pathway is common in melanoma and may be essential in B-Raf/MEK inhibitor resistance. Moreover, co-targeting the MAP kinase and PI3K/Akt/mTOR pathways may be required to combat N-Ras-driven melanomas [105]. Candidate targets for melanoma therapy include PI3K, Akt, mTOR (and dual PI3k/mTOR), and the mTOR complexes mTORCH1/2, and there are a variety of small-molecule inhibitors to these components currently under investigation (see Fig. 5.3). Examples include the PI3K inhibitor BKM120, the dual PI3K/mTOR inhibitor BEZ235, the Akt inhibitor MK2206, and several mTOR inhibitors including the rapamycin analogs temsirolimus, everolimus, and deforolimus. Preclinical studies

have demonstrated some efficacy of these drugs on the growth and survival of melanoma cell lines and other cancers, and many of these are in clinical trials, but, to date, there is no efficacious single-agent therapy in this group for melanoma patients [106–108]. There is more optimism for use of these drugs in combination regimens.

#### 5.4.1.6 GNAQ/GNA11 Inhibition

While *GNAQ* and *GNA11* mutations appear to be drivers for uveal melanoma oncogenesis, targeting of these mutated proteins has not been effective. These molecules are  $\alpha$  subunits of G proteins, and like the story for Ras, this is a difficult molecular/pharmacologic “space” to manipulate. Additionally, *GNAQ* is an important molecule in cardiomyocyte development and survival, raising potential toxicity concerns [109]. As these molecules potentially feed into the MAP kinase and PI3K/Akt/mTOR pathways, targeting of downstream signaling molecules, such as MEK and mTOR, has been the strategy of choice [110].

### 5.4.2 Immunotherapy

Immunotherapy has been an explosive field, attaining recent success in the treatment of advanced melanoma. This is a vast area of research and includes vaccines, adoptive cell therapy, cytokine therapy, and immunomodulatory agents, with available reviews on the topic [111]. A thorough discussion of immunotherapy is beyond the scope of this text, but a few strategies are briefly mentioned here.

High-dose IL-2 (aldesleukin, Proleukin) has been available for over a decade and is FDA approved for the treatment of advanced melanoma. It can induce partial responses in subsets of patients but has little effect on overall survival and is associated with significant toxicity [1]. More recent data have suggested that melanomas with *NRAS* mutations respond better to IL-2 than tumors with wild-type *NRAS* [33]. Ipilimumab (Yervoy) is a monoclonal antibody that acts as an immunomodulator. Along with vemurafenib, it was FDA approved in 2011 for the treatment of advanced melanoma, ending the long drought

since IL-2 [112]. Ipilimumab binds to CTLA-4 (cytotoxic T-lymphocyte antigen 4), which is a molecule on the cell membrane of a subset of T cells. These regulatory T cells ( $T_{reg}$ ) act to reign in the immune response. By blocking the activity of these cells, specifically the  $B7 \leftrightarrow$  CTLA-4 interaction, the disinhibited and unleashed immune system becomes more effective at combating melanoma. In the phase III study comparing ipilimumab with or without the glycoprotein 100 (gp100) peptide vaccine compared to the gp100 vaccine alone, patients receiving ipilimumab (either group,  $\pm$ gp100) had a median overall survival of 10.0 months compared with 6.4 months in the gp100-only group [113]. One potential problem with this type of immune-activating therapy is that it is not specific to melanoma cells and will have immune-related adverse events. Examples involving the skin, gastrointestinal tract, liver, and endocrine system have been described. Diarrhea and infections are particularly problematic. Tremelimumab is also a CTLA-4 blocker with a slightly longer half-life and therefore can be less-frequently dosed. It is not FDA approved but is currently being investigated in clinical trials [114].

More recently, lambrolizumab (MK-3475) was FDA approved as a breakthrough therapy following positive results in a single-arm phase IB clinical trial [115]. Lambrolizumab is an antibody directed against the programmed cell death 1 (PD-1) pathway. The PD-1 receptor on activated T cells normally binds to ligands on antigen-presenting cells, shutting down the immune response. Lambrolizumab interferes with this process and, like ipilimumab, causes a heightened immune response with antitumor activity. In the phase IB trial, 38 % of patients ( $n=135$ ) showed a confirmed antitumor response (across all dose cohorts), and the median progression-free survival was longer than 7 months. There was mild toxicity, mostly immune related, associated with treatment. Nivolumab is another drug in the pipeline, a PD-1 receptor blocker with similar action.

Ipilimumab and lambrolizumab are monoclonal antibodies and can theoretically be used irrespective of the melanoma's histologic

subtype, UV exposure, or mutational status. *There is no current molecular testing required.* Moreover, as these are immunomodulators, they act by a completely different mechanism than the small-molecule signaling pathway inhibitors, making them attractive options for combination therapy or for patients who relapse from or fail to qualify for B-Raf or MEK inhibitor therapy.

### 5.4.3 Resistance to Therapy and Clinical Relapse

Resistance to therapy is the albatross for most single-agent therapeutic regimens, not isolated to melanoma. Resistance can come in the form of primary resistance or secondary resistance. Primary, or de novo, resistance refers to the therapy's ineffectiveness at the time of initial therapy, despite a predicted response given the mutational status of the tumor. This is likely mediated by additional mutations or oncogenic drivers other than the targeted area of the protein or on a different protein. Secondary resistance refers to adaptive resistance following an initial response to the targeted therapy. As the targeted biologic driver is quenched, a new mutation is created, in either the same molecule, another molecule in the same pathway, or one in a different pathway altogether, leaving us playing the futile game of "whack-a-mole."

#### 5.4.3.1 B-Raf Inhibitor Resistance

As the vemurafenib BRIM2 and BRIM3 trials showed us, treatment with a single-agent B-Raf<sup>V600E</sup>-mutant inhibitor, while effective, is no definitive cure. After an initial dramatic tumor response to inhibitor therapy, in many cases, the melanoma recurs, with a median progression-free survival of 5.3 months. Additionally, almost half of the patients with BRAF V600E-mutated melanoma had no objective responses, suggesting pre-therapy resistance, or primary resistance, mechanisms were already in place.

In melanoma patients treated with vemurafenib, with recurrence of their tumor, there are a number of possible mechanisms for resistance:

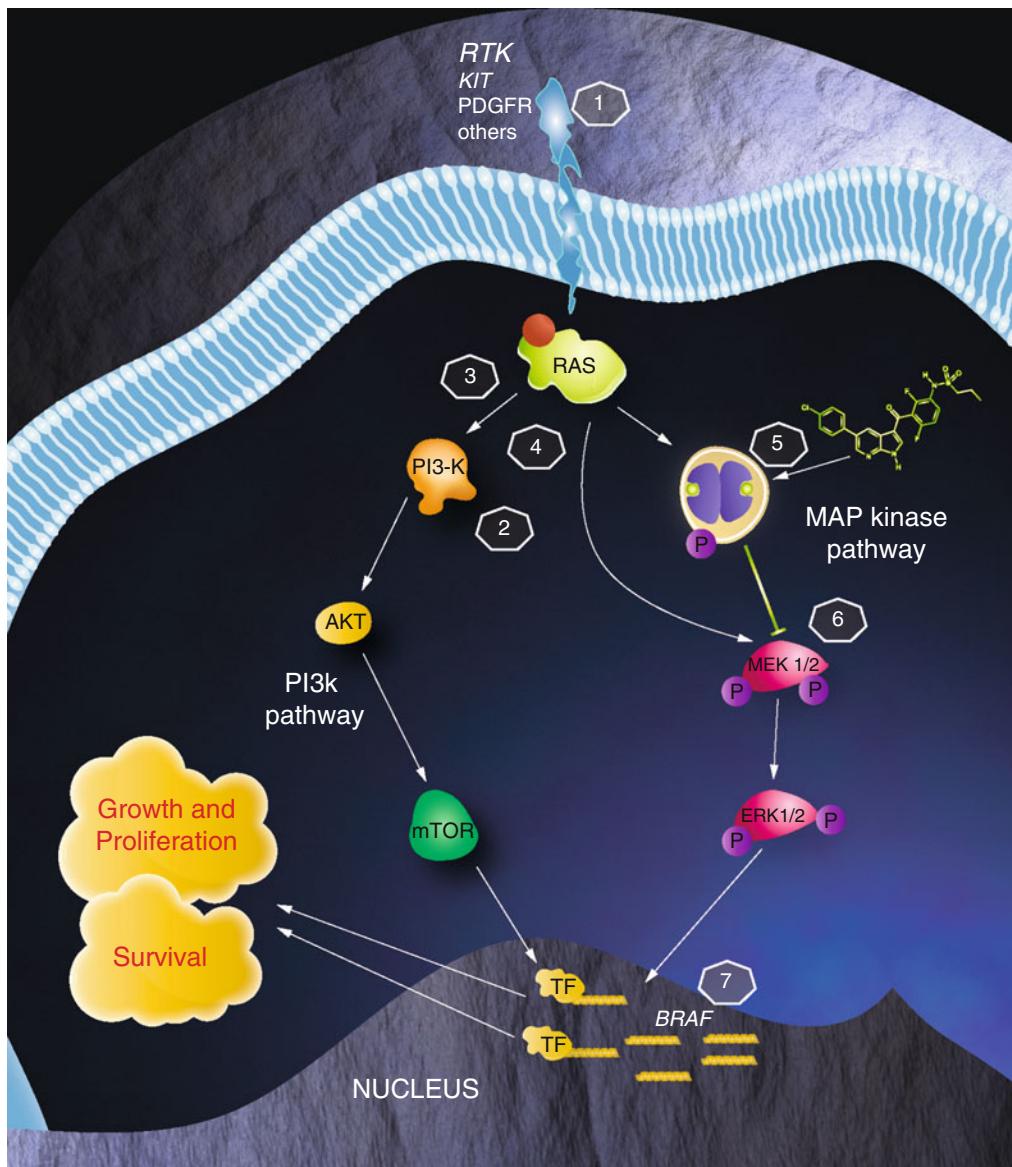
secondary mutation in *BRAF* to block drug binding or otherwise bypass the drug effect, activating mutations downstream of B-Raf, upstream activating mutations that can bypass B-Raf, activating mutations in parallel signaling pathways, amplification of signaling molecule genes, etc. (Fig. 5.6). Interestingly, it appears that a secondary mutation in *BRAF* that blocks binding of the drug, for example, does *not* typically occur [116]. This is somewhat of a surprise because this mechanism of secondary mutation of the drug target has been observed in other systems, such as with *KIT* in relapsed GISTs and with *BCR-ABL* in CML treated with tyrosine kinase inhibitors [117, 118]. There are increasing data to support B-Raf inhibitor resistance via upregulation of N-Ras, PI3K, or surface tyrosine kinase receptors such as PDGFR- $\beta$  or IGF-1R, acquired mutations in *NRAS* or *MAP2K1* (MEK1) (C121S), splice variants of *BRAF*, and amplification of *BRAF*, among others [116, 119–122].

#### 5.4.3.2 Kit Inhibitor Resistance

Years of experience with treating CML and GISTs has taught us a lot about resistance to RTK inhibitors. Unlike with B-Raf inhibitor resistance, which appears to be more commonly achieved by activation or secondary mutations in downstream or parallel signaling molecules, *KIT* usually undergoes a secondary mutation in the Kit protein itself. This secondary mutation may evade inhibition by preventing binding by the inhibitor (facilitated by an exon 11 mutation in the juxtamembrane region) or by activating the kinase domains (exon 13) independent of the status of the juxtamembrane region [97]. Indeed, in melanoma, like in CML and GISTs, secondary *KIT* mutations appear to be the main mechanisms of resistance. In preclinical melanoma studies, A829P and T670I mutations allow melanoma cells to become resistant to imatinib and some later-generation Kit inhibitors [123]. Overcoming resistance may be possible by using combinations of receptor tyrosine kinase inhibitors, dual inhibition of Kit and the PI3K/Akt/mTOR pathway, or other permutations on this theme as described below.

#### 5.4.4 Combination Therapy and Emerging Therapeutic Strategies

Combination therapy is a natural next step to mitigate monotherapy resistance. This has proven to be an effective strategy in other scenarios, perhaps most notably with the success of HAART triple therapy in the assault on the HIV virus. As viruses and cancers share a remarkable ability to mutate and become genetically diversified to the point of evading therapy, it is reasonable to anticipate similar success with similar therapeutic approaches. Simultaneous targeting of multiple checkpoints along series or parallel circuits may provide a greater initial tumor response and may minimize resistance. Our understanding of the cross talk between molecular signaling pathways and our uncovering of resistance mechanisms have provided the rationale behind combination therapy [119, 124]. For example, Villanueva et al. developed a model of B-Raf inhibitor resistance by chronic treatment of melanoma cells in vitro. These investigators identified flexible switching of Raf between its isoforms (and thus bypassing blocked mutant B-Raf), as well as enhanced activity of the PI3K/Akt/mTOR pathway as potential acquired resistance mechanisms [119]. There were no secondary mutations that developed in *BRAF*, and there were no detectable novel mutations or changes in copy number for *KIT*, *NRAS*, or *PTEN*. In their model, they were also able to show that use of inhibitors to IGF-1R/PI3K and MEK could overcome the resistance and induce cell death. Similar concepts are being translated into clinical trials. A recent phase I/II clinical trial of B-Raf inhibitor/MEK inhibitor combination therapy has yielded promising results [125]. Flaherty et al. performed an open-label trial, randomly assigning 162 stage IV melanoma patients to receive dabrafenib (150 mg, twice daily) plus trametinib (1 or 2 mg daily) or dabrafenib monotherapy. Patients on the combination B-Raf inhibitor+MEK inhibitor regimen had a median progression-free survival of 9.4 months compared to 5.8 months for B-Raf inhibitor monotherapy. Additionally, fewer patients developed squamous cell carcinoma when on combination therapy



**Fig. 5.6**  $B\text{-Raf}^{V600E}$  inhibitor resistance mechanisms.  $B\text{-Raf}^{V600E}$  inhibitors block the normal propagation of signaling from RAS to MEK (green molecule and line). There are several postulated mechanisms for resistance to mutant-B-Raf inhibitor therapy, occurring in isolation or in combinations: (1) upregulation or activation of receptor

tyrosine kinases such as PDGFR, (2) upregulation of PI3K, (3) upregulation of N-Ras, (4) activating mutations of *NRAS*, (5) *BRAF* splice variants that enhance dimerization, (6) acquired mutations in MEK, (7) *BRAF* amplification, and others. Secondary mutations in *BRAF* to prevent drug binding do not appear to play a major role

(7 % vs. 19 %). Similar combinations including dabrafenib + trametinib versus vemurafenib, and GDC-0973 + vemurafenib versus vemurafenib are in phase III trials at the time of this publication.

Strategies behind combination therapies are not limited to the MAP kinase and related signaling pathways. In fact, there is theoretical

(and realized) benefit to using therapies with completely different mechanisms of action. There are data to suggest that chemotherapy and/or B-Raf (and/or MEK) inhibitor therapy may cause melanoma to be more immunogenic, perhaps by exposing melanoma-specific antigens upon melanoma cell death. This would predict

**Table 5.2** Select ongoing clinical trials with inhibitors to signaling pathways for advanced melanoma

Study	Phase
<i>B-Raf inhibitor studies</i>	
Vemurafenib±GDC-0973 (MEK inhibitor) for V600-mutated melanoma	III
Dabrafenib + trametinib (MEK inhibitor) versus vemurafenib for V600E/K-mutated melanoma	III
Dabrafenib ± trametinib (MEK inhibitor) for V600E/K-mutated melanoma	III
Vemurafenib±bevacizumab (anti-VEGF) for V600-mutated melanoma	II
LGX818+combination of MEK162 (MEK inhibitor), LEE011 (CDK4/6 inhibitor), BGJ398 (fibroblast growth factor receptor inhibitor), BKM120 (PI3K inhibitor), INC280 (c-Met inhibitor) for V600-mutated melanoma	II
Dabrafenib for V600E/K-mutated melanoma with and without brain metastases	II
Vemurafenib+ ipilimumab (immunomodulator) for V600-mutated melanoma	II
RAF265	I/II
Vemurafenib+ decitabine (hypomethylating agent) for V600-mutated melanoma	I/II
Vemurafenib+ BKM120 (PI3K inhibitor) for V600E/K-mutated melanoma	I/II
Dabrafenib ± trametinib (MEK inhibitor) in combination with ipilimumab (immunomodulator) for V600E/K-mutated melanoma	I
<i>Kit inhibitor studies</i>	
Masitinib versus dacarbazine (cytotoxic chemotherapy) for <i>KIT</i> (juxtamembrane)-mutated melanoma	III
Imatinib mesylate for <i>KIT</i> -mutated melanoma	II
Nilotinib for <i>KIT</i> -mutated or amplified melanoma	II
Sunitinib for <i>KIT</i> -mutated or amplified melanoma	II
Dasatinib for acral lentiginous, mucosal, or chronically sun-damaged melanoma with or without <i>KIT</i> (juxtamembrane)-mutations	II
Sunitinib for primary mucosal and acral lentiginous melanoma	II
Imatinib mesylate+ ipilimumab (immunomodulator) for advanced cancers	I
<i>Other combination therapy studies</i>	
MEK162 (MEK inhibitor) versus dacarbazine for <i>NRAS</i> (Q61)-mutated melanoma	III
Pimasertib (MEK inhibitor) versus dacarbazine for <i>NRAS</i> -mutated melanoma	II
Selumetinib (MEK inhibitor) for <i>BRAF</i> - or <i>NRAS</i> -mutated melanoma	II
Selumetinib (MEK inhibitor)+MK2206 (Akt inhibitor) for <i>BRAF</i> -mutant-inhibitor-resistant melanoma	II
MEK162 (MEK inhibitor)+LEE011 (CDK4/6 inhibitor) for <i>NRAS</i> -mutated melanoma	I/II
Temsirolimus (mTOR inhibitor)+Bryostatin 1 (protein kinase C modulator) for melanoma	I
SAR260301 (PI3K $\beta$ inhibitor)±vemurafenib for <i>BRAF</i> -mutated melanoma	I

As of January 2014

a potentially synergistic effect by using a combination approach of a B-Raf inhibitor followed by ipilimumab therapy, and these trials are currently in phase I/II (preliminary data has shown some limitations with this approach due to liver toxicity) [72, 126, 127]. A rationale also exists for trying various combinations of the above with anti-angiogenesis therapy (bevacizumab, an anti-VEGF antibody), anti-apoptosis therapy (oblimersen/Genasense, a bcl-2 antisense molecule), other immunotherapies (lambrolizumab), possibly autophagy modulators, and others. Table 5.2 highlights select clinical trials for advanced melanoma, ongoing at the time of this publication. Due to the rapidly evolving nature of this area,

the reader is directed to [www.clinicaltrials.gov](http://www.clinicaltrials.gov) and other sites for up-to-date lists and status reports of melanoma clinical trials [72, 128, 129].

## 5.5 Practical Considerations for Ordering and Performing Molecular Tests

### 5.5.1 Targeted Mutation-Specific Molecular Assays

While, in theory, a whole litany of tests could be performed to assess the activity and/or mutational status of all the molecules in the above

signaling pathways, only a few are performed on clinical samples at this time, namely, mutational analyses of *BRAF*, *NRAS*, and *KIT*. The reason for lack of testing of other signaling molecules is due to one, or a combination, of the following reasons: there is no defined diagnostic, prognostic, or therapeutic purpose for testing; the target does not have an effective inhibitor therapy available; the target does not have a known melanoma-specific mutation amenable to assay; and an assay is not available on formalin-fixed paraffin-embedded tissue, among others. Currently, testing for *BRAF*, *NRAS* and *KIT* mutations is performed to identify patients likely to respond to available inhibitor therapy. There is no current role for testing for the purpose of diagnosis.

All of these mutational analyses require purified tumor DNA from a patient sample, obtained in the same fashion, regardless of the molecular target. Once tumor DNA is obtained, these analyses differ by how they target their respective genes and potentially their sequencing methods. Tissue is procured from a paraffin block or slide(s) (see Chap. 3 for an overview and schematic of this process). Only small amounts of DNA are needed. Technically, because the DNA is amplified, only a few cells are needed. In practice, having abundant tumor-rich DNA is preferred to allow for repeat testing, if needed, and to prevent interpretation challenges, which are inherent with extremely low levels of DNA. Most laboratories strive for at least a 5-mm-diameter focus of tumor (size of a pencil eraser), with at least 20 % tumor cells. For small specimens, multiple unstained slides may be used in order to ensure there is sufficient tissue for testing. The limits of detection of the most commonly used sequencing assays require at least 5 % mutant alleles (10 % tumor cells) (Sanger sequencing is an exception and may require up to 20 % mutant alleles/40 % tumor). We try to obtain more pure DNA to account for any overestimation of tumor percentage through the microscope as well as potential tumor molecular heterogeneity. Obtaining abundant and pure tumor DNA is not usually a problem for primary tumors, but may be an issue with heavily inflamed tumors or small metastatic deposits. In these cases, dissecting out

the area of highest tumor purity is required. Once the DNA is extracted, it is amplified using a PCR-based assay. The mutational status is evaluated by a variety of different methods, including Sanger 2X bidirectional sequencing, allele-specific PCR, TaqMan real-time PCR, pyrosequencing, PCR with mass spectroscopy, and next-gen (high throughput, deep) sequencing, among others. An overview of select common methods is provided in Chap. 3.

Clinicians often query if a new biopsy or any special handling is required, but this is not the case. Testing is performed on formalin-fixed, paraffin-embedded tissue, and therefore no new biopsy is required. The sample can be from primary tumors, recurrences, or metastases so long as there is sufficient tumor burden. The most current available patient material is preferred as it is likely the best representation of the tumor's current mutational profile and thus likely best predicts response to therapy. In the case of *BRAF* mutations, Dong et al. suggest using metastatic over primary tumor, when there is a choice, as the former has a higher frequency of *BRAF* mutations and may better reflect the genome of the systemic disease, thus better predicting a therapeutic response [130]. It is well recognized that tumors may be molecularly heterogeneous, both within an individual and within a single lesion [131]. This raises questions regarding the efficacy of inhibitor therapy in this context, as subpopulations of tumor lacking the targeted defect will be selected. While criteria for therapeutic eligibility are constantly evolving, currently, detection of any level of oncogenic driver mutation (*BRAF*, *KIT*, etc.) is considered significant. Quantitative molecular methods and immunohistochemistry may become more attractive assays if molecular heterogeneity of tumors is deemed biologically and therapeutically relevant.

### 5.5.1.1 BRAF Mutational Analysis

Identification of melanoma patients with *BRAF* V600 mutations qualifies them for B-Raf mutation-specific inhibitor therapy, which appears to modestly improve overall survival, emphasizing the importance of a quality diagnostic assay [76]. Because *BRAF* mutations

**Table 5.3** Mutational frequency at select loci in cutaneous melanoma

	Amino acid substitution	Base substitution(s)	Frequency (%) <sup>a</sup>
<i>BRAF</i>	V600E	1799T>A	90
	V600K	1798/1799GT>AA	8
	V600R	1798/1799GT>AG	1
	V600E(2)	1799/1800TG>AA	0.5
	Other	Variable	Variable
<i>NRAS</i>	Q61R	182A>G	40
	Q61K	181C>A	31
	Q61L	182A>T	9
	G12D	35G>A	4
	G13R	37G>C	2
	G13D	38G>A	2
	Other	Variable	Variable
<i>KIT</i>	L576P	1727T>C	38
	K642E	1924A>G	17
	V559A	1676T>C	8
	V560D	1679T>A	4
	W557R	1669T>C	4
	V559D	1676T>A	2
	Other	Variable	Variable

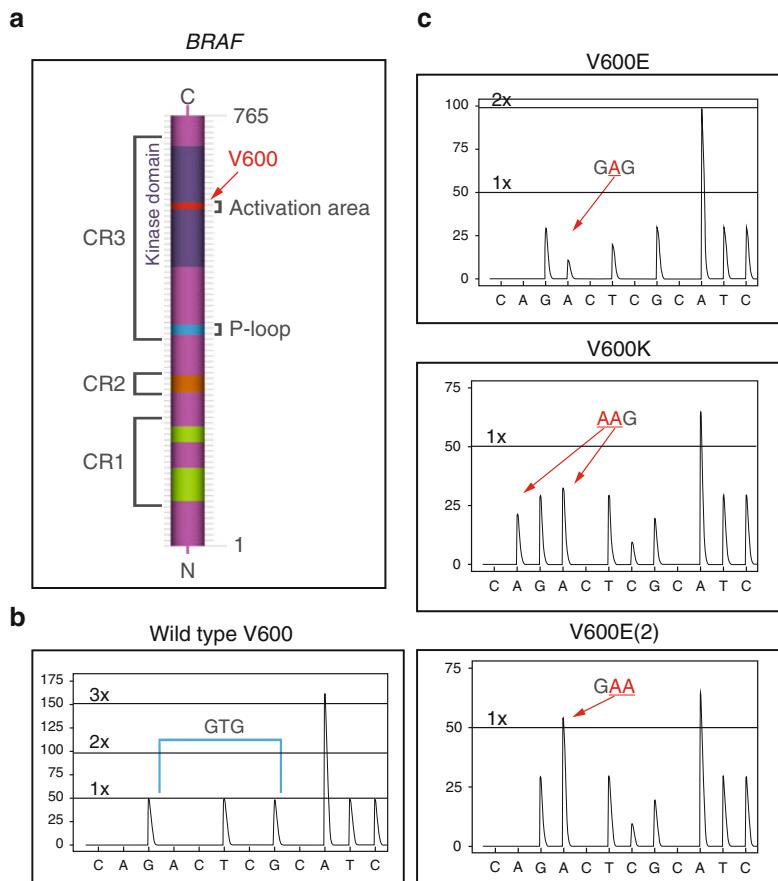
<sup>a</sup>Mutation frequency refers to the reported frequency of the mutation compared to all mutations in that gene for cutaneous melanoma. For *BRAF*, it is in reference to V600 only. V600 accounts for 95 % of all *BRAF* mutations (Data taken from the COSMIC database [46]). Lists of other identified mutations also available at [www.vicc.org/research/shared/translational/services/snapshot.pdf](http://www.vicc.org/research/shared/translational/services/snapshot.pdf) [133]

commonly occur in melanoma and there is a fairly consistent activating mutation, testing for *BRAF* mutations has become commonplace for patients with advanced melanoma. Testing is often requested and performed, regardless of anatomic site or histologic subtype.

Approximately 50–60 % of melanomas have an activating mutation in their *BRAF* gene [44, 48]. Up to 95 % of those mutations involve V600, and 90 % of those are specifically V600E (p.V→E, bp.1799 T→A), estimating that roughly half of all melanoma patients will be positive for B-Raf<sup>V600E</sup> and thus be candidates for these therapies. This percentage would slightly increase in patients selected for superficial spreading melanomas or melanomas on skin with intermittent sun exposure. For this reason, V600 is the primary focus of all *BRAF* mutational assays. In addition to V600E, there are minor mutations at this locus, including V600K (8 %), V600R (1 %), V600E(2) (V600E by the complex p.V→E mutation bp.1799/1800TG→AA) (0.5 %), and a mix of others (<1 %) (note these percentages

were calculated from the COSMIC database, but reports vary) [46, 132, 133]. Less is known about the biology of these alternate mutations and their responses to individual B-Raf inhibitor therapies. Therefore, the ability to detect these mutations should be under consideration when ordering or designing a mutant-*BRAF* detection assay (Table 5.3 and Fig. 5.7).

There are many ways to effectively detect mutant *BRAF*. Commonly employed methods include Sanger sequencing, pyrosequencing, PCR with mass spectrometry (Sequenom MassARRAY), allele-specific or TaqMan real-time PCR, and single nucleotide extension assays. Of the above, only two methods are currently FDA approved for guiding therapy in the advanced melanoma patient. The first to be FDA approved was the cobas 4800 BRAF V600 Mutation Test (Roche Molecular Systems, Pleasanton, CA). This is a real-time PCR assay. Briefly, it uses two differentially colored fluorochrome-labeled probes to wild-type and mutant sequences in the V600 region. PCR primers are designed to amplify



**Fig. 5.7** The *BRAF* locus and mutational analysis. The *BRAF* gene has three conserved regions (CR1, CR2, CR3) with its homologues *ARAF* and *CRAF* (a). Within CR3, there is a kinase domain. The most common mutation in *BRAF* occurs at V600, which lies in an activation area within the kinase domain, leading to constitutive activation of B-Raf. The wild-type sequence at codon 600 is GTG, encoding for the amino acid valine, or V (b, pyrosequencing tracing, reading left to right). The most common *BRAF* mutation in melanoma is a T → A at base pair position 1799, resulting in the V600E, or valine → glutamate,

mutation (c, top panel). A portion of the “T” spike is moved over to “A” (only a portion due to tumor heterogeneity). A result is considered positive for the mutation if the mutation peak accounts for >5 % of the total alleles. V600K, or valine → lysine, is the second most common mutation at this codon (c, middle panel). It is a double mutation. Due to the redundancy in the genetic code, V600E can also occur by alternate mutations (c, bottom panel). Other mutations can occur at V600 with lower frequency (not shown)

wild-type and mutant-specific PCR amplicons while releasing the two different detectable fluorochromes, accordingly. This test is potentially limited by the purity (or percentage) of the mutant sequence in the DNA sample, the integrity of the DNA, and its inability to identify and distinguish non-V600E mutations. Investigators involved in the FDA-approval process report better performance characteristics for this assay in comparison to standard Sanger sequencing using

both nonclinical samples and clinical samples in the vemurafenib trials [134, 135]. These authors suggest that Sanger sequencing would miss up to 13 % of melanoma patients that may have benefited from the drug, while the false-negative rate for the cobas assay was only 1 %. These studies also revealed some degree of cross-reactivity by the cobas assay for other mutations, including V600K, V600D, and alternative V600E mutations, although these reactions are less efficient

since the mutant probe is designed to exactly match the bp1799T>A mutant sequence. This latter issue is significant because retrospective analysis in the vemurafenib trial identified ten patients in the treatment arm with a V600K mutation. An objective response to vemurafenib was observed in four of ten, suggesting these patients may also benefit from therapy. It remains unclear if V600K-mutated melanoma patients (or other variants) will respond equally to vemurafenib or other similar agents.

More recently, the FDA approved another companion test, the THxID BRAF test (bioMérieux, Inc., Grenoble, France), for the management of the advanced melanoma patient. This test is approved for two purposes: V600E detection to qualify these patients for dabrafenib (B-Raf<sup>V600E</sup> inhibitor) and V600E and V600K detection to qualify these patients for trametinib (MEK inhibitor). THxID BRAF is also a real-time PCR assay (see Chap. 3 for description of THxID BRAF and a comparison with the cobas assay, and see below Sect. 5.5.3).

#### <sup>2</sup>CPT coding

81210 (V600E testing); 81479 (unlisted molecular procedure code) for all others

### 5.5.1.2 NRAS

Like with *BRAF*, there are mutational hotspots in *NRAS* amenable to testing (see Table 5.3 and Fig. 5.8). Q61 on exon 2 is the most commonly affected residue in cutaneous melanoma, followed by G12 and G13 on exon 1 [46, 133]. Interestingly, G12 is the most common residue mutated in *KRAS*. Q61R, Q61K, and Q61L are the most common *NRAS* mutations found in melanoma, accounting for up to 80 % reported. The tight localization of mutations on *NRAS* allows for a fairly straightforward detection assay. With mutations localized to only two areas, testing can be performed using a multitude of differ-

ent approaches and platforms, similar to *BRAF*. There is no FDA-approved assay for *NRAS* mutations, primarily because there is not a direct N-Ras inhibitor companion therapy yet available. Because of this, the utility of the *NRAS* mutational assay is not straightforward, and *NRAS* mutational analysis is not as widely used as *BRAF* mutational analysis. With that said, however, N-Ras may play a role in the paradoxical activation of the MAP kinase pathway in wild-type B-Raf cells exposed to B-Raf<sup>V600</sup> inhibitor therapy. Therefore, *NRAS* mutations could potentially activate wild-type B-Raf melanoma cells or increase the risk of cutaneous malignancies in patients on inhibitor therapy. *NRAS* mutations may also be important in resistance to B-Raf inhibitor therapy and may qualify individuals for clinical trials. Additionally, the presence of *NRAS* mutations has been associated with a better response to high-dose IL-2, further supporting the argument for testing in some populations [33]. Several commercial laboratories offer a *NRAS* mutational assay, either in isolation or as a panel. More and more laboratories are offering a melanoma mutation panel, to include *KIT* and *BRAF* and others, using a next-gen sequencing platform.

#### <sup>3</sup>CPT coding

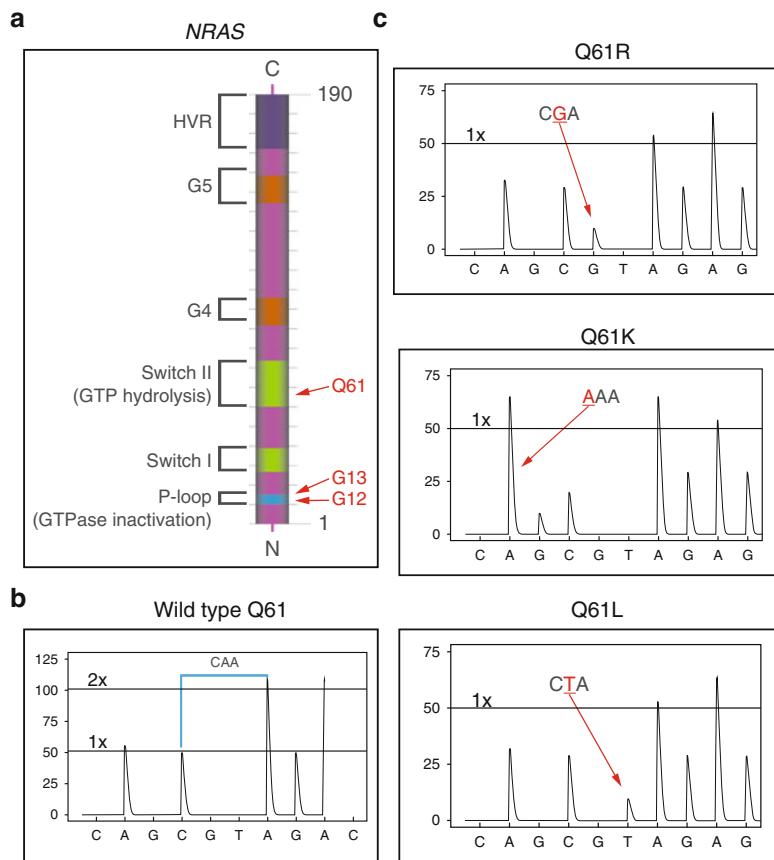
81404 (exon 1 and 2 sequencing); 81275 (listed as KRAS codons 12 and 13 analysis); 81479 (unlisted code) for all others, which is the unlisted molecular procedure code (or 81275)

### 5.5.1.3 KIT

As recognition that subtypes of melanoma have alterations in *KIT* and may respond to inhibitor therapy, the demand for testing has increased [56, 103]. The association between *KIT* mutations and decreased overall survival provides further rationale for testing [58]. Due to the high prevalence of *KIT* mutations in acral lentiginous

<sup>2</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [136]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

<sup>3</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [136]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).



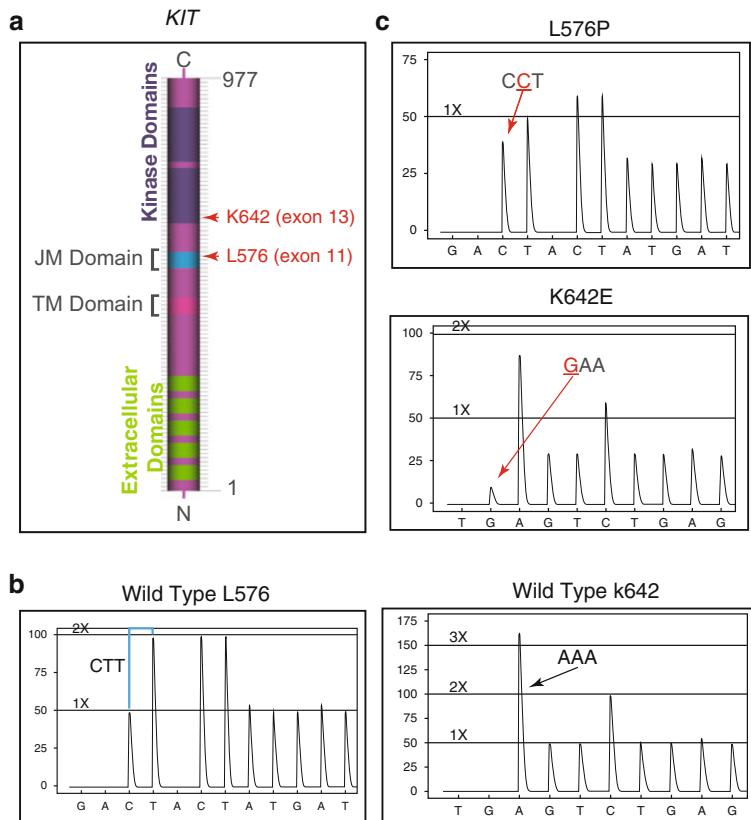
**Fig. 5.8** The *NRAS* locus and mutational analysis. The *NRAS* gene has five main domains—P-loop, Switch region I, Switch region II, G4, and G5 (a). Q61 is the most commonly mutated codon in melanoma. This glutamine residue is in the Switch II domain, which is important for GTP hydrolysis. Glycine, or G, residues at positions 12 and 13 are also commonly mutated. These fall within the P-loop domain and are important for inactivation of the GTPase. All these mutations result in a constitutively activated N-Ras. The wild-type sequence at codon 61 is CAA, encoding for the amino acid glutamine, or Q (b).

pyrosequencing tracing, reading left to right, the “A” peak is double the size due to two successive “A’s”). The most common mutations in melanoma are A → G at base pair 182, C → A at position 181, and A → T at position 182, resulting in Q61R (glutamine → arginine), Q61K (glutamine → lysine), and Q61L (glutamine → leucine), respectively (c, top, middle, and bottom panels). A result is considered positive for the mutation if the mutation peak accounts for >5 % of the total alleles. G12 and G13 mutations occur at a lower frequency, and there are other minor mutations in *NRAS* (not shown)

melanoma, primary mucosal melanoma, and melanoma of chronically sun-damaged skin, it is not inappropriate to test for *KIT* mutations in these populations. Because there is a potentially effective treatment for *KIT*-mutated melanomas, some clinicians will request *KIT* status for all melanomas (usually in the context of a melanoma mutation panel), even if the yield is low.

A *KIT* mutational assay is slightly more challenging to perform than assays for *BRAF* and *NRAS* as mutations are more widely spread along

several exons, specifically exons 9, 11, 13, and 17 [46, 133]. The more exons that are involved, the more complex the assay must be and may require complete sequencing of one or more entire exons. Fortunately, there are emerging data that only select mutations, or mutational hotspots, may be significant in their ability to predict a response to tyrosine kinase inhibitor therapy [59]. The structure of *KIT* can be broken down into four segments: an extracellular domain (exons 1–9), juxtamembrane domain (exon 11), proximal tyrosine kinase



**Fig. 5.9** The *KIT* locus and mutational analysis. The *KIT* gene is made up of extracellular domains, a transmembrane (TM) domain, a juxtamembrane (JM) domain, and proximal and distal kinase domains (a). Mutations have been reported all along the *KIT* gene but cluster in exons 11 and 13, corresponding to the juxtamembrane and proximal kinase domains, respectively. Mutations at these foci result in a constitutively activated Kit protein, a receptor tyrosine kinase. Codons 576 and 642 encode for leucine

and lysine, respectively, and are the most commonly mutated in melanoma (b, left and right panels, respectively). The T → C mutation at base pair position 1727 is the most common *KIT* mutation, resulting in L576P, or leucine → proline (c, top panel). The A → G mutation at base pair position 1924 is the second most common, resulting in K642E, or lysine → glutamate (c, bottom panel)

domain (exons 12–16), and the distal tyrosine kinase domain (exons 18–21) (see Table 5.3 and Fig. 5.9). The therapeutically relevant mutational hotspots appear to fall within the juxtamembrane domain (exon 11) and, less so, within the extracellular domain (exon 9) or kinase domain (exon 13). Kit inhibitor therapy appears to be more efficacious for melanomas with activating (not silent) *KIT* mutations at these foci, and not with amplifications, underscoring the importance of appropriate patient selection for therapy. Direct sequencing has been the assay of choice for *KIT* mutations in order to detect the wide number and breadth of defects, but as the number of therapy-

meaningful mutations becomes smaller and more focused, other detection methods, such as pyrosequencing, may become more commonplace.

#### <sup>4</sup>CPT coding

81402 (hotspot analysis), 81404 (full-gene sequencing); 81479 (unlisted code) for all other methods

<sup>4</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [136]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

### 5.5.1.4 Others

*PIK3CA* has several well-characterized mutations in human cancers, but to date, these appear to be rare in melanoma, despite the commonly observed overall activation of the PI3K/Akt/mTOR pathway. Testing is not routinely performed for *PIK3CA*, but as PI3K inhibitor therapy has entered into recent melanoma clinical trials and mutational activation of the PI3K/Akt/mTOR pathway may be a mechanism of therapy resistance, mutational status of *PIK3CA* may gain importance. The tumor suppressor *PTEN* is challenging to assay because it can be inactivated by a variety of methods (point mutation, deletion, loss of heterozygosity, epigenetic mechanisms, etc.). Molecular testing requires entire gene sequencing plus deletion analysis. *PTEN* sequencing is performed for some genetic disorders such as Cowden disease, but not melanoma. Sequencing *PTEN* in melanomas will not likely become commonplace until an effective therapy (reconstituting inhibition of PI3K) becomes available. Immunohistochemistry may have some future utility in assessing *PTEN* expression levels. Currently, *PTEN* testing has no practical role in the evaluation of the melanoma patient.

Testing for *GNAQ* and *GNA11* activating mutations is fairly straightforward and can be achieved by a variety of methods including direct sequencing, pyrosequencing, next-gen sequencing, and allele-specific or single base pair extension techniques. The recurrent mutations in uveal melanoma and blue nevi occur with the following relative frequency GNAQ Q209>GNA11 Q209>>GNAQ R183>GNA11 R183. The most common mutation at Q209 is Q209L, but other variants occur, including Q209P. Widespread use of a *GNAQ/11* mutant detection assay has been hampered by lack of a well-defined application. The mutations may have prognostic value, but as the mutations are present in benign nevi and an effective inhibitor is not yet available, testing is not routinely performed.

### <sup>5</sup>CPT coding

81403 (*GNAQ* hotspot analysis); 81479 (unlisted procedure code) for all others, including *PIK3CA*, *AKT*, and *PTEN* (unlisted for melanoma)

## 5.5.2 Immunohistochemistry

Immunohistochemistry has the ability to determine expression level of proteins, typically providing functional data as opposed to direct molecular data. This has been successfully employed to predict responses to therapy in a variety of different tumor types, including breast and lung cancer, among others [137, 138]. In preliminary studies, antibodies specific for B-Raf<sup>V600E</sup> such as VE1 have shown promise in their ability to detect tumor cells harboring the corresponding genetic defect and have shown good interobserver reproducibility [139, 140]. There are still questions, however, regarding antibody sensitivity, cross-reactivity with other V600 mutations, and the ability of an immunohistochemical result to predict response to inhibitor therapy [141]. Using immunohistochemistry as a surrogate method to determine patient eligibility for therapy is attractive as it can be readily adopted by most pathology laboratories, can reduce turnaround time by a week or more, and can potentially be cost-saving. Currently, however, until true equivalence with the molecular method becomes established and/or it becomes FDA approved for predicting response to B-Raf inhibitors, it should not be used as a substitute. Due to the time-sensitive nature of metastatic melanoma, some institutions immediately perform immunohistochemistry, to support initiating the patient on inhibitor therapy, and then later confirm the result with the molecular test. This is not routine practice, however, and

<sup>5</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [136]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

may have implications for third-party payment of medical costs.

Regarding Kit (CD117), there is currently no role for immunohistochemistry in the evaluation of melanoma. In fact, loss of Kit expression has been reported with melanoma progression, specifically invasion and metastasis, adding to the confusion [142]. Additionally, expression of Kit by immunohistochemistry does not correlate with mutational/amplification status nor efficacy of Kit inhibitor therapy [57, 143]. Immunohistochemical markers for other signaling pathway proteins, such as PTEN, EGFR, hormone receptors, and more, are also available. There are even extensive (and expensive) panels packaged for commercial use. To date, these have no role beyond a theoretical one for diagnosis of melanoma on clinical samples or predicting responses to targeted therapy.

#### <sup>6</sup>CPT coding

88342 (per antibody)

### 5.5.3 Companion Testing: The New Reality?

As an aside, it is worthwhile discussing some of the issues surrounding companion testing, which refers to the linkage of a test to the FDA approval of therapies. Vemurafenib is FDA approved by the Center for Drug Evaluation and Research “for the treatment of patients with unresectable or metastatic melanoma with BRAF V600E mutation as detected by an FDA-approved test.” Specifically, that test is the cobas 4800 BRAF V600 Mutation Test. Companion testing is becoming more commonplace albeit surrounded by controversy. Companion testing offers the benefit of standardizing testing and arguably cleaning up the clinical trial data. After all, if different types of tests are

performed, with slightly different performance characteristics, the data on therapeutic efficacy could theoretically become skewed. Indeed, discrepant results in the reported frequency of *BRAF* mutations may be due to the detection method employed [144]. Another benefit, depending on the eye of the beholder, is the protection of the investment by the assay developer in terms of funneling testing to them. Opponents of companion testing would argue that this stifles innovation and may have a negative effect on patient care. The enormous cost of taking an assay through FDA approval is prohibitive for smaller laboratories. Furthermore, there is no guarantee that a companion test is the best test or the most cost-effective test. A recent comparison analysis of *BRAF* mutation detection by the cobas test, Sanger sequencing, pyrosequencing, TaqMan RT-PCR, and competitive amplification of differentially melting amplicons (CADMA) revealed 100 % concordance among all tests for melanoma with >10 % tumor [145]. The cobas test, pyrosequencing, and Sanger sequencing performed less well with lower tumor density samples. Other studies have reported the superiority of pyrosequencing over other methods [141]. Even if a companion test is determined to have worse performance characteristics than other methods, the developer may have no incentive to switch or improve upon their already FDA-approved assay. Even minor performance-enhancing tweaks often require resubmittal to the FDA and therefore a developer may not feel it is worth the effort.

Because the concept of companion testing is relatively new, regarding the Zelboraf/cobas 4800 BRAF V600 Mutation Test story, there are currently more questions than answers: (1) How effective is the cobas 4800 BRAF V600 Mutation Test at detecting and discriminating the different V600 mutations, and is this even clinically important? (2) Do insurance companies deny payment for the drug if an alternative mutation-detection method is used, even if the test may be better and/or less expensive (the cost for 1 year of vemurafenib+dacarbazine is estimated at \$300,000 vs. \$30,000 for dacarbazine alone)? (3) As newer-generation-B-Raf inhibitors become

<sup>6</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [136]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

FDA approved with a different, perhaps better, companion test, will all laboratories then need to purchase another platform or even carry both platforms depending on which drug the oncologist wants to prescribe (these types of platforms may cost \$50,000–100,000 each)? (4) Since *BRAF* mutational analysis is also important in colorectal carcinoma and papillary thyroid carcinoma, will laboratories need to perform the identical test on three different platforms depending on tumor type (currently melanoma is the only one with an FDA-approved companion test, but this could change)?

Even with all these questions, it appears that companion testing is here to stay. There are more and more examples over a cross section of oncology. After the Zelboraf/cobas 4800 *BRAF* V600 Mutation Test, more companion tests for melanoma have surfaced. Tafinlar is now FDA approved for advanced melanoma patients with *BRAF* V600E mutations as detected by the THxID *BRAF* test. And Mekinist is now FDA approved for advanced melanoma patients with *BRAF* V600E or V600K mutations as detected by the same THxID *BRAF* test. As controversy continues to swarm around this topic, some have advocated that alternative testing methods be considered acceptable if they can demonstrate performance equivalence and not require the FDA-approval process for use. The lack of agreement on this topic and continued use of a multitude of different *BRAF* assays were captured in a recent College of American Pathologists (CAP) survey. Laboratories were asked which *BRAF* mutation-detection method they were using, with the following results: 28 % Sanger sequencing, 21 % allele-specific PCR, 17 % pyrosequencing, 17 % commercial kit, 7 % PCR with melting curve analysis, and the rest “other” [146]. Like with many topics in molecular dermatology, the story on companion testing is in evolution.

## 5.6 Summary

The potential applications for molecular testing in melanoma span from pre-diagnosis to post-therapy (Table 5.4). They can be used to identify

**Table 5.4** Current and potential uses of molecular testing in melanoma

Purpose	Test
Assess risk	Mutational analysis of patient's germline DNA
Diagnosis	FISH and CGH on primary tumor <sup>a</sup> Mutational analysis of signaling molecules
Prognosis	Gene expression arrays to assess risk for metastasis <sup>a</sup> FISH and CGH to assess copy number changes of prognostic markers <sup>a</sup> Mutational analysis of signaling molecules <sup>a</sup> Microstaging of sentinel lymph nodes by RT-PCR
Develop new classification systems	<sup>a</sup> Combination of FISH, CGH, and mutational assays
Determine candidacy for therapy	Mutational analysis of signaling molecules in tumor
Identify resistance/recurrence	Mutational analysis of signaling molecules in tumor <sup>a</sup> Minimal residual disease/recurrence of tumor in peripheral blood by RT-PCR

<sup>a</sup>Primarily investigative

carriers of melanoma-related gene mutations and variants, aid in the diagnosis of melanoma, better stratify a patient's risk of progression, and guide therapy of primary or resistant tumors, among others. The significance of molecular testing has accelerated since the discovery of oncogenic drivers of melanoma and the inception of personalized mutant-specific therapy. Intense efforts to identify and evaluate more oncogenic drivers and therapeutic targets continue. New technologies to detect driving mutations, such as whole-genome, whole-exome, and now whole-transcriptome sequencing, are extremely powerful at generating immense data quickly and are revolutionizing the field. This field of theranostics is evolving so quickly that entire websites are now devoted toward educating patients and physicians, allowing them to enter the mutational status of the melanoma and deliver prognostic information, as well as direct them to ongoing clinical trials [128].

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# Leukemia and Lymphoma. Part I.

## Mycosis Fungoides and Sézary Syndrome: Using Molecular Tools to Aid in the Diagnosis, Staging, and Therapy for Mycosis Fungoides and Sézary Syndrome

### Contents

6.1	Introduction .....	134
6.2	Diagnosis .....	135
6.2.1	Clinical Features.....	135
6.2.2	Histology .....	135
6.2.3	Immunohistochemistry.....	135
6.2.4	The Need for Molecular Testing .....	138
6.2.5	Molecular Studies.....	138
6.2.6	Diagnostic Algorithms for MF/SS .....	147
6.3	Staging and Prognosis.....	148
6.3.1	Assessing Prognosis by PCR .....	148
6.3.2	Assessing Prognosis by FISH and aCGH .....	151
6.4	Therapy .....	151
6.5	Practical Considerations for Ordering, Performing, and Interpreting Molecular Tests.....	151
6.5.1	Assay Selection and Design .....	152
6.5.2	Interpretation of the PCR TCR Gene Rearrangement Assay .....	156
6.6	Summary .....	161
	References .....	162

### Key Points

- The diagnosis of early MF/SS can be challenging as there is significant clinical and histologic overlap with benign/reactive inflammatory conditions.
- The diagnosis of MF/SS is a multi-pronged approach—integrating clinical, histologic, immunophenotypic, and molecular data.
- For MF/SS, T-cell clonality is determined by the molecular analysis of rearranged T-cell receptor genes, most commonly *TRG* ( $\gamma$ ), *TRB* ( $\beta$ ), or both. Rearrangement of these genes is a normal biologic process during T-cell development.
- Clones, as determined by T-cell gene rearrangement studies, may be observed in reactive inflammatory processes, and conversely, malignant infiltrates may fail to have a detectable clone.
- PCR is the most commonly used method for assessing clonality in MF/SS. There is extreme variability in assay design and data interpretation.
- Assay selection and interpretation of data in the context of the individual MF/SS patient are the responsibility of the clinician, dermatopathologist, and molecular diagnostician.
- While the primary role for molecular testing in MF/SS is for diagnosis, there

are other current and potential applications. Molecular assays have been incorporated into the TNMB staging/prognosis workup of the MF/SS patient. Molecular testing for prognostic biomarkers is in the investigative stage.

- New therapeutic strategies for MF/SS are centered on immune modulation. Theranostic applications have not yet been realized, but are being investigated.

## 6.1 Introduction

Mycosis fungoides (MF) and Sézary syndrome (SS) have a rich history with regard to their discovery and significant scientific advances, well chronicled in various reviews [1, 2]. Because of their historically intertwining relationship and similar cell of origin, MF and SS are considered together in this chapter. The name “mycosis fungoides” (loosely translated into “mushroomlike fungal disease”), coined by the French dermatologist Jean-Louis-Marc Alibert in 1806, conjures up wild images of how the original patient must have presented (and viewing the original sketches would leave any historian satisfied). In 1862, Pierre-Antoine-Ernest Bazin described the natural progression of MF, from patch to plaque to tumor stage [3]. The contributions by these two individuals led to MF’s alternative eponymic designation—Alibert-Bazin disease. Louis-Anne-Jean Brocq was another important player in the history of MF, annotating the first reports of the related condition, parapsoriasis, in 1902 [4].

For over a century after Alibert’s initial encounter, the diagnosis of MF had relied almost exclusively on clinical parameters. By the latter half of the nineteenth century and beginning of the twentieth century, microscope usage became more routine, and histomorphologic diagnostic criteria for cutaneous disease became more developed. In 1938, Albert Sézary and Yves Bouvrain documented a woman with edematous plaques and pruritus who progressed to

erythroderma [5]. This woman was biopsied, revealing an epidermotropic population of irregular small cells, and a blood smear revealed “monster cells.” Sézary incorporated blood smears into diagnostic criteria to characterize paramycosis hémotrope, which would later be designated Sézary syndrome. It wasn’t until the 1970s that the T cell was determined to be the cell of origin for MF and SS. The early 1980s incorporated immunohistochemistry into the MF/SS workup and the mid-1980s brought molecular testing for T-cell gene rearrangements into the diagnostic armamentarium [6]. Today, integration of clinical, histopathologic, immunophenotypic, and molecular data is required to arrive at a diagnosis of MF or SS. These cutaneous lymphoproliferative diseases are now prototypes for this multi-pronged diagnostic approach.

Assessing T-cell infiltrates in cutaneous eruptions for clonality, specifically in MF and SS, is arguably the first clinical application for molecular diagnostics in dermatology and dermatopathology. Clonality assays, in this context, are unique in molecular diagnostics because they do not target genetic events that have gone awry (mutations, translocations, etc.), but take advantage of a normal biologic process—the somatic recombination of DNA within the developing T cell. And because clonal expansion is a normal T-cell response to antigen, clonality does not always equal malignancy. Interpretation is not always black or white, emphasizing the importance of reviewing results in the context of the entire patient.

Diagnosis of MF and SS remains a commonly encountered challenge in dermatology and dermatopathology. Accurate diagnostic criteria and testing not only are required to establish a diagnosis of MF in an individual but are also needed to tabulate meaningful epidemiologic data and prognostic information, select appropriate therapy, interpret responses to therapies, and qualify individuals for therapeutic clinical trials. While molecular assays have now become fully integrated into the routine diagnosis of early MF and SS, and have even been incorporated into commonly used diagnostic algorithms [7], there still remain many questions on how to implement

these tests for routine use. Molecular assays have also worked their way into staging and prognostic criteria [8], and future applications include a possible role in therapy and pharmacogenomics, among others. This chapter summarizes the current role for molecular testing in the evaluation of the MF/SS patient, with emphasis on its current primary role—diagnosis. Limitations of the assays are emphasized, and practical considerations for assay design and interpretation of results in the context of the whole patient are discussed.

## 6.2 Diagnosis

### 6.2.1 Clinical Features

MF is the most common cutaneous T-cell lymphoma, accounting for 65 % of cases [8–10]. The true incidence is difficult to determine, as many cases are underdiagnosed and reporting is variable, but an estimated 1,000 new cases of MF are diagnosed in the United States each year. Typically, the cell of origin is a post-thymic CD4+/CD45RO+ helper T cell (less commonly CD8+) that has homing properties to the skin. The male to female ratio is 2:1, and it primarily affects the adult and elderly populations, although rare pediatric cases do occur. SS originates from a similar cell and affects a similar demographic but is more rare, accounting for <5 % of cutaneous T-cell lymphomas. While MF is fairly indolent, SS is aggressive with a 5-year survival of only 10–20 %.

MF often progresses through patch → plaque → tumor stages, although exceptions exist. Patch-stage MF is comprised of multiple variably sized (usually >5 cm) erythematous plaques, most commonly located on skin with low ultraviolet exposure or a “bathing suit” distribution (Fig. 6.1). Skin lesions may have epidermal atrophy, telangiectasias, and variable pigmentation (poikilodermatous). As the disease progresses, skin lesions become larger and thicker with possible ulceration and tumor formation. Erythroderma, lymphadenopathy, and visceral organ involvement are late findings.

SS is defined by circulating neoplastic cells (specifically, >1,000 Sézary cells/mm<sup>3</sup> and/or >20 % Sézary cells) resulting in erythroderma and generalized lymphadenopathy and thus is a distinct cutaneous T-cell lymphoma with a leukemic component [11–13]. SS patients have generalized disease at presentation [14]. MF may progress to erythroderma with circulating neoplastic cells, but this “secondary” SS should remain separate from and not be confused with “true,” or “primary,” SS, as they have different clinical courses.

### 6.2.2 Histology

Entire monographs have been written on the histologic criteria for diagnosis of MF and SS [15, 16]. Briefly, when “histologically behaving” like it should, MF is a top-heavy (involving epidermis and superficial dermis) epidermotropic cutaneous T-cell lymphoma (Fig. 6.2). Early lesions have a mild lichenoid or band-like infiltrate, often with characteristic stromal changes. Cytologically atypical cells migrate into the epidermis, both tagging along the epidermal-dermal junction and forming Pautrier microabscesses in more superficial levels of the epidermis. These cells are atypical, with enlarged, irregular nuclei and perinuclear halos. At times, the nuclei fold upon themselves, creating a cerebriform appearance. More advanced lesions will have denser and deeper infiltrates and may transform into larger cells. SS infiltrates are often histologically subtle, despite patients’ striking clinical presentations.

### 6.2.3 Immunohistochemistry

Immunohistochemistry has proven to be a powerful ancillary tool in the diagnosis of MF and SS, which have similar immunoprofiles. In most cases, the neoplastic cell is CD4+ (see Fig. 6.2). A subset of cases will be CD8+/CD4−. Aberrant loss of normal T-cell markers, such as CD7 and others, adds further credence to the presence of a true malignant infiltrate. Transformed cases may

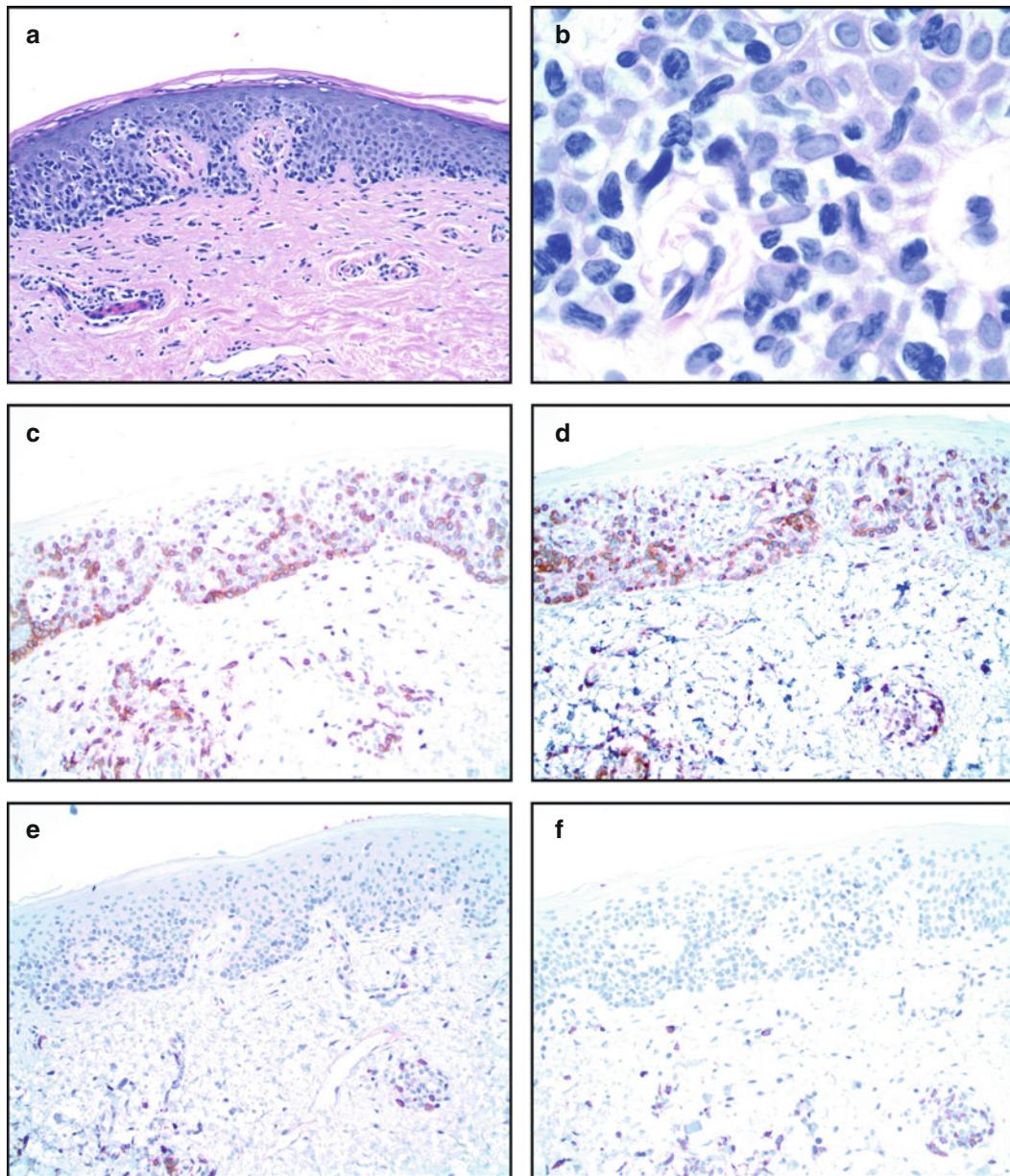


**Fig. 6.1** Clinical features of mycosis fungoides (MF) and Sézary syndrome (SS). MF often progresses through patch → plaque → tumor stages. Areas not routinely exposed to ultraviolet light, the so-called “bathing suit” distribution, are the most commonly involved. A 52-year-old man has multiple erythematous patches over the buttocks and thighs (**a**). A 59-year-old female has coalescing

scaly erythematous patches and plaques on her trunk (**b**). An elderly male has an erythematous and focally ulcerated tumor on his lower extremity, typical of tumor stage MF (**c**). SS often rapidly progresses and presents as erythroderma, as with this 60-year-old man (**d**) (Several images courtesy of Dr. Stephen Weis, Ft. Worth, TX, and Dr. Alan Menter, Dallas, TX)

express CD30. When present, the following immunohistochemical data can be quite helpful: CD4:CD8 >10:1, aberrant loss of CD7 (loss in >50 % of the infiltrate seen in 75 % of MF/SS; some algorithms require <10 % staining in T

cells), loss of CD26 (62 % of cases), loss of CD5 (22 % of cases), and loss of other T-cell antigens such as CD2, CD3, and CD4 when the epidermal CD4:CD8 ratio is elevated (>10:1) (all uncommon) [17, 18].



**Fig. 6.2** Histologic features of mycosis fungoides. In conventional MF, lymphocytes extend into the epidermis, in a pattern of epidermotropism (a, H&E, 200 $\times$  original magnification). These cells align along the epidermal-dermal junction as well as extend into more superficial layers of the epidermis, forming Pautrier microabscesses. The lymphocytes are cytologically atypical, with enlarged

hyperchromatic and convoluted (cerebriform) nuclei and perinuclear halos (b, H&E, 1,000 $\times$ ). By immunohistochemistry, the epidermal infiltrate is positive for CD3 (c, CD3, 200 $\times$ ) and CD4 (d, CD4, 200 $\times$ ). There are virtually no epidermal CD8+ cells (e, CD8, 200 $\times$ ), and CD7 expression appears aberrantly lost (f, CD7, 200 $\times$ )

### 6.2.4 The Need for Molecular Testing

Diagnosis of late-stage MF is not problematic, but establishing this diagnosis early in its course is one of the most frustrating exercises in dermatology—for the patient, the dermatologist, and the dermatopathologist. For patients, in particular, it is often hard to understand why a team of doctors “think” they might have a malignancy but are not sure, leaving the patient in a state of diagnostic purgatory.

Early MF diagnosis is plagued by its vague clinical presentation, which can be quite protean, overlapping with many reactive inflammatory dermatoses. These include eczema, psoriasis, lymphomatoid papulosis, pityriasis lichenoides et varioliformis acuta (PLEVA), and drug eruptions, among other papulosquamous disorders. Moreover, MF and the reactive/inflammatory dermatoses may have similar responses to conservative treatment, such as topical steroids, further complicating the clinical picture. The histology can be equally ambiguous, and there is often interpretation variability, even among panels of experts [16, 19]. There are many well-described histologic mimickers of MF, mirroring the clinical differential diagnosis [20–24]. Further compounding problems with diagnosis are well-described clinical and pathologic variants of MF—hypopigmented, purpuric, bullous, follicular, palmoplantar, granulomatous, pagetoid reticulosis, and others—all of which lie “outside the box” of clinically and histologically conventional MF. There are also various poorly defined, nebulous chronic conditions, like small- and large-plaque parapsoriasis, which may or may not be precursors to MF (Fig. 6.3). Indeed, it is the persistence and/or progression of lesions in MF that is often the first clue to something more sinister, and the diagnosis is often made retrospectively.

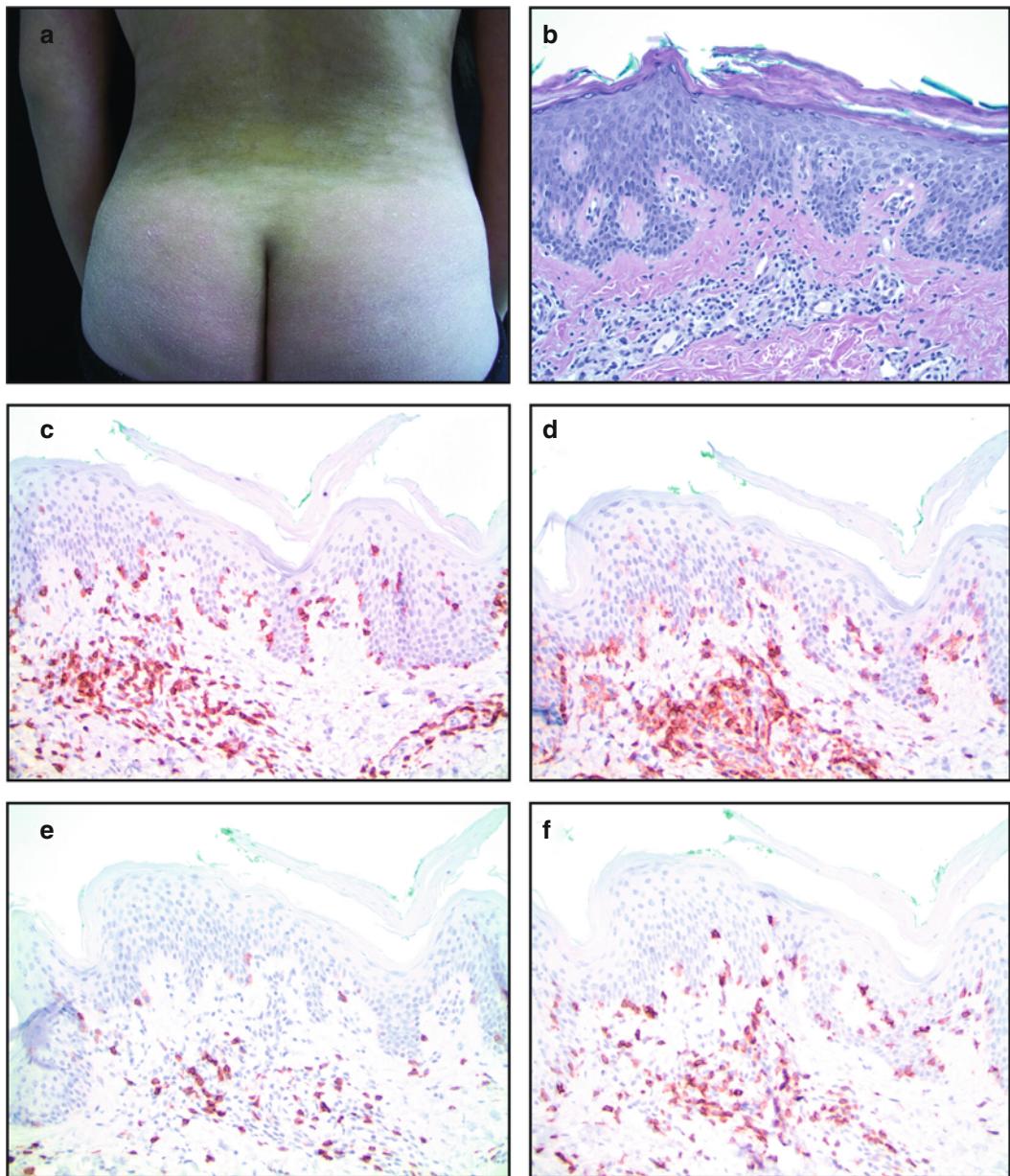
Immunohistochemistry can be helpful but is not a panacea [17, 18, 25, 26]. Definitive immunomarkers for clonality are lacking in T-cell processes, and there has been minimal advancement in this area for several decades. In B-cell

processes, clonal populations can be more easily identified (by immunohistochemistry and/or flow cytometric analysis) for the following reasons: There are more clearly immunophenotypically defined populations of B cells, and expansion of certain populations (such as the follicle center cell) would be distinctly abnormal and suggestive of clonality; kappa and lambda expression can act as a surrogate for clonality when expression is restricted to one or the other; and B-cell infiltrates, in general, are less common in cutaneous inflammatory (nonneoplastic) conditions (see Chap. 7).

Immunohistochemical evaluation of T-cell populations remains limited to subjective assessment of CD4:CD8 ratios (the best available immunosurrogate for clonality) and variable T-cell antigen loss. Often times, multiple biopsies, sometimes up to seven, from different anatomic sites and points in time, are required to establish a diagnosis. In the erythrodermic patient, the diagnosis may have to be made from blood or lymph nodes. *As there is no pathognomonic histologic or immunophenotypic alteration in MF or SS and the interobserver variability in the evaluation of these criteria is suboptimal, additional diagnostic tools, such as molecular assays, are needed.*

### 6.2.5 Molecular Studies

Permutations of molecular assays for the diagnosis of MF and SS have been around for several decades. These continue to evolve, acting as a supplement to histology and immunohistochemistry, promising to improve upon their diagnostic accuracy. The most common assay—T-cell gene rearrangement analysis—began by utilizing Southern blot techniques and now almost exclusively uses PCR-based methods (see Sect. 6.5 for discussion on various techniques). As the following discussion exposes, despite this assay’s relatively long existence in the diagnostic armamentarium of MF and SS and its refinement over the years, limitations remain as do questions on how to best implement this assay for routine use.



**Fig. 6.3** Ambiguous cases. Diagnosis of MF early in its course is a commonly encountered problem and remains a great challenge for the dermatologist and dermatopathologist. One example is this 15-year-old girl who presented with a several-year history of hypopigmented patches and plaques involving her lower trunk and buttocks (a). These have a fingerlike, or digitate, appearance on the flanks. This finding is associated with the poorly defined entity parapsoriasis, which may or may not be a precursor to MF. The biopsy reveals a moderate mononuclear cell

infiltrate in the superficial dermis and extending into the epidermis. There is slight tagging along the epidermal-dermal junction, but epidermotropism is not present (b, H&E, 200 $\times$  original magnification). Overt cytologic atypia is also not observed. The infiltrate primarily contains CD3+ T cells (c, CD3, 200 $\times$ ). In the epidermis, the vast majority of these are CD4+ (d, CD4, 200 $\times$ ), with only few CD8+ cells present (e, CD8, 200 $\times$ ). CD7 expression is retained (f, CD7, 200 $\times$ )

### 6.2.5.1 T-Cell Gene Rearrangement and Determination of Clonality

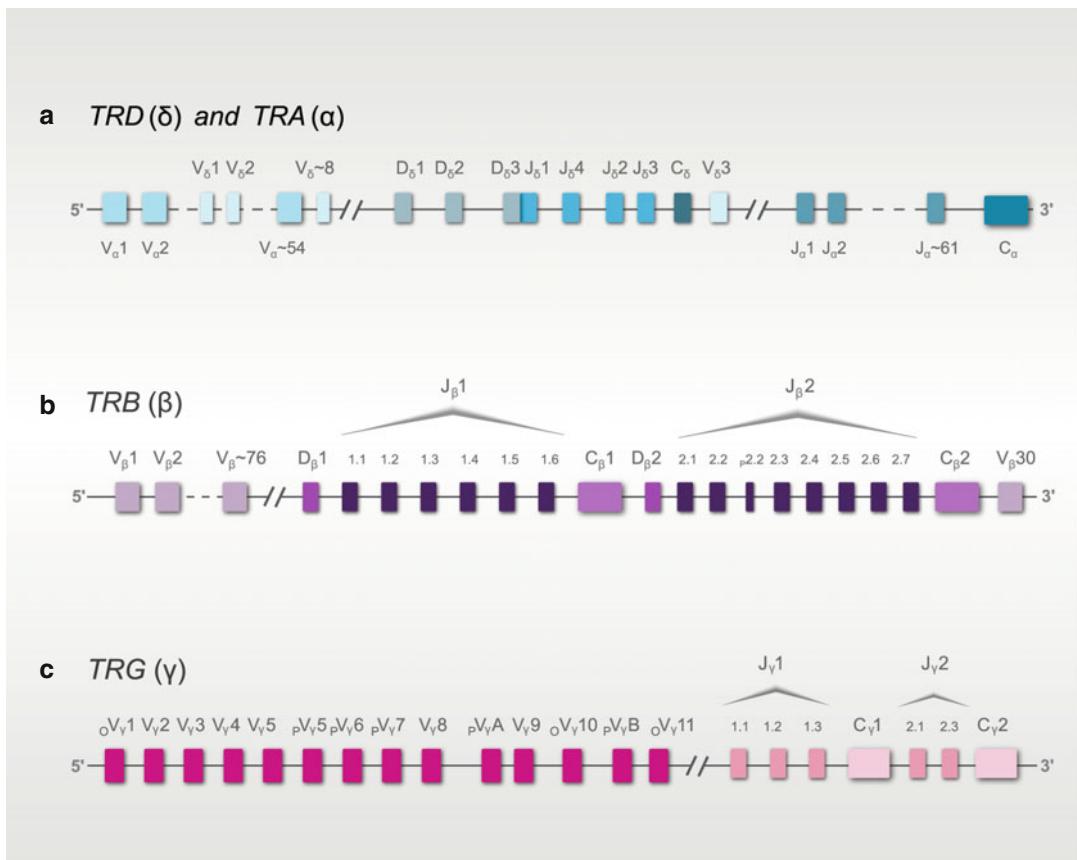
As determined from the Human Genome Project, the human genome contains approximately 25,000 genes [27]. This number appears grossly inadequate for generating sufficient diversity among T-cell receptors (TCR) and B-cell receptors (immunoglobulins) required to combat, or target, the seemingly infinite number of potential foreign antigens. It would appear that the entire human genome must be dedicated to the creation of different TCRs and immunoglobulins. This conundrum can be solved by the process of somatic recombination, which uses a “cut-and-paste” method to recombine a limited number of variable, diversity, and joining gene segments at TCR and immunoglobulin gene loci to logarithmically increase the antigen-recognition diversity of the corresponding receptor proteins. Somatic recombination occurs in lymphocytes and no other human cells. Clonality is determined by evaluating relative proportions of somatic recombination events among lymphocyte populations. The following discussion focuses on T-cell gene rearrangement (see Chap. 7 for discussion on B-cell clonality, immunoglobulin gene rearrangement and testing).

In normal T-cell development, within the thymus, for any given T cell, gene segments rearrange with the purpose of generating a functional TCR that is capable of recognizing and reacting to a foreign antigen, but not a self-antigen. There are four loci—*TRD* ( $\delta$ ) on 14q11.2, *TRG* ( $\gamma$ ) on 7p14, *TRB* ( $\beta$ ) on 7q34, and *TRA* ( $\alpha$ ) on 14q11.2—that rearrange in that exact sequential order, until a functional TCR protein is created [28–30]. The TCR- $\gamma$  chain pairs with the TCR- $\delta$  chain to form TCR $^{\gamma\delta}$  on  $\gamma/\delta$  T cells, and the TCR- $\alpha$  chain pairs with the TCR- $\beta$  chain to form TCR $^{\alpha\beta}$  on the more common CD4+ or CD8+  $\alpha/\beta$  T cells. These receptors couple with CD3 on the T-cell surface membrane, to form functional TCR complexes.

*TRD* ( $\delta$ ), *TRG* ( $\gamma$ ), *TRB* ( $\beta$ ), and *TRA* ( $\alpha$ ) differ in the nature and quantity of rearrangeable gene segments (Fig. 6.4). The *TRA* ( $\alpha$ ) and *TRG* ( $\gamma$ ) loci are made up of variable and joining (V-J) gene segments, adjacent to constant (C) regions. *TRB* ( $\beta$ ) and *TRD* ( $\delta$ ) have variable, diversity, and joining (V-D-J) gene segments, adjacent to constant (C) regions. Therefore, the complete TCR $^{\alpha\beta}$  and TCR $^{\gamma\delta}$  contain an antigen-binding domain dictated by exactly one V-J sequence and one V-D-J sequence. At the *TRG*

( $\gamma$ ) locus, one gene segment is randomly selected from the V region and one from the J region. These segments recombine by forming a complex rolling circle formation, splicing out the interval DNA and leaving the upstream and downstream DNA intact. This newly formed recombined DNA is transcribed to primary RNA transcripts. These transcripts are modified to mRNA by splicing out all the interval genetic material and ultimately combining the V-J and C segments. The V-J-C mRNA transcripts are translated to the TCR- $\gamma$  chain protein. At virtually the same time, a similar process occurs at the *TRD* ( $\delta$ ) locus (with recombination of V, D, J, and C). If the recombination events of *TRG* ( $\gamma$ ) and *TRD* ( $\delta$ ) are successful at forming a functional surface TCR $^{\gamma\delta}$ , the  $\gamma/\delta$  T cell leaves the thymus seeking its antigen. If not, the other allele at the failed locus will make an attempt. If recombination of both alleles fails for either the *TRG* ( $\gamma$ ) or *TRD* ( $\delta$ ) locus, the cell becomes committed to the TCR $^{\alpha\beta}$  lineage, and segments from the TCR- $\beta$  chain attempt to rearrange (*TRB* ( $\beta$ ) actually begins its rearrangement at around the same time as *TRG* ( $\gamma$ ) and *TRD* ( $\delta$ ), while *TRA* ( $\alpha$ ) rearrangement occurs much later). At the *TRB* ( $\beta$ ) locus, first, the D and J gene segments are randomly selected and recombine, and then a V segment is selected and recombines with D-J (Fig. 6.5). The recombined DNA is transcribed to primary RNA transcripts. These transcripts are modified to mRNA containing the V-D-J-C union, and the mRNA transcripts become translated into the TCR- $\beta$  chain protein. If the T cell reaches this stage, it will continue on to rearrange a V-J on the *TRA* ( $\alpha$ ) locus, ultimately forming the TCR- $\alpha$  chain. Similar to the other loci, if recombination fails to generate a functional protein, rearrangement of the other allele is attempted.

The V, D, and J segments of a single locus can rearrange into many combinations, referred to as combinatorial diversity (Table 6.1). Overall receptor diversity takes into account the product of the combinatorial diversity of the two loci that make up the receptor (e.g., *TRA* ( $\alpha$ ) and *TRB* ( $\beta$ )) as well as imprecise joining of gene segments, potentially altering the amino acid sequence. Regarding the latter, during the recombination of gene segments, there can be slight modifications at the junctions, such as an insertion of extra random nucleotides (e.g., “N-nucleotide addition”). All of these factors contribute to the lymphocyte



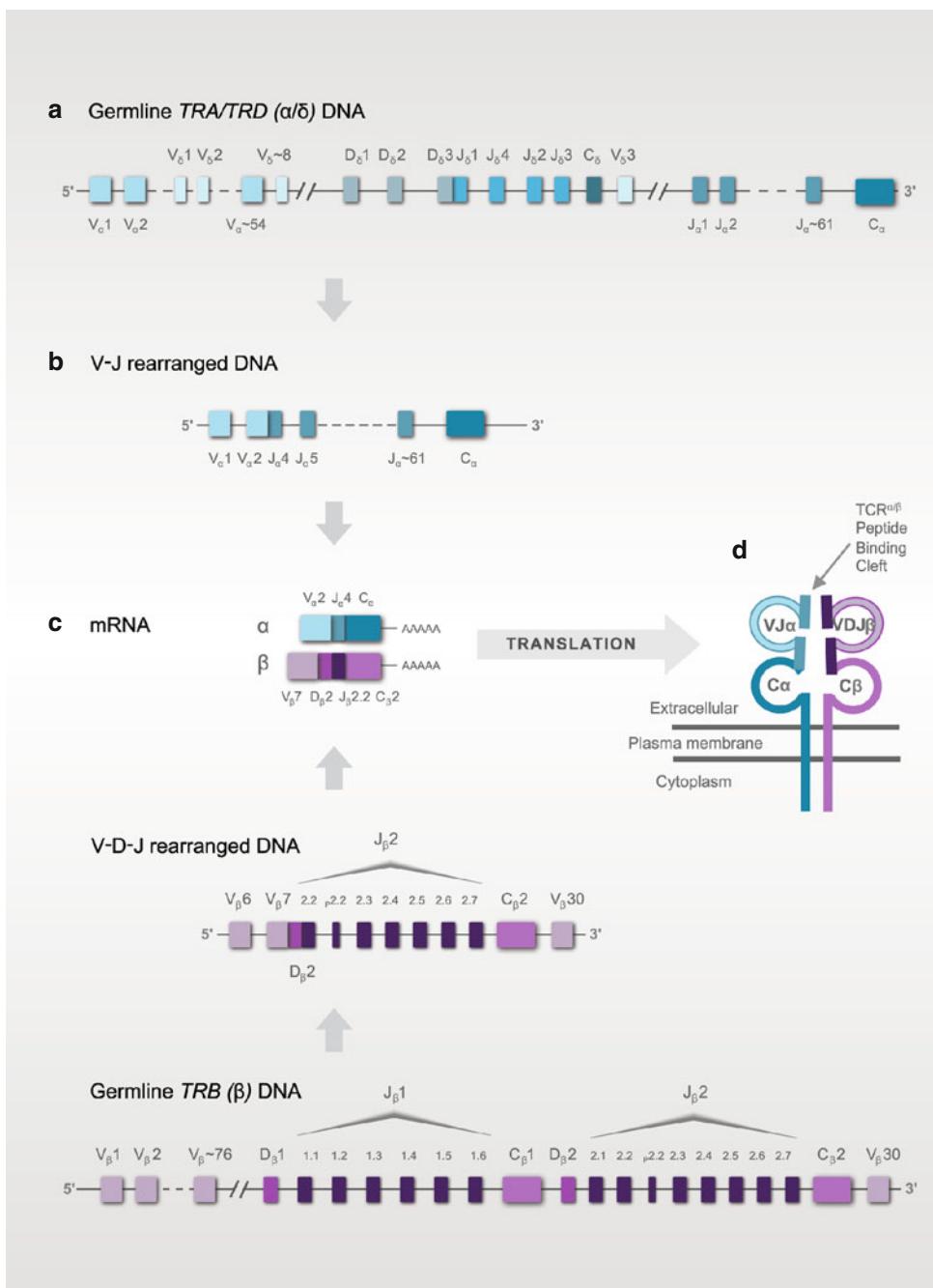
**Fig. 6.4** TCR gene loci. *TRA* ( $\alpha$ ), *TRD* ( $\delta$ ), *TRB* ( $\beta$ ), and *TRG* ( $\gamma$ ) loci are shown. *TRD* (upper labels) resides within *TRA* (lower labels) at the same locus (a). The V regions for *TRA* and *TRD* overlap. The *TRA* V and J regions contain many pseudogenes and orphans (nonfunctional genes located outside main chromosomal locus), and some genes are shared between loci (not included for clarity). *TRB* contains approximately 76 V gene segments, each with a leader sequence (not shown), followed by almost a duplication of D-J-C segments (b). The *TRB* V region has

many pseudogenes and orphans (not included for clarity). *TRG* has 14 V gene segments, many of which are pseudogenes and orphans (indicated by a "P" or "O," respectively) (c). Transcription occurs in the 5' → 3' direction (with the exceptions of V $_{\delta}$ 3 and V $_{\beta}$ 30 which are located outside the normal variable regions and have inverted sequences). V segments for *TRA* and *TRB* are numbered sequentially for clarity (For up-to-date loci maps and gene designations, refer to the international ImMunoGeneTics database at [www.imgt.org](http://www.imgt.org) [31]).

army's ability to recognize a seemingly infinite number of antigens with a limited number of genes. The total amino acid sequence diversity for antigen recognition is estimated at  $10^{15}$  permutations for  $TCR^{\alpha\beta}$  and  $10^{18}$  for  $TCR^{\gamma\delta}$  [32].

Following recombination of gene segments, the surface T-cell receptor proteins are formed. Because of the stepwise rearrangement of alleles, all surface TCRs for a given cell have only one, a unique, antigen-recognition site (the exception to this is *TRA* ( $\alpha$ ), which, on occasion, may express both rearranged alleles). This process is termed *allelic exclusion* and is somewhat unusual, because with most genes, both allelic copies are expressed.

For a given naïve T cell, all the surface TCRs recognize a single epitope of a single antigen, but because there are many permutations of gene segment recombinations occurring in all the developing T cells within the thymus, the entire circulating T-cell population has a vast array of different T-cell receptors, a "repertoire," capable of recognizing many different potential invading pathogens/antigens/epitopes. In a normal inflammatory response to antigen, lymphocytes that have their surface antigen receptor engaged (the B or T cell recognizes the antigen) will undergo *clonal expansion*, meaning the cells will multiply, maintaining virtually identical TCR recombinations and surface



**Fig. 6.5** T-cell receptor somatic recombination. This schematic highlights the steps involved in rearrangement of the *TRA* ( $\alpha$ ) and *TRB* ( $\beta$ ) loci to form a surface TCR $^{\alpha\beta}$  protein. *TRA* ( $\alpha$ ) contains V and J gene segments, interrupted by the *TRD* ( $\delta$ ) locus (a). During somatic recombination, a randomly selected  $V_{\alpha}$  gene segment recombines with a  $J_{\alpha}$  gene segment, deleting the interval genetic material (b). This interval material contains the entire *TRD* ( $\delta$ ) locus, which would have already attempted rearrangement (not shown). The newly recombined DNA is transcribed into primary RNA transcripts (not shown). These transcripts are modified

to mRNA, splicing out all interval genetic material to achieve the V-J-C union (with a poly-A tail) (c). A similar process is taking place at the *TRB* ( $\beta$ ) locus. *TRB* ( $\beta$ ) is slightly different in that it also must recombine a  $D_{\beta}$  gene segment, first to a  $J_{\beta}$  segment, and then to a  $V_{\beta}$  segment (bottom half of figure). Once mRNA transcripts for the TCR- $\alpha$  and TCR- $\beta$  chains are generated, they are translated into the TCR- $\alpha$  and TCR- $\beta$  proteins and expressed on the surface of the TCR $^{\alpha\beta}$  T cell (d). A similar process occurs at the *TRG* ( $\gamma$ ) and *TRD* ( $\delta$ ) loci (prior to completion of *TRA* ( $\alpha$ ) and *TRB* ( $\beta$ ) recombination) to form the TCR $^{\gamma\delta}$  (not shown).

**Table 6.1** Number of gene segments and potential rearrangements from different TCR loci

TCR locus		Genes <sup>a</sup>	Rearrangeable genes <sup>a</sup>	Functional rearrangeable genes	Combinatorial diversity (max V×D×J)
TRA ( $\alpha$ )	V	54	54	45	2,300
	J	61	61	50	
	C	1	1	1	
TRB ( $\beta$ )	V	76	67	48	2,300
	D	2	2	2	
	J	14	13	13	
	C	2	2	2	
TRG ( $\gamma$ )	V	14	9	6	30
	J	5	5	5	
	C	2	2	2	
TRD ( $\delta$ )	V	8	8	8	96
	D	3	3	3	
	J	4	4	4	
	C	1	1	1	

Numbers of genes are approximate and include pseudogenes and orphans, data compiled from several sources [31–33]

<sup>a</sup>Rearrangeable genes have the appropriate flanking recombination signal sequences (RSS)

proteins. Lymphocyte activation and clonal expansion occur with help from other cells in the immune response by direct binding of cells through surface receptors and/or by cytokine release. The process in which only the cells that recognize antigen become activated and proliferate is termed *clonal selection*. In most infections or benign inflammatory diseases, there are numerous different antigens, many of which are complex with multiple epitopes. This results in numerous clonal expansions or, effectively, a polyclonal response.

In T-cell neoplasia, there is also clonal expansion, but this is due to molecular oncogenic events as opposed to the process described above. In this monoclonal “response,” all cells harbor virtually identical TCR rearrangements and surface proteins. If any single V-J or V-D-J rearrangement appears overrepresented among a population of T cells, this is evidence for clonality. As should be clear from the discussion, clonality does not necessarily equal malignancy. Overrepresentation of V-J or V-D-J recombinations by molecular analysis could potentially occur in reactive processes when the number of recognized antigens/epitopes is few and finite, resulting in clonal expansion of only one or few clones. These may be true clonal populations but biologically normal. If the sample assayed is focal enough to pick up a small clonally expanded population of cells (or even one cell), this will give the appearance of a neoplastic clone,

or pseudoclone. Clonality may also be present and detected in preneoplastic but biologically irrelevant processes, as these conditions may never progress to true malignancy. These latter examples of detectable clones in nonmalignant conditions have been assigned various terms over the years, including clonal dermatitis, abortive or latent lymphoma, and T-cell dyscrasia, among others.

### 6.2.5.2 Limits of Detection: Finding the “Sweet Spot”

The primary problem with histologically diagnosing early MF is detecting the presence of a neoplastic population within a background of reactive inflammatory cells. In early lesions, there may only be a few neoplastic cells within the epidermis, accounting for <5 % of the cellularity (and total DNA content) in the sample (note the limit of detection for Southern blot is 5 %), leaving the dermatopathologist to search for the proverbial “needle in the haystack.” Studies that looked at longitudinal progression of lymphoma have observed that clones are detected in the skin, blood, and lymph nodes with increased frequency as the disease progresses, supporting the notion that false-negative results are, at least in part, a function of limits of detection. Molecular tools are attractive because of their inherent high analytical sensitivity (low limits of detection). Southern

blot and PCR techniques have reported limits of detection of 5 and 1 % neoplastic cells, respectively, and these limits can be pushed even further by certain manipulations of the sample [34, 35]. While this is likely sufficient in most cases, it introduces a new problem of potentially being *too* sensitive. Molecular testing for clonality takes advantage of TCR gene segment recombination, as described above, which naturally occurs in normal T-cell development. And because clonal expansion is a normal process for lymphocytes in inflammatory conditions, clones can and are detected in nonneoplastic conditions if the sample lacks a significant polyclonal background. This scenario is termed “pseudoclonality.” Taken to an extreme, based on amplification of DNA, a “clone” will be detected if the sample contains DNA from only one cell.

Therefore, there is a conceptual “sweet spot” for a molecular assay. The assay is sensitive enough to identify neoplastic cells within a polyclonal background, but not too sensitive to identify a pseudoclone. This “sweet spot,” however, is poorly defined. To improve upon the analytical sensitivity, some enhancement techniques have been introduced to PCR-based assays, among them manual microdissection of the epidermis and laser capture microdissection. Manual microdissection usually entails separation of the epidermis and superficial dermis from the remainder of the tissue, either on slides or from the block, by a razor blade, glue, or other coarse separation means. Laser capture microdissection uses a specialized suction apparatus to pull microscopically visualized small aggregates of cells off of a slide. In a recent proof of concept study by Yang et al., laser capture microdissection was compared with standard phenol-chloroform extraction from full-tissue sections [35]. Using early suspected MF ( $n=31$ ), established MF ( $n=10$ ), and reactive dermatoses ( $n=9$ ) samples, the laser capture microdissection technique had improved sensitivity over standard full-tissue-section DNA extraction. This was true for the established MF group (100 % vs. 80 %) and the suspected early MF group (80.7 % vs. 35.5 %),

using multiple primer pairs, and no false positives were detected in the inflammatory group. Of course, not everyone has access to or even wants a laser capture microdissecting device, but the concept is sound. Regarding the issue of becoming “too sensitive,” there are no guidelines. Typical PCR-based assays recommend 0.5–2 µg of total DNA as a template per reaction, and so long as there is a significant lymphocytic population in the sample (which should always be analyzed by a pathologist to flag sparse infiltrates as potentially problematic), the issue of pseudoclonality can be minimized, even when using enhancement techniques such as manual and laser capture microdissection.

### 6.2.5.3 Limitations of the Molecular Assay

Molecular tools have been available and used for the diagnosis of MF and SS for several decades and have been an important addition to the diagnostic checklist for MF, but not without controversy [17, 33–42]. It is well known that clonal analysis for early MF suffers from less-than-ideal sensitivity and specificity parameters. Unambiguous MF, confirmed by progression or other means, does not always have a detectable clone, and, conversely, clones are commonly reported in inflammatory dermatoses within the clinical differential diagnosis of MF. For example, regarding the latter, clones have been detected in 10–32 % of benign inflammatory conditions—pityriasis lichenoides et varioliformis acuta (PLEVA), pityriasis lichenoides chronica (PLC), lichen planus, psoriasis, eczema, morphea, discoid lupus erythematosus, drug eruptions, and up to 70 % of cases of lymphomatoid papulosis, among others [38, 43–49].

As evident from Table 6.2, the utility of T-cell gene rearrangement analysis for the diagnosis of MF/SS is extremely variable [17, 33, 34, 36, 38–40, 45, 51–57]. In established MF patients, clonality is detected in 40–90 % of cases in these select studies. And as expected, in early MF, the performance of PCR assays wanes, with sensitivities in the 10–80 % range. Wide variations in performance characteristics have been the

**Table 6.2** Select MF/SS TCR gene rearrangement studies with performance characteristics

Authors (year)	Samples (CTCL/total)	Material	Method	Primer coverage <sup>a</sup> (directed to TCR- $\gamma$ unless specified)	Sensitivity (%) clonality in CTCL)	Sensitivity (%) early MF/SS)	Specificity
Wood et al. (1994) [34]	68/185	Frozen	Nested PCR/DGGE	V1-8/J1, J2; V9/J1, J2	90	75 (9/12)	94
	17/17	Frozen	Southern	N/A	59	—	—
Theodorou et al. (1995) [38]	141/211 <sup>b</sup>	Frozen	PCR/DGGE	All V/J	66	52 (29/56)	94
Liebmann et al. (1997) [36]	38/51	FFPE	PCR/PAGE	All V/J	63 ( $\gamma$ )	78 (18/23)	100
Delfau-Lanue et al. (1998) [50]	104/144	Frozen	PCR/DGGE	All V/J	40	14 (6/43)	100
Kohler et al. (2000) [51]	51/87	FFPE	PCR/HD	V1-8/J1, J2	72.5	68 (13/19)	100
Murphy et al. (2000) [52]	22/37	FFPE	Nested PCR/SSCP	All V/J1, J2	77	77	100
Klemke et al. (2002) [53]	14/14 <sup>c</sup>	FFPE	Nested PCR/GeneScan	V1-8/All J	67	67	—
Ponti et al. (2005) [17]	194/547	Frozen	PCR/HD	All V/J	84	71 (20/28)	98
Ponti et al. (2008) [54]	203/270	Frozen	PCR/HD	All V/J	67	60	95
			PCR/GeneScan	All V/J	84	79	100
Goeldel et al. (2010) [45]	77/157 <sup>b</sup>	Frozen	PCR/GeneScan	All V/J	77	75	86 %
Zhang et al. (2010) [55]	69/202	FFPE	PCR/DGGE	BIOMED-2 <sup>d</sup>	74	70	86 %
			PCR/GeneScan	$\gamma$ BIOMED-2 <sup>d</sup>	64	30	84
				$\beta$ BIOMED-2 <sup>f</sup>	74 <sup>e</sup>	84 <sup>e</sup>	
				$\gamma$ OR $\beta$ BIOMED-2	74 <sup>e</sup>	83 <sup>e</sup>	
				$\gamma$ AND $\beta$ BIOMED-2	90 <sup>e</sup>	74	
					49	73 <sup>e</sup>	
					58 <sup>e</sup>	93 <sup>e</sup>	

*Abbreviations:* DGGE denaturing gradient gel electrophoresis, PAGE polyacrylamide gel electrophoresis, HD heteroduplex, SSCP single-strand conformational polymorphism, GeneScan fluorescently tagged amplicons separated by capillary electrophoresis and GeneScan data interpretation

<sup>a</sup>Primer coverage is as reported in the publication. “All V” refers to all rearrangeable V segments (those with recombination signal sequences). Terminology for J regions may vary ( $J\gamma 1.1 = J\gamma P1$ ,  $J\gamma 1.2 = J\gamma P$ ,  $J\gamma 1.3 = J\gamma P2$ ,  $J\gamma 2.1 = J\gamma P2$ ,  $J\gamma 2.3 = J\gamma 2$ )

<sup>b</sup>Not limited to MF/SS

<sup>c</sup>Only early MF cases analyzed

<sup>d</sup>BIOMED-2 primers for TRG include V $\gamma$ 2-5, 7-11, all J except J $\gamma$ 1.2 (J $\gamma$ P) [33]

<sup>e</sup>Oligoclonality interpreted as positive

<sup>f</sup>BIOMED-2 primers for TRB include three reaction mixes: 23 V $\beta$ +6 J $\beta$ 1 + 3 J $\beta$ 2 primers, 23 V $\beta$ +4 J $\beta$ 2 primers, and 2D $\beta$ +13 J $\beta$  primers [33]

primary criticism for the routine use of molecular assays in the diagnosis of MF and SS. The reasons for such variations are manifold but can largely be attributed to differences in study design (including sample size and populations of patients studied), pre-analytical variables such as sample quality and fixation/storage methods of material, the nature of the assay employed, and interpretation of results, among others. This lack of standardization in assay design has impaired the research community's ability to compare studies in the literature and has clouded the true utility of molecular information in the diagnosis of MF/SS.

Standardization not only offers the benefit of being able to compare data across studies but is imperative for clearly defining diagnostic criteria, staging, and therefore management, as well as accurately assessing responses to therapy with recurrence and minimal residual disease. While most agree that standardization is a good idea, few agree on which method to employ. There have been a few recent large studies and assay comparison studies to help better define the true role of molecular clonality determination for the diagnosis of MF/SS and possibly make a case for use of their method.

Ponti et al. analyzed 547 cutaneous biopsies, 194 from cutaneous T-cell lymphoma patients and 353 from patients with confirmed benign inflammatory disease [17]. They used a multiplex PCR assay, targeted to *TRG* ( $\gamma$ ), with heteroduplex analysis. Using this approach, they reported an overall sensitivity of 84 % and specificity of 98 %, with a diagnostic accuracy of 93 %. When looking at early MF patients (i.e., those with a clinical stage of T1), the sensitivity was 71 % (20/28). And conversely, with erythrodermic patients, the sensitivity was 92 % and specificity was 100 %. Additionally, there were no significant differences in the sensitivity/specificity parameters between cutaneous T-cell lymphoma with a classic CD4+ MF immunophenotype and those with CD8+, TCR $\gamma\delta$ +, or CD45RA+ immunophenotypes. While these are some of the most impressive performance characteristics published on this topic, one of the limitations of this study was that all the patient samples were

cryopreserved, not formalin fixed and paraffin embedded (FFPE).

In an attempt to standardize clonality assays for B-cell and T-cell lymphomas, a European BIOMED-2 Concerted Action collaborative study developed highly sensitive and specific multiplex PCR assays [33]. This landmark study reported excellent performance characteristics, and, subsequently, versions of these assays, including the EuroClonality/BIOMED-2 multiplex PCR kit, have become commercially available and are widely used (TCRG+TCRB Gene Clonality Assays and IdentiClone, InVivoScribe Technologies, Inc., San Diego, CA). In the BIOMED-2 study, for T-cell malignancies, the authors report a sensitivity of 89 % for *TRG* ( $\gamma$ ) and 94 % for *TRB* ( $\beta$ ) [33]. Of note, these performance characteristics correspond to analysis of fresh or cryopreserved tissue. FFPE samples were also studied, but the assays did not perform as well using these tissue sources. Additionally, very few of the T-cell lymphoma/leukemia samples in the original studies were from MF/SS patients.

Zhang et al. addressed these latter issues by analyzing 202 FFPE samples (69 MF/SS) using the commercially available EuroClonality/BIOMED-2 assay [55]. They compared performance characteristics of the clonality assays for *TRG* ( $\gamma$ ), *TRB* ( $\beta$ ), and combinations of the two. To summarize their findings, *TRG* ( $\gamma$ ) and *TRB* ( $\beta$ ) as stand-alone assays performed similarly, both detecting 64 % of MF/SS cases. This could be pushed to 74 % if oligoclonal patterns were interpreted as positive (see Practical Considerations section). In early MF cases (clinical T1), which arguably are the ones more in need of diagnostic help, the sensitivity was less (30 % for *TRG* ( $\gamma$ ) and 60 % for *TRB* ( $\beta$ )). If clinical stage T2 was included with T1 in the early MF group, the sensitivity for each test was 62 %. The specificities were around 84 % and were not impacted by interpreting oligoclonal patterns as positive. There was 77.2 % concordance between the *TRG* ( $\gamma$ ) and *TRB* ( $\beta$ ) stand-alone tests. If sequential testing of *TRG* ( $\gamma$ ) and *TRB* ( $\beta$ ) was performed, the sensitivity could be pushed even further, to 90 %. And other studies suggest this may be

pushed even higher with multiple biopsies and/or enhanced microdissection techniques [35, 58]. The data from Zhang et al. are probably the closest to reality (currently) for evaluation of clonality on FFPE tissue in suspected MF/SS patients using this commercially available test.

In light of these data, despite a litany of studies and methods to evaluate for clonality in MF/SS, there remain significant limitations to the use of these assays for diagnostic purposes. Sensitivities and specificities in the high 90s have been reported in some studies, but when using the test for diagnosis in the most common setting—early MF/SS with ambiguous clinical, histologic, and/or immunophenotypic features, on FFPE tissue—and when trying to minimize cost and complexity of the test (i.e., using *TRG* ( $\gamma$ ) only), one may still miss up to two-thirds of malignant cases!

#### 6.2.5.4 Other Molecular Methods for Diagnosis

T-cell gene rearrangement studies are the most commonly used assays for diagnosis of MF/SS, but there are other ones on the horizon. Chromosomal abnormalities occur in MF and SS, with complex karyotypes occurring in approximately 50 % of patients [59–62]. Espinet and Salgado recently used array comparative genomic hybridization (aCGH) to look for chromosomal copy number changes associated with MF and SS [60]. MF is associated with gains in 7q, 17q, and 8q and losses in 9p, 13q, 17p, and 10q. Gains in 8q and 17q, and losses in 17p and 10q, are also associated with SS. Chromosomal copy number alterations are potentially useful in future diagnostic algorithms for MF/SS but have not yet been implemented for clinical use. Other potential diagnostic molecular tests still in the investigative stage include microRNA (miRNA) expression profile analysis [63, 64] and high-throughput genomic transcription profiling [65], among others. As with other areas of molecular diagnostics, these next-gen (high throughput, massively parallel) sequencing assays will likely play a prominent role in the diagnosis of MF/SS in the future. Complete sequencing of the TCR genes and other areas of the T-cell genome (or

transcriptome) will generate much more data than current gene rearrangement assays, likely inserting next-gen sequencing into future diagnostic algorithms. Commercial offerings of next-gen sequencing for this purpose are beginning to surface (e.g., LymphoTrack DeepSeq TCRG Assay, InVivoScribe Technologies, San Diego, CA, and others) [66, 67].

### 6.2.6 Diagnostic Algorithms for MF/SS

In order to more clearly define early MF, facilitate its diagnosis, and perhaps reduce interobserver variability intrinsic to early MF, algorithmic methods have been proposed [7, 68, 69]. These are often based upon various weighted combinations of clinical, histologic, immunophenotypic, and now molecular findings.

Guitart et al. proposed one such diagnostic algorithm [69]. This is a point-based algorithm using histologic features only. Major criteria are awarded 0–3 points each and include density of the infiltrate and the degree of epidermotropism and cytologic atypia. Minor criteria are awarded 0, 1, or 2 points based on the presence or absence of dermal fibroplasia, relative atypia of the intraepidermal component, and relative absence of background inflammation. Based on points, it was proposed that one of the following four diagnoses be issued: some dermatitis or specific inflammatory diagnosis (0–2 points); atypical lymphocytic infiltrate, mycosis fungoides cannot be excluded (3–4 points); atypical lymphocytic infiltrate, suggestive of mycosis fungoides (5–6 points); or mycosis fungoides (7+ points). This algorithm is limited to the evaluation of only the classic histologic type of MF.

The algorithm developed by Guitart et al. is still used by many dermatopathologists today. Of note, it does not take into account immunophenotypic or molecular data, perhaps due to reported less-than-ideal sensitivity/specificity data. This fact makes it attractive by many of its users yet is the main lightning rod for its critics. Leading up to the latest lymphoma staging and classification revisions [8], the International Society for

Cutaneous Lymphoma (ISCL) proposed a revised multipronged algorithm to take into account immunohistochemical and molecular data [7]. Like its progenitors, the ISCL algorithm was designed to standardize minimum criteria for diagnosis of MF, aid in the diagnosis of early classical presentations of MF, and standardize the evaluation for recurrence. They use a point-based system, requiring 4 points for a diagnosis of MF. There are two major criteria, one clinical and one histologic, each awarded 2 points. For the clinical criteria, 2 points are awarded if the patient has persistent and/or progressive patches or thin plaques with two of the following: non-sun-exposed location, size/shape variation, and poikiloderma. If only one, not two, from this list is met, only 1 point is awarded. For the major histologic criteria (2 points), there must be a superficial lymphoid infiltrate with the following: epidermotropism without spongiosis and lymphoid atypia. If there is only one of these present, 1 point is awarded. An additional 1 point could be “earned” if a molecular clone is detected. Another point is scored if immunophenotypic criteria are met, as defined by any or all of the following: (1) <50 % CD2+, CD3+, and/or CD5+ T cells, (2) <10 % CD7+ T cells, and (3) epidermal-dermal discordance of CD2, CD3, CD5, or CD7 (antigen loss confined to epidermis).

Comprehensive algorithmic approaches may help standardize the diagnosis of early MF. Many dermatopathologists use the ISCL approach or variants, but no uniformly accepted system has been adopted. Barriers to widespread implementation include variation in the availability of clinical information for the dermatopathologist, in the immunomorphologic interpretation of “atypia” or antigen loss (requiring “retraining” in some cases), in the availability or nature of immunohistochemical clones, and in the availability or type of molecular assays, to name a few. Furthermore, there is disagreement in the best system or algorithm to implement, as some are more comprehensive but onerous, and there is disagreement in the relative importance, or weight, of individual criteria [70] (see Practical Considerations—incorporation of molecular data for more on this topic of algorithms).

## 6.3 Staging and Prognosis

There are several ways molecular testing can potentially impact prognosis in the MF/SS patient: (1) detecting clones in the blood, lymph nodes, or visceral organs at diagnosis; (2) detecting clones in multiple, anatomically separate skin biopsies at diagnosis; (3) detecting clones in skin or blood while in clinical remission; and (4) analyzing prognostic biomarkers within the tumor.

### 6.3.1 Assessing Prognosis by PCR

#### 6.3.1.1 The Role of Molecular Detection of Tumor in the TNMB Staging System

Through a large international collaborative effort between the International Society for Cutaneous Lymphoma (ISCL), European Organization for Research and Treatment of Cancer (EORTC), and World Health Organization (WHO), the (proposed revisions to) current staging for MF and SS is based upon the size and quantity of skin lesions and presence or absence of tumor in the lymph nodes, blood, and/or visceral organs. This is captured in the tumor-node-metastasis-blood (TNMB) staging system (Table 6.3) [8, 9, 71]. Prognosis and recommended therapeutic strategies are stage dependent.

Regarding lymph node status, histopathology remains the standard for determining the presence or absence of an involved node, but recent data suggest that molecular identification of clones in lymph nodes may be predictive of which patients will progress. In three select studies using Southern blot to evaluate lymph nodes, no clones were detected in histologically negative nodes, and clones were detected with increasing frequency (13 % LN2, 83 % LN3, 100 % LN4 using the NCI-VA classification scheme) as tumor burden increased, supporting a correlation between histologic and molecular status. Patients with a detectable clone had a worse prognosis [8, 72]. Similar conclusions were drawn in studies using PCR-based assays [73]. These types of data have prompted the inclusion of the molecular data (clonality as determined by Southern blot or PCR)

**Table 6.3** The tumor-node-metastasis-blood (TNMB) staging of MF/SS

TNM classification of mycosis fungoïdes and Sézary syndrome [8]	
<i>Primary tumor (T)—skin</i>	
T1	Limited patches <sup>a</sup> , papules, and/or plaques <sup>b</sup> covering less than 10 % of the skin surface. May further stratify into T1a (patch only) versus T1b (plaque±patch)
T2	Patches, papules, or plaques covering 10 % or more of the skin surface. May further stratify into T2a (patch only) versus T2b (plaque±patch)
T3	One or more tumors <sup>c</sup> ( $\geq 1\text{-cm}$ diameter)
T4	Confluence of erythema covering 80 % or more of body surface area
<i>Regional lymph nodes (N)</i>	
N0	No clinically abnormal peripheral lymph nodes <sup>d</sup> , biopsy not required
N1	Clinically abnormal peripheral lymph nodes, histopathology Dutch grade 1 nor NCI LN0-2
N1a	Clone negative <sup>e</sup>
N1b	Clone positive <sup>e</sup>
N2	Clinically abnormal peripheral lymph nodes, histopathology Dutch grade 2 or NCI LN3
N2a	Clone negative <sup>e</sup>
N2b	Clone positive <sup>e</sup>
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
<i>Distant metastasis (M)—visceral</i>	
M0	No visceral organ involvement
M1	Visceral involvement (must have pathology confirmation <sup>f</sup> and organ involved should be specified)
<i>Distant metastasis (M)—blood</i>	
B0	Absence of significant blood involvement: 5 % or less of peripheral blood lymphocytes are atypical (Sézary) cells <sup>g</sup>
B0a	Clone negative <sup>e</sup>
B0b	Clone positive <sup>e</sup>
B1	Low blood tumor burden: more than 5 % of peripheral blood lymphocytes are atypical (Sézary) cells but do not meet the criteria of B2
B1a	Clone negative <sup>e</sup>
B1b	Clone positive <sup>e</sup>
B2	High blood tumor burden: 1,000/ $\mu\text{L}$ Sézary cells <sup>g</sup> or more with positive clone <sup>e</sup>

<sup>a</sup>For skin, patch indicates any size skin lesion without significant elevation or induration. Presence/absence of hypo- or hyperpigmentation, scale, crusting, and/or poikiloderma should be noted

<sup>b</sup>For skin, plaque indicates any size skin lesion that is elevated or indurated. Presence or absence of scale, crusting, and/or poikiloderma should be noted. Histologic features such as folliculotropism or large cell transformation (>25 % large cells), CD30+ or CD30-, and clinical features such as ulceration are important to document

<sup>c</sup>For skin, tumor indicates at least one 1-cm diameter solid or nodular lesion with evidence of depth and/or vertical growth. Note total number of lesions, total volume of lesions, largest size lesion, and region of body involved. Also note if histologic evidence of large cell transformation has occurred. Phenotyping for CD30 is encouraged

<sup>d</sup>For node, abnormal peripheral lymph node(s) indicates any palpable peripheral node that on physical examination is firm, irregular, clustered, fixed, or 1.5 cm or larger in diameter. Node groups examined on physical examination include cervical, supraclavicular, epitrochlear, axillary, and inguinal. Central nodes, which are not generally amenable to pathologic assessment, are not currently considered in the nodal classification unless used to establish N3 histopathologically

<sup>e</sup>A T-cell clone is defined by PCR or Southern blot analysis of the T-cell receptor gene

<sup>f</sup>For viscera, the spleen and liver may be diagnosed by imaging criteria

<sup>g</sup>For blood, Sézary cells are defined as lymphocytes with hyperconvoluted cerebriform nuclei. If Sézary cells are not able to be used to determine tumor burden for B2, then one of the following modified ISCL criteria along with a positive clonal rearrangement of the TCR may be used instead: (1) expanded CD4+ or CD3+ cells with CD4:CD8 ratio of 10 or more and (2) expanded CD4+ cells with abnormal immunophenotype including loss of CD7 or CD26

into the revised TNMB staging. Complete staging within the N1 and N2 categories requires molecular status, which subdivides these substages into two groups—for example, N1a (clone negative) and N1b (clone positive). Note that the true impact of nodal disease, *only* detectable by molecular means, is not completely understood. It may be determined that clone-positive lymph nodes will shift the survival curve to the next higher stage (i.e., N2b=N3), much like ulceration does in the staging of melanoma. More multivariate analyses are needed to determine the independent prognostic value in this setting, and in the meantime, molecular data is required for complete staging, mainly for data-collecting purposes.

Blood involvement by circulating neoplastic cells has independent prognostic value and thus is an important component to the staging criteria. The blood (B0–B2) stage is determined by quantifying the extent of circulating neoplastic cells. The most facile way to do this is by performing a Sézary count on a peripheral blood smear, looking for atypical cells with convoluted nuclei. Because Sézary counts are subjective with interobserver variability, many centers have moved to flow cytometric analysis to determine degree of blood involvement. Flow cytometry is more objective, evaluating for cell size, cell complexity, and variable loss of expression of CD3, CD4, CD7, and CD26 on CD4+ T cells, with loss of CD26 considered the most specific [18]. Flow cytometric analysis is more objective than Sézary counts but may still be complicated by subjective variability of antigen expression and the possible presence of more than one clone. There have been a number of studies exploring the impact of a circulating clone, as detected by molecular analysis, on MF/SS patient prognosis. The results have been variable. In general, clonality (PCR positivity) in the blood can be detected in MF/SS patients, including those with early-stage disease, but has also been observed in the blood in a portion of patients with benign inflammatory disease and also in the elderly [74–76]. Moreover, when a clone is detected in the blood, it is not uncommonly different from the cutaneous clone, questioning its meaning. And when the circulating clone is identical to the cutaneous clone, progres-

sion is usually clinically obvious, questioning its utility [77]. The preponderance of data, however, supports molecular status as being important in determining which patients will progress [17, 78]. PCR positivity increases with skin stage, overall clinical stage, and degree of histologic involvement [79]. In a study by Fraser-Andrews et al. (60 patients), detecting a clone in the blood impacted overall survival. In MF patients, blood PCR positivity shifted the median survival from 72 to 16 months. For SS patients, median survival went from 41.5 to 16.5 months [78]. Because of these data, molecular status within the blood modifies the B (blood) substage for complete TNMB staging. B0 is defined as absence of significant blood involvement ( $\leq 5\%$  Sézary cells) and B1 is defined as  $>5\%$  Sézary cells, but not meeting criteria for B2. Stage B2 is defined as a positive clone (as determined by PCR or Southern blot) and either  $>1,000$  Sézary cells/mm<sup>3</sup> or 1 of the following 2: increased CD3+ or CD4+ cells with CD4:CD8  $>10:1$  or increased CD4+ cells with an abnormal phenotype (CD4+/CD7- $\geq 40\%$  or CD4+/CD26+  $\geq 30\%$ ). Note that stage B2 utilizes molecular criteria in its definition and stages B0 and B1 are subdivided based on molecular status—for example, B0a (negative clone) and B0b (positive clone) [8].

Molecular data are also recommended when evaluating bone marrow, in certain circumstances, to fully assess the M status in TNMB staging of the MF/SS patient. Patients should have their bone marrow evaluated when they qualify for blood stage B2 or have otherwise unexplained hematologic abnormalities [8]. As with lymph node and blood evaluation, however, evaluation of the bone marrow still has some unanswered questions on the independent prognostic value of a molecularly detected clone. PCR may be “too sensitive,” picking up pseudo-clones or biologically irrelevant clones. Flow cytometric analysis of lymph nodes, blood, and bone marrow is considered, by many, superior to molecular techniques in identifying true biologically meaningful populations, particularly since antigen expression can be compared to the cutaneous population [80]. Logic tells us that clones at distant sites would portend a worse prognosis

than a completely negative workup, and, for now, molecular data remains recommended for complete staging of MF/SS patients, even if primarily for purposes of data collection.

### 6.3.1.2 Other Prognostic Applications for PCR Assays

While staging remains the primary focus for utilizing molecular data for the purposes of stratifying patients based on risk of progression, there are other potential uses. For example, there is evidence that patients with multiple skin biopsies harboring the same T-cell clone have a higher risk of progression [81]. Vega et al. analyzed skin biopsies from 15 MF patients. Biopsies were taken from different involved areas on the same day. After a mean follow-up period of 8 years, 12/15 had disease progression. Of this group, 10/12 had evidence of the same clone in multiple biopsies. In the clinically stable group, 1/3 had the same clone in simultaneous biopsies, reaching statistical significance ( $p=0.04$ ) in the correlation between identical clones in simultaneous biopsies and disease progression.

Molecular testing may also be used for disease monitoring, for example, searching for minimal residual disease or recurrences in patients who achieve clinical remission [79]. Since the frequency of PCR positivity in blood increases with increasing clinical stage over populations of MF/SS patients, there is an implication that molecular status would predict recurrence/progression. This narrative has not been so straightforward, however. Detection of clones by PCR has been reported in the blood of up to 40 % of MF/SS patients in clinical remission [82, 83]. This would argue that molecular testing plays no role in minimal residual disease monitoring, but this sentiment may evolve with different technologies and different strategies, such as molecularly quantifying clonal burden over time.

### 6.3.2 Assessing Prognosis by FISH and aCGH

Fluorescence in situ hybridization (FISH) and array comparative genomic hybridization (aCGH) can examine targets in the tumor genome

for changes in copy number of genes and/or chromosomes. With MF and SS, as with other tumors (e.g., melanoma, Chap. 4), genetic copy number changes not only can aid in diagnosis of tumors but may also predict tumor behavior and thus patient prognosis [84]. Copy number alterations in 9p21.3 (*CDKN2A*, *CDKN2B*, *MTAP*), 8q24.21 (*MYC*), and 10q26qter (*MGMT*, *EBF3*) have all been associated with poor prognosis in the MF/SS patient [60]. These are not yet routinely used to assess tumor behavior in the clinical setting.

## 6.4 Therapy

There have been recent advances in the treatment for MF/SS. In addition to standard topical and chemotherapeutic options, there are numerous new therapies centered on immune modulation. Several immunotherapies have been developed to combat the neoplastic T cell ( $\pm$  normal counterpart casualties). These include but are not limited to antibodies to IL-2R/CD25 (denileukin diftitox), CD4 (zanolimumab), CD30 (brentuximab vedotin), and CD52 (alemtuzumab). As with many immune modulators, minimizing immune-related adverse events is a challenge with these approaches. To date, recurrent activating mutations of oncogenic drivers have not been well described for MF/SS; therefore, targeted inhibitor therapy is not a prominent current treatment modality for these patients. A search for candidate therapeutic targets, however, is underway using intensive high-throughput sequencing analyses. A current list of ongoing clinical trials for MF/SS can be found at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) [85].

## 6.5 Practical Considerations for Ordering, Performing, and Interpreting Molecular Tests

Understanding the different testing strategies, their limitations, and interpretation of results in the context of the whole MF/SS patient is the responsibility of all members of the care team—specifically, the dermatologist (or other clinician), dermatopathologist/pathologist, and molecular

diagnostician (or other individual interpreting the test result). Moreover, it is important to remember that care providers (usually) have a choice on which test to order and where to send the specimen. This is important given the variations among assays and interpretation philosophies between laboratories. The T-cell gene rearrangement study is not a sodium level but the epitome of a clinicopathologic correlation assay. There needs to be a continuous and meaningful dialogue among those ordering the test and those performing and interpreting the test in order to maximize patient care.

## 6.5.1 Assay Selection and Design

### 6.5.1.1 Southern Blot Versus PCR-Based Assays

There are currently two main categories of molecular tests used to detect clonal TCR gene rearrangements—*Southern blot* and *PCR-based assays*. These are both named as acceptable methods in the latest recommended revisions to the TNMB staging of MF and SS for the purposes of molecular staging [8]. *Southern blot techniques were commonly used to identify clones but have largely been replaced in the last decade by newer generations of PCR-based assays.* Reasons for this include the following: Southern blot has a limit of detection around 5 % tumor cells, requires  $10^5$ – $10^6$  cells for analysis, has a 1- to 2-week turnaround time, cannot be performed on formalin-fixed and paraffin-embedded (FFPE) tissue, and may require handling of toxic substances such as radioactivity [34]. With PCR techniques, the analytical sensitivity can be pushed to 0.1 % tumor cells by dilution studies and 1 % in patient samples, and because of this, much less starting material is required [38, 86]. The turnaround time and minimal toxic exposure when performing PCR are also more favorable than Southern blot. While Southern blot may still be used at some centers [87], it is fading as a preferred method, and the remainder of this discussion is focused on PCR-based assays (see Chap. 3, Methods, for an overview of amplification and non-amplification molecular methods).

### <sup>1</sup>CPT coding

81340 (*TRB β*) and 81342 (*TRG γ*) for PCR-based assays; 81341 (*TRB β*) for direct probe assays (e.g., Southern blot); 81479, which is the unlisted molecular procedure code, for all others.

### 6.5.1.2 Variations in PCR-Based Assays

The main variables between PCR-based methods include the *sample selection* (frozen vs. FFPE, ± microdissection), the *targeted locus* (e.g.,  $\gamma$  vs.  $\beta$ ), the *primer selection* (primer design and number required), and the *detection method* of PCR amplicons. This is not a complete list, however, as variations exist at pretty much any point in the pre-analytical → analytical → post-analytical process, including but not limited to purifying/dissecting tumor, DNA extraction method, and number of PCR reaction tubes employed.

### Sample Selection

Many PCR-based assays have been optimized on fresh or cryopreserved material (see Table 6.2). Historically, because many immunohistochemical antibodies could not be performed on FFPE tissue, many centers apportioned and cryopreserved tissue for this purpose. This was the preferred stock of tissue to use for molecular studies to minimize the potential DNA degradation and amplification inhibition concerns of FFPE tissue; therefore, cryopreservation served a dual purpose. While fresh tissue is fairly routinely obtained in the diagnostic workup of most leukemia and lymphoma patients, this is not true for the MF patient. Diagnosis of MF continues to rely upon a skin biopsy, and collection of fresh tissue introduces logistical challenges. Currently, most if not all routine immunohistochemical antibodies for the MF/SS workup are available for FFPE tissue, abrogating this specific need for cryopreservation. Therefore, if the performance characteristics are comparable to those using

<sup>1</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [88]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

cryopreserved samples, assays optimized for FFPE tissue are preferred in the evaluation of the MF patient. The FFPE process remains a significant pre-analytical variable. Many laboratories will perform a test of DNA integrity by amplifying control targets of different amplicon sizes. The ability to amplify 300 base pairs predicts sufficient DNA quality to perform most PCR gene rearrangement assays. Additionally, most laboratories will use at least two concentrations of DNA from the FFPE sample in amplification reactions to assess for the presence of inhibitors. These procedures can reduce the number of false-negative and ambiguous results.

Laboratories also vary on the degree of enrichment of the sample. Some laboratories use complete full-slide tissue sections, others manually microdissect the specimens, and some may even use laser capture devices to ultra-enrich the tumor sample (primarily restricted to the research setting). *Manual microdissection offers several benefits and is recommended practice.* First of all, during reevaluation of the slide for microdissection, the dermatopathologist/pathologist has an opportunity to review the biopsy. This second set of eyes may abort unnecessary testing in cases of granuloma annulare or other MF mimickers submitted for molecular analysis. Cases with low lymphocyte counts can be flagged for the data interpreter to be vigilant for pseudoclonality. Also, by dissecting off the epidermis and superficial dermis, the neoplastic population is enriched compared to the background polyclonal infiltrate, improving assay sensitivity. The deeper dermis and subcutis would not be involved in early MF/SS and only add volumes of polyclonal T-cell DNA and unrearranged (non-lymphoid) DNA to the reaction mix. High levels of nontarget DNA have been known to inhibit PCR reactions, and therefore, eliminating non-lymphoid DNA from the reaction may have added benefit.

### Targeted Locus

All T-cell gene rearrangement assays are predicated on the events of somatic recombination of T-cell receptor loci during normal T-cell development (refer to Figs. 6.4 and 6.5). These assays amplify DNA by using primers to conserved

sequences in the V, D, and/or J regions that flank the recombined DNA. Four loci, *TRA* ( $\alpha$ ), *TRB* ( $\beta$ ), *TRG* ( $\gamma$ ), and *TRD* ( $\delta$ ), are potentially rearranged in the neoplastic T cell and are candidates for assay design.

Because a T-cell malignancy will have unique rearrangements of their TCR gene loci, these rearrangements will be overrepresented among the many potential rearrangements observed in a polyclonal population. Therefore, for a TCR locus to be an effective target in a clonality assay, it must be capable of generating sufficient diversity through recombination of its gene segments in order to “see” a true neoplastic clone (taken to an extreme, if there is only one possible recombination event at a locus, all cells with rearrangements will appear clonal). Conversely, if a TCR locus is too complex, with many gene segments and many possible recombination events, the complexity of testing becomes prohibitive. *TRB* ( $\beta$ ) and *TRG* ( $\gamma$ ) fall into the middle range.

Because most cases of MF/SS are CD4+  $\alpha/\beta$  T cells, early molecular assays (including Southern blot) targeted the  $\beta$  locus. *TRB* ( $\beta$ ) targeting is possible and still used, but it can be a more cumbersome technique with questionable benefits over *TRG* ( $\gamma$ ) [55]. *TRG* ( $\gamma$ ) is now the most commonly used target for T-cell clonality studies [87]. Reasons for this include the following: *TRG* ( $\gamma$ ) has the ability to generate enough diversity through recombination to detect a meaningful clone, when present; *TRG* ( $\gamma$ ) is not too complex, and therefore, the total number of possible recombinations is manageable to assay; *TRG* ( $\gamma$ ) has relatively good sequence homology flanking gene segments, therefore minimizing the number of PCR primers and reactions required in a multiplex assay; the PCR amplicons are less than 300 base pairs, allowing use on FFPE material and obviating the need for RT-PCR of RNA transcripts (the amplicons are slightly smaller than *TRB* ( $\beta$ ) amplicons and therefore more likely to amplify with poor-quality DNA) [34, 89]; *TRG* ( $\gamma$ ) is rearranged before *TRB* ( $\beta$ ) during development and is therefore present in a (marginally) higher percentage of tumors (whether or not  $TCR^{\gamma/\delta}$  is actually expressed); and the performance characteristics of the *TRG* ( $\gamma$ ) assay have an attractive combina-

tion of high sensitivity and specificity relative to the other loci. Although *TRG* ( $\gamma$ ) has many advantages as an assay target, *TRB* ( $\beta$ ) still plays a role in some settings, with some centers continuing to use *TRB* ( $\beta$ ) or a *TRB* ( $\beta$ )/*TRG* ( $\gamma$ ) combination algorithm to maximize test performance (see interpretation section below) [33, 43, 55]. A *TRD* ( $\delta$ ) assay is available commercially, but because the *TRD* ( $\delta$ ) locus is completely spliced out with *TRA* ( $\alpha$ ) recombination, it is only useful for analyzing immature (lymphoblastic) and TCR $\gamma/\delta$  T-cell tumors. The *TRD* ( $\delta$ ) assay has no practical advantage over *TRG* ( $\gamma$ ) or *TRB* ( $\beta$ ) assays in the evaluation of MF/SS.

### PCR Primer Selection

Different assays use different PCR primers. When considering primer selection, recall that only a subset of gene segments are capable of rearranging (e.g., due to the presence of recombination signal sequences), and the actual number of *functional* gene segments that have the potential to rearrange is even less (refer to Table 6.1) [32, 33]. Ideally, one of the four TCR loci would be rearranged in all T-cell clonal processes, and within that locus, there would be sequence homology 5' to all the V gene segments, capable of binding to a single well-designed PCR primer. Likewise, in this same ideal scenario, there would be sequence homology 3' of all the J gene segments, also capable of binding to a single well-designed PCR primer. With only two primers required, the conditions for the PCR reaction could easily be determined and optimized, and all rearrangements would be detected. Of course, this scenario does not exist. There is not perfect sequence homology flanking the gene segments, and therefore multiple primers are required to “cover” all the possible rearrangement permutations.

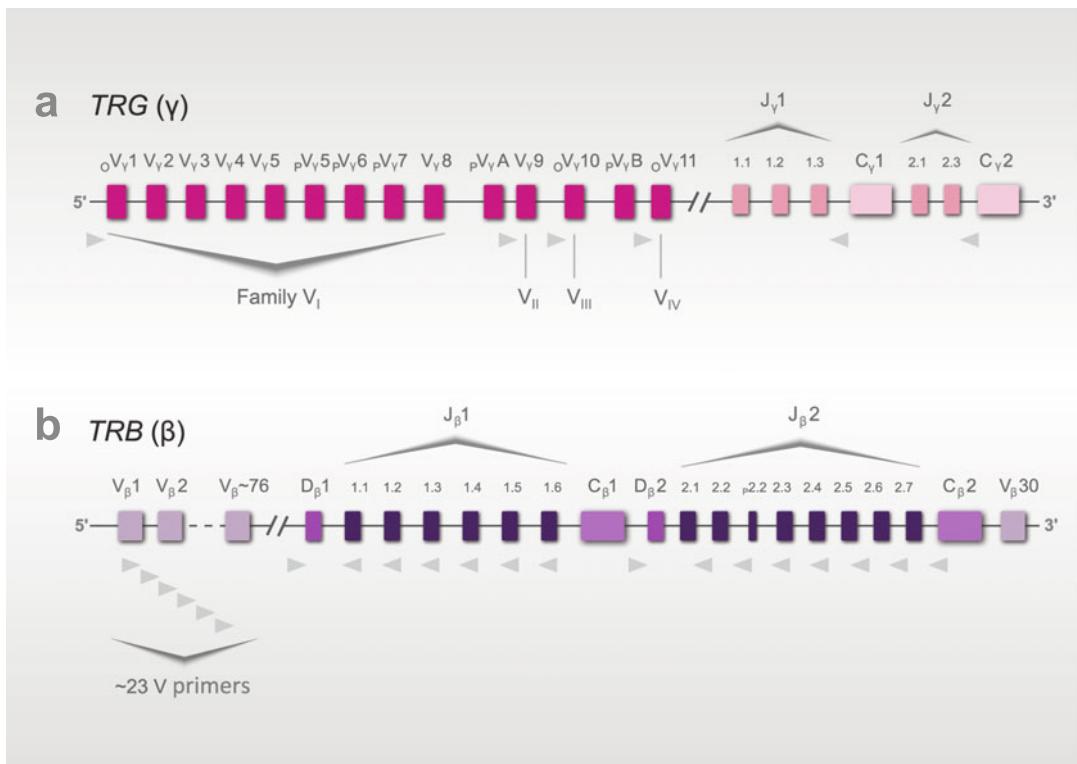
As an example, to illustrate the importance of sequence homology, the EuroClonality/BIOMED-2 multiplex PCR kit (InVivoScribe) that targets *TRG* ( $\gamma$ ) only requires 6 total primers to cover virtually all possible rearrangements (Fig. 6.6). The same company’s kit targeting *TRB* ( $\beta$ ) requires 38 different primers. There is a trade-off between designing primers capable of detecting as many permutations as possible, to increase sensitivity, and minimizing the complexity of the assay. The more primers in

the reaction mix, the more risk for competing reactions and failed reactions due to suboptimal annealing conditions. *TRG* ( $\gamma$ ) has good sequence homology flanking the relatively limited number of V-J gene segments. Indeed, some assays have been designed that include primers to all possible functional *TRG* ( $\gamma$ ) rearrangements [17]. The above-mentioned InVivoScribe kit covers all rearranged V segments and all but one J segment (J $\gamma$ 1.2, which is only rarely reported to rearrange [33]). Others have focused on the more common rearrangements reported for MF/SS, in order to reduce the assay’s complexity (see Table 6.2). For example, the V $\gamma$ I family (V $\gamma$ 1-8, which has sequence homology capable of being “covered” by a single primer) and J $\gamma$ 1.3/J $\gamma$ 2.3 are rearranged in up to 80 % of MF/SS tumors. And for *TRB* ( $\beta$ ), J $\beta$ 1 is preferentially rearranged [45, 54, 90]. Another strategy to deal with the requirement for an increasing number of primers is to separate the primers into groups based on optimal PCR reaction conditions and perform reactions in multiple separate reaction tubes, or “mixes.” This minimizes the complexity of the reactions within a single tube but adds to labor and the cost of reagents. Most T-cell gene rearrangement assays use a multiplex/multi-tube approach.

### Detection Methods

Once PCR amplicons are generated, there are multiple ways to detect clonal populations. Amplicons can be separated by their size, sequence, melting point, fluorescent tags, etc. All these methods can be effective, but interpretation of the data, at times, can be challenging.

The most common method for PCR amplicon analysis is by size discrimination. This can be achieved by standard gel electrophoresis and newer technologies such as capillary electrophoresis with GeneScan (or GeneMapper) analysis. With capillary electrophoresis/GeneMapper technology, the amplicons fluoresce due to the incorporation of fluorescently labeled primers. Because different reaction mixes and colored primers are used, the family of rearranged gene segments can be identified. These additional data allow for tracking and comparing individual clones and performing population/incidence studies. The analytical sensitivity approaches



**Fig. 6.6** Primer design strategy at the *TRG* (γ) and *TRB* (β) loci. For *TRG* (γ), *V<sub>γ</sub>* segments are arranged in families, based on sequence homology (a). For example, *V<sub>γ</sub>*1–*V<sub>γ</sub>*8 (family *V<sub>I</sub>*) can be potentially covered by a single primer. An example of a primer design strategy is depicted (arrowheads). By designing primers to conserved regions flanking *V<sub>γ</sub>* and *J<sub>γ</sub>* gene family members, most if not all possible *TRG* rearrangements can be amplified. In contrast, the *TRB* (β) locus has many more gene segments and

less sequence homology in the flanking regions (b). To cover all potential rearrangements, many more primers are required (arrowheads) [33]. *TRG J<sub>γ</sub>* segments have multiple designations in the literature (*J<sub>γ</sub>*1.1=J<sub>P1</sub>, *J<sub>γ</sub>*1.3=J<sub>γ</sub>1, *J<sub>γ</sub>*2.1=J<sub>P2</sub>, *J<sub>γ</sub>*2.3=J<sub>γ</sub>2). *P* pseudogene, *O* orphom (For up-to-date maps and sequence information of these loci, refer to the international ImMunoGeneTics database at [www.imgt.org](http://www.imgt.org) [31])

0.1 % (although, practically, primary tumor samples should still have over 20 % tumor cells to achieve a result with confidence). It is technically straightforward, without using toxic reagents (ethidium bromide, polyacrylamide, radioactivity). It is semiquantitative.

DGGE (denaturing gradient gel electrophoresis) is a fractionization of the PCR amplicons. Fractionization is based on size and nucleotide sequence (specifically, the polymorphic N sequence at the V-J junction of *TRG* (γ)). A clone is represented by a discrete band on the gel, while polyclonal amplification appears as a smear.

SSCP (single-strand conformation polymorphism) uses changes in the three-dimensional conformation of the denatured (single-strand)

amplicons to identify clones. The three-dimensional conformations, or shapes, are sequence dependent. These denatured amplicons can be separated by gel or capillary electrophoresis.

In heteroduplex analysis, PCR amplicons are denatured and reannealed, allowing for separation and identification of clones based on nucleotide sequence instead of size, similar to SSCP and DGGE, with comparable sensitivity and specificity parameters. Separation is usually performed on polyacrylamide gels. Heteroduplex analysis is highly reproducible. It is less expensive than capillary electrophoresis/GeneMapper.

Whichever detection method is used, laboratories must be familiar with the associated pre-analytical, analytical, and post-analytical

variables. Heteroduplex analysis, SSCP, and DGGE, in particular, can be technically challenging with variations in assay conditions—buffers, temperatures, run times, etc.—highly influencing results. All of these assays have interpretive challenges.

### 6.5.1.3 Selection of a PCR-Based T-Cell Clonality Assay

There are many different permutations to assay design, varying by any or all the elements described above. Understanding the nuances is important not only for individuals *performing* the tests but also for those *ordering* the tests.

For a laboratory adding T-cell gene rearrangement studies to their diagnostic menu, there are several important considerations. There are advantages and disadvantages to each assay design, not limited to just the above discussion on sample selection, target and primer selection, and detection method. Others include the overall performance characteristics of the assay, cost, and ease of use, just to name a few. One assay may make more sense for a large commercial laboratory, while another is more practical for a small hospital or boutique operation. *Of note, there are no FDA-approved assays for T-cell gene rearrangement analysis.* All of these assays are lab developed but may or may not include commercially available reagent kits with operation recommendations. When considering bringing up one of these laboratory-developed or “home-brew” tests, it is important to recognize that there may be intellectual property surrounding one or multiple methods. For example, InVivoScribe Technologies, Inc. (San Diego, CA) owns several patents on PCR-based detection of clonality [91].

One approach to avoid the hassle of primer design (and intellectual property issues, for that matter) is to use a commercially available kit. InVivoScribe offers kits for *TRG* ( $\gamma$ ) (two different assays), *TRB* ( $\beta$ ), *TRG* ( $\gamma$ ) + *TRB* ( $\beta$ ), and *TRD* ( $\delta$ ). These tests have been well studied and are now used worldwide [33, 92]. Of course, the disadvantage, as with any commercial product, is the elevated cost. Laboratories with low volumes of T-cell clonality studies will undoubtedly perform them at a monetary loss. Most assays

require at least three controls (no target, negative control, positive control) whether there is a single patient sample or 50 samples in the “run.” At times, the loss may be justified given the higher level of service provided to the patient by this close-knit integration of biopsy and molecular results.

According to a recent College of American Pathologists (CAP) survey, there is a pretty wide spectrum of T-cell gene rearrangement assays being offered by laboratories [87]. *TRG* ( $\gamma$ ) is the most commonly used target (twice as common as *TRB* ( $\beta$ ), but often both are used in combination). Of 120 laboratories responding to this recent survey using *TRG* ( $\gamma$ ) as the target, 49 (41 %) used InVivoScribe’s BIOMED-2 protocol, 20 (17 %) used InVivoScribe’s alternate *TRG* assay, 45 (38 %) used their own laboratory-developed assay, and 6 (5 %) used a different method. Interestingly, the CAP survey used a T-cell leukemia cell line as a test sample, and the percentage of laboratories reporting a positive result strikingly ranged from 20 to 100 %, depending on the assay used.

For those ordering T-cell clonality studies, understanding the subtleties between assays can be equally important. *Different testing centers may employ different methods, and therefore results cannot necessarily be correlated.* For example, a positive clone detected on a diagnostic biopsy using *TRG* ( $\gamma$ ) as a target at testing center A may be followed up by a negative clonality assay using *TRB* ( $\beta$ ) as a target (or using a different *TRG* ( $\gamma$ ) assay) at testing center B, even though the disease is progressing. Moreover, even when the same assay and target are used, results vary widely from laboratory to laboratory and technician to technician [33, 35, 87, 93–95].

### 6.5.2 Interpretation of the PCR TCR Gene Rearrangement Assay

Interpretation of the TCR gene rearrangement assay has three tiers: interpretation of data from each reaction, an overall interpretation of the molecular assay (compiling data from all reactions for a given sample), and incorporation of the molecular result into the context of all clinical,

**Table 6.4** Potential sources of false-positive and false-negative TCR gene rearrangement studies

False positives	False negatives
Carryover contamination	Contaminated or poor-quality DNA, poor amplification <sup>a</sup>
Switched specimen	PCR inefficient or fails due to inhibitors (heparin, EDTA, divalent cation chelators, others intrinsic to paraffin) <sup>a</sup>
Limited DNA sample—pseudoclonaity and oligoclonality	Tumor below limit of detection
Clonal expansion of normal, nonmalignant inflammatory process	Specific targeted gene rearrangements undetectable due to primer selection
Immunosuppressed patient—pseudoclonaity and oligoclonality	Targeted gene rearrangement and/or primer sites deleted or absent during T-cell development
Premalignant but stable disease (T-cell dyscrasia, “clonal dermatitis,” etc.)	TCR loci unarranged (germline)
Other biologically irrelevant clones	TCR locus with trans-rearrangement (e.g., V <sub>γ</sub> -J <sub>β</sub> )
Incorrect clinical diagnosis (follow-up too short, patient actually does have MF, so result is true positive)	Antitumor response resulting in oligoclonality Tumor with secondary rearrangement, resulting in oligoclonality Incorrect clinical diagnosis (patient has eczema, etc., so result is true negative)

EDTA ethylenediaminetetraacetic acid

<sup>a</sup>Results in failed reaction, not false-negative result

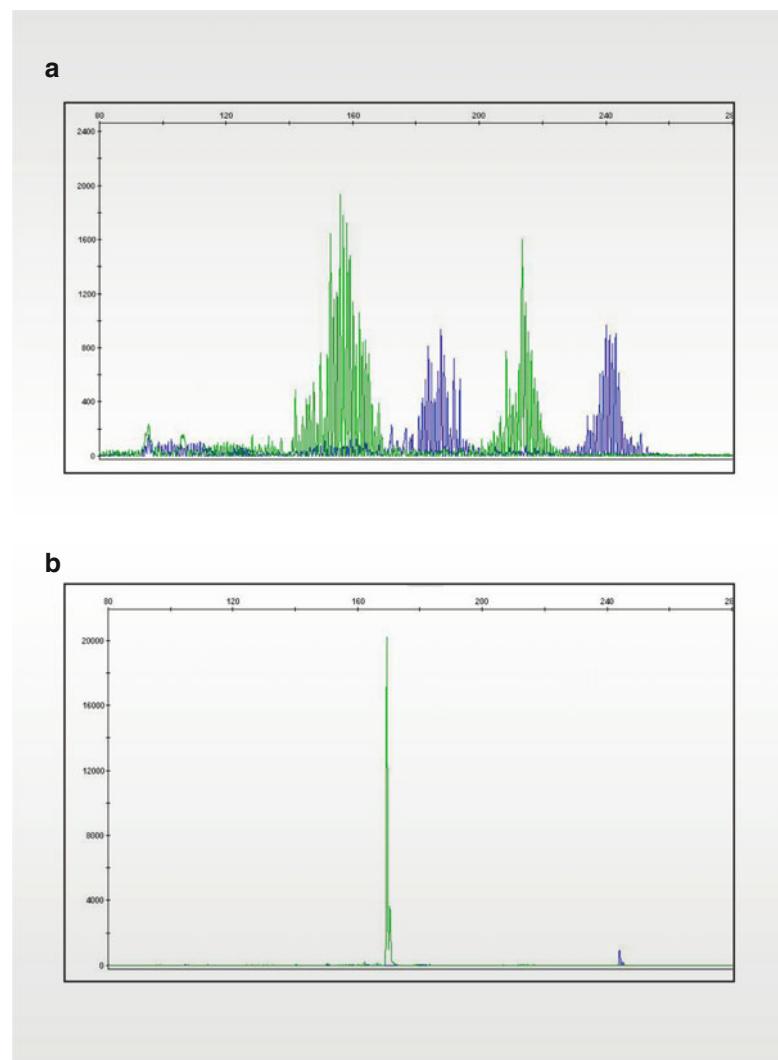
histologic, and immunohistochemical data. The individual tiers of interpretation may be performed by different people (usually the first two by molecular diagnosticians and/or pathologists and the third by pathologists and/or clinicians, but these lines can be blurred). False-positive and false-negative results are common and must be considered at all interpretation tiers. There may be biologic or technical reasons for these misleading results (Table 6.4).

### 6.5.2.1 Reaction Data (Tiers 1 and 2)

Most PCR-based assays (the good ones, anyway) use multiple primer sets in multiplex reactions, for the reasons described above. Each reaction generates data, which must be interpreted individually (tier 1). Outside of the category of “failed reactions,” each reaction should yield one of the following results: clonal (positive), polyclonal (negative), or some indeterminate category, such as “peak noted” or “suspicious.” Failed reactions are facts of life, possibly due to variable fixation techniques and poor-quality DNA, for example. Failed reactions often require re-extraction of the sample’s DNA and repeat analysis. Performing the analysis in duplicate is good practice for resolving ambiguous results.

With polyclonal populations, the amplicons will vary in size, in a vague Gaussian distribution, due to the numerous and random V-J and V-D-J rearrangements represented in the mix (Fig. 6.7). Clonal populations have an overrepresented TCR rearrangement, and when present, the PCR amplification of this clone dominates within the mix. In these cases, there is little or no background polyclonal tracing. Neoplastic populations may have rearrangement of one allele, causing a single peak, or, in approximately 40 % of tumors, rearrangement of two alleles, resulting in two peaks [54]. In many cases, however, the data fall somewhere in between (Fig. 6.8). At times, a spike or spikes is/are seen rising above a polyclonal tracing. This could be a spurious, biologically irrelevant finding but may also represent a true low-level clone within a background polyclonal population. Pseudoclonaity is another interpretive challenge. Pseudoclonaity refers to the detection of a biologically irrelevant “clone,” possibly by amplifying DNA from a single cell or small population of T cells. This spurious finding can often be identified by its lack of reproducibility in duplicated or repeated reactions. Sometimes “clones” are also observed just outside of the normally acceptable amplicon size

**Fig. 6.7** Interpretation of TCR gene rearrangement studies—polyclonal and clonal. In these examples, DNA is extracted from microdissected FFPE tissue from suspected MF patients. Fluorescently labeled primers are grouped into two separate PCR reactions (mix A and mix B, EuroClonality/BIOMED-2 protocol, InVivoScribe), and the amplicons are separated by capillary electrophoresis based on amplicon size (with GeneMapper technology). Blue and green tracings correspond to different groups of fluorescently labeled primers within the multiplex reaction. The x-axis corresponds to amplicon size and the y-axis to fluorescence intensity (quantity). A polyclonal population of T cells will have Gaussian spread of amplicon sizes due to the numerous different rearrangements represented in the reaction (a). A clonal population of T cells will have an overrepresented rearrangement, indicated by a peak, or spike, with a low-level or suppressed polyclonal background (b)

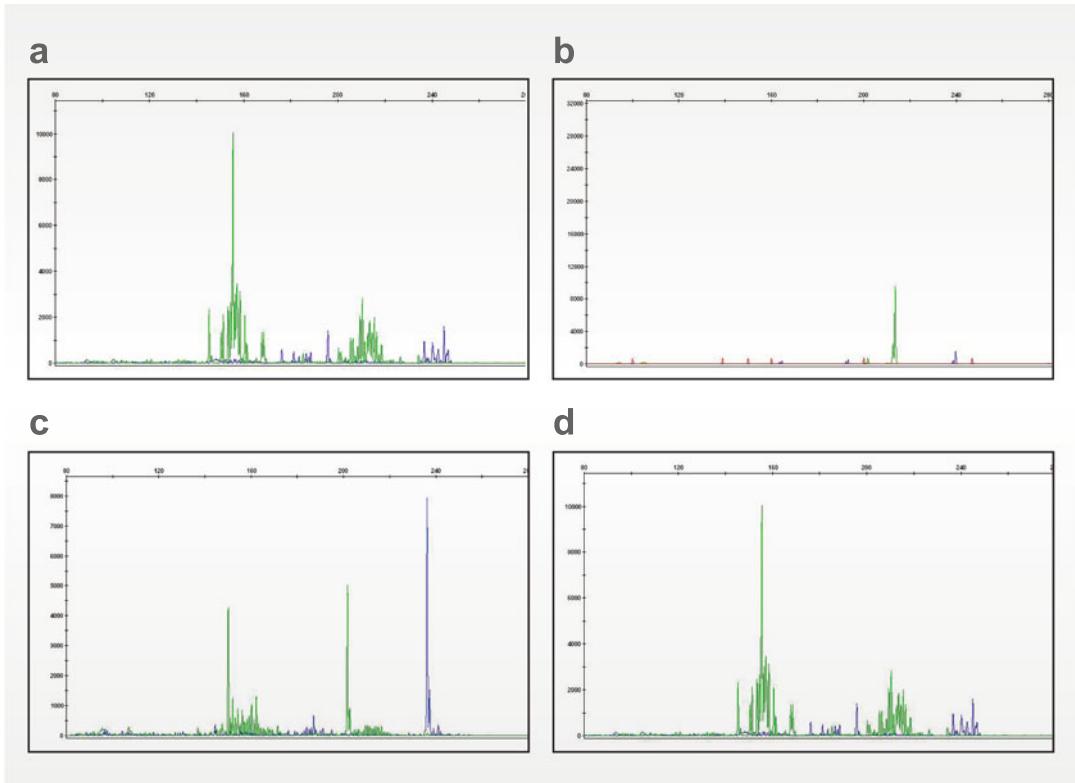


range. In all these circumstances, interpretation can be challenging and may lead to the unsatisfactory recording of “peak noted” or “suspicious,” requiring further studies.

Once the individual reactions are interpreted, the results must be incorporated into a final molecular report (tier 2). The final report takes into account the quantity and quality of all potential clonal peaks, as well as possible additional data, such as the clinical, histologic, and immunophenotypic findings. Again, no matter which assay is employed, there will be at least three categories of final results—*clonal (positive)*, *polyclonal (negative)*, and a middle category. This middle category may include “*indeterminate*,” for cases with con-

spicuous populations but do not otherwise meet criteria for clonality, or possibly “*oligoclonal*.”

While true neoplastic clonal processes will often have one (one allele rearranged) or two (both alleles rearranged) discrete peaks, sometimes there are more than two. Oligoclonality refers to the presence of multiple discrete peaks, which would be interpreted as clonal if there were only one or two per tracing (see Fig. 6.8). There are several possible reasons for oligoclonality. It could be a “false positive,” resulting from sampling, limited DNA input, or DNA degradation. Repeat extraction, analysis, and/or sampling may yield a true polyclonal (negative) result. However, oligoclonality may be a sign of true clonality [55, 96]. Multiple



**Fig. 6.8** Interpretation of TCR gene rearrangement studies—intermediate (or indeterminate) categories. Similar to Fig. 6.7, DNA is extracted from microdissected FFPE tissue from suspected MF patients. Fluorescently labeled primers are grouped into two separate PCR reactions (mix A and mix B, EuroClonality/BIOMED-2 protocol, InVivoScribe), and the amplicons are separated by capillary electrophoresis based on amplicon size (with GeneMapper technology). Blue and green tracings correspond to different groups of fluorescently labeled primers within the multiplex reaction. The x-axis corresponds to amplicon size and the y-axis to fluorescence intensity

clones may exist in bi-lineage tumors or if the tumor has a secondary rearrangement of their TCR locus. This latter event certainly occurs in the case of T-cell leukemia, and likely in MF/SS, albeit more rare of an event as these are neoplasms of post-thymic T cells. Another possibility for oligoclonality is the presence of a “clonal” antitumor response in the tested tissue. While the antitumor T-cell clone may not be a true neoplasm, its presence may still aid in the identification of underlying MF/SS. Laboratories will often report oligoclonality as such, but due to the uncertainty of its meaning, final interpretations may differ in their level of concern for a neoplasm.

(quantity). Tracings may not always fall into clear-cut clonal or polyclonal patterns. Often, there is a spike, or peak transcending a polyclonal background, but not meeting criteria for clonality (a). Similar prominent peaks may occur with low levels of DNA or low amplification, a finding associated with pseudoclonality (b). The oligoclonality pattern has multiple (>2) peaks (c). This may occur in reactive or truly malignant processes. The final sample is from blood of a healthy volunteer (d). This individual recently complained of an upper respiratory infection but has no clinical evidence for a lymphoproliferative disorder, emphasizing the importance of pretest probability

It is important to note that there is no standard definition for what constitutes a clone. Some individuals measure the size of the peak, the ratio of the peak size to the next highest or third highest peak, the ratio of the peak size to the top of the polyclonal tracing, or some variation on this theme. Criteria may vary depending on if the sample is for diagnosis or for monitoring minimal residual disease or recurrence when a patient’s “signature” clone is already known. Furthermore, most individual laboratories use the same interpretive criteria for analyzing fresh/frozen and FFPE tissue and the same criteria for skin, lymph node,

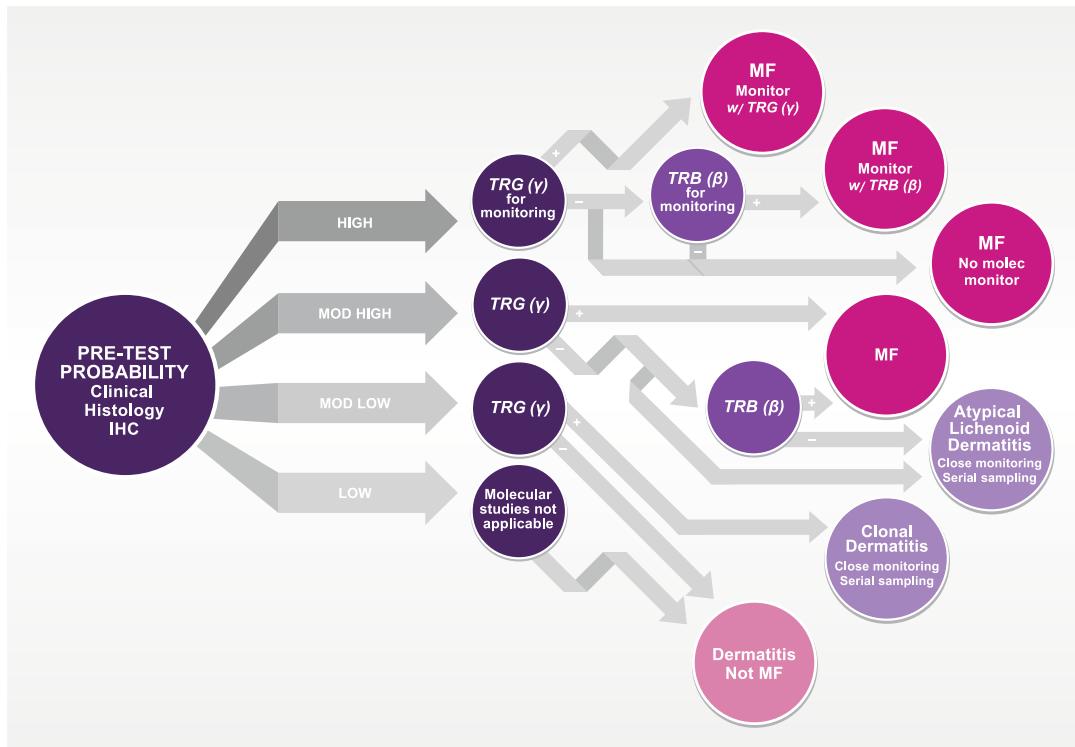
blood, and bone marrow samples. There are some published guidelines on interpretation [92], but it is unclear if these are appropriate practices for all sample types. For example, normal blood samples are often more “spiky” than skin on capillary electrophoresis tracings, possibly indicating the normal presence of small clonally expanded subpopulations. Large studies on these topics are lacking, and because of this, *there is no standardization across laboratories for interpretation of specimens from early MF/SS patients, and results may vary between laboratories or even between individual interpreters within the same laboratory.*

Final molecular interpretations often rely upon analysis of molecular data only. This is especially true for reference laboratories that usually receive no additional information on the patient. While it is important for all laboratories to analyze the data in isolation *initially*, final interpretation can be enhanced, or shaped, in light of the entire patient picture. Ideally, the pathologist and interpreter of the data have a close professional relationship, or they are even the same person. This practice may resolve a subset of cases in the less-than-desirable “indeterminate” category. For example, in patients with a very low pretest probability of having MF/SS (clinician thought eczema, biopsy unimpressive, etc.), a borderline “indeterminate” case can be downgraded to “negative.” This clinicopathologic correlation is good practice for molecular interpretation, but it is a *requirement* for rendering a final biopsy interpretation. A recent experience highlights this latter point. A volunteer donated blood for the purposes of validating a T-cell gene rearrangement assay. The results from the assay are shown in Fig. 6.8d. The tracing is suggestive of clonality, yet this individual had no clinical evidence for a lymphoproliferative disorder. Upon further questioning, he admitted to a recent upper respiratory infection. Submitting samples for T-cell clonality analysis without screening for pretest probability will yield an increased number of false-positive results.

### 6.5.2.2 Incorporation of Molecular Data into the Evaluation of the MF/SS Patient (Tier 3)

There are several main uses of molecular TCR data in the context of MF/SS patients—to aid diagnosis, to stage the patient, and to follow patients’ disease progression longitudinally. For clonality studies to be useful for diagnosis, there must be a relatively high pretest probability [42]. For example, in patients with clear benign inflammatory conditions, such as eczema and psoriasis, T-cell clonality studies will only cloud the picture, as 10–30 % of these patients will have a PCR-detectable clone [43, 49]. Furthermore, many extensive and expensive MF workups for benign lichenoid keratoses (when clinical information, such as a single papule on the neck, was poorly transferred or ignored) could be avoided. After all, if the test is positive, it must be explained. Similarly, in patients with clear or known MF/SS, either by clinical or histomorphologic criteria, up to one-third will not have a PCR-detectable clone, also requiring explanation. In cases of tumor stage or erythrodermic MF, or SS, clonality detection approaches 100 % [17]. In these patient groups, molecular testing for diagnosis may not be indicated. However, there is a clear argument for performing these tests for other reasons. Comparing tracings from cutaneous biopsies at diagnosis with those from blood, lymph node, and bone marrow specimens can help when looking for occult disease. Also, longitudinally monitoring the patient for disease recurrence is much easier if there is a previous clonality test with a “clonal signature” on that patient to use for comparison. In these cases, a correctly sized peak, no matter how prevalent within a polyclonal background, can be meaningful.

There is no single or right way to incorporate molecular data into the diagnosis of the MF/SS patient, but any is acceptable so long as the basic principles are applied: Molecular analysis is not a stand-alone test, molecular data concordant with the pretest probability should carry significant diagnostic weight, and, accordingly, molecular data discordant with the pretest probability should carry significantly less diagnostic weight. Algorithmic approaches to the diagnosis of MF



**Fig. 6.9** Algorithm for incorporating molecular data. For MF/SS, the *TRG ( $\gamma$ )* assay is the preferred initial molecular test due to its performance parameters, small amplicon size,

and relatively low complexity. *TRB ( $\beta$ )* testing may have utility in some scenarios. Pretest probability for the disease will shape the testing strategy and interpretation of data

have been previously proposed (see previous section on diagnostic algorithms) [7, 69], but interestingly, either these do not incorporate molecular data at all, or the molecular data carries little weight and is interpreted in a vacuum, without regard to assay design or pretest probabilities.

Perhaps an improved diagnostic algorithm is the following (Fig. 6.9). After an initial assessment of the pretest probability for MF—as determined by clinical, histologic, and/or immunophenotypic parameters, by an algorithm [69] or other means—*TRG ( $\gamma$ )* rearrangement studies are performed. Any well-designed assay can be used. The interpretive weight of the *TRG ( $\gamma$ )* rearrangement test result is guided by the pretest probability. Discordant results may advocate a second round of molecular testing, using a different target, like *TRB ( $\beta$ )*. In the event the results remain discordant (or *TRB ( $\beta$ )* testing not performed at all), diagnoses such as “atypical lichenoid der-

matitis” (high pretest probability but no clone) or “dermatitis with detectable T-cell clone” (low pretest probability but positive for clone) or similar verbiage would be appropriate. In these cases, recommendations would include the following: close monitoring of the patient, serial sampling with molecular studies (at 6-month intervals from untreated areas), and sampling multiple anatomic sites (which has been shown to increase sensitivity/specifity [58]). Similar diagnostic algorithms have been proposed [55, 92].

## 6.6 Summary

Gene rearrangement analysis is arguably the first molecular diagnostic assay used in dermatology, and it continues to be the primary molecular method used in diagnostic algorithms for mycosis fungoïdes and Sézary syndrome. Because

clonal expansion is a normal response by lymphocytes exposed to antigen, clonality and malignancy are not synonymous. A significant subset of patients with reactive inflammatory conditions may have a detectable clone, and, conversely, due to limitations of the molecular assays, clones may not be detected in a significant subset of MF/SS patients. PCR-based assays are the most commonly used methods to assess clonality. These assays have significant limitations, with extreme variations in performance characteristics, assay design, and interpretation of data. The diagnosis of MF/SS relies upon the incorporation of the molecular data with the clinical, histologic, and immunohistochemical features. Placing sole or primary emphasis on any one of these will lead to misdiagnosis in some cases. This same mantra can be applied to non-MF/SS hematopoietic malignancies discussed in the next chapter.

Applications of gene rearrangement analysis expand beyond diagnosis. Molecular evaluation of the lymph nodes, blood, and bone marrow is now required for complete tumor staging of the MF/SS patient, even if this is primarily for data-collecting purposes. These assays can also be used to monitor patients for occult disease and early relapse during or after therapy. Other molecular assays, including FISH, aCGH, microRNA expression profile analysis, and high-throughput sequencing, are not currently routinely used on clinical samples but have enormous potential to become incorporated into future molecular diagnostic menus and impact the MF/SS patient's entire management—from diagnosis to therapeutic decision making.

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# Leukemia and Lymphoma. Part II: Primary Cutaneous B-Cell Lymphoma and Other Non-MF/SS Hematopoietic Tumors

7

## Contents

7.1	<b>Introduction.....</b>	168
7.2	<b>Determination of Clonality in B-Cell Infiltrates .....</b>	169
7.2.1	Immunohistochemistry and Flow Cytometry .....	169
7.2.2	Molecular Studies .....	169
7.3	<b>Diagnostic Applications for Molecular Testing.....</b>	173
7.3.1	Primary Cutaneous B-Cell Lymphomas .....	174
7.3.2	Non-MF/SS Primary Cutaneous T-Cell Lymphomas.....	179
7.3.3	B-Cell Versus T-Cell Lymphoma.....	181
7.3.4	Other Hematopoietic Tumors Primarily and Secondarily Involving the Skin.....	181
7.4	<b>Other Applications for Molecular Testing .....</b>	184
7.4.1	Prognosis.....	184
7.4.2	Therapy .....	186
7.5	<b>Practical Considerations for Ordering, Performing, and Interpreting Molecular Tests.....</b>	186
7.5.1	Gene Rearrangement Assays .....	186
7.5.2	Other Molecular Methods for the Diagnosis and Management of the Cutaneous Leukemia/ Lymphoma Patient .....	193
7.6	<b>Summary.....</b>	195
	<b>References.....</b>	195

## Key Points

- Molecular testing has a role in the diagnosis of primary cutaneous B-cell and T-cell tumors and other leukemias/lymphomas primarily or secondarily involving the skin.
- The diagnosis of primary cutaneous B-cell lymphoma requires a multi-pronged approach—integrating clinical, histologic, immunophenotypic, and molecular data.
- B-cell clonality is determined by the molecular analysis of the rearranged immunoglobulin heavy chain (*IGH*) +/- light chain (*IGK* and *IGL*) genes.
- Somatic recombination of the TCR and Ig loci are normal biologic events during T-lymphocyte and B-lymphocyte development, respectively.
- Non-B/non-T cutaneous hematopoietic neoplasms, such as the NK lymphomas, Langerhans cell tumors, and the blastic plasmacytoid dendritic cell neoplasm, are not derived from lymphocytes, and the cells from these tumors have TCR and Ig loci in the germline configuration.
- Clones, as determined by gene rearrangement studies, may be observed in reactive inflammatory processes, and, conversely, malignant infiltrates may fail to have a detectable clone.

- Currently, standard PCR is the most commonly used molecular method for assessing clonality in lymphocytic infiltrates. Newer methods such as high-throughput sequencing are emerging.
- Besides B-cell and T-cell gene rearrangement studies, other molecular assays, such as FISH, are used to diagnose hematopoietic tumors, especially leukemias and lymphomas that secondarily involve the skin.
- Molecular assays have prognostic and theranostic applications for hematopoietic tumors, but these are not routinely used for primary cutaneous lymphomas.

ular test used for diagnostic purposes, taking advantage of a unique property of human lymphocytes—somatic recombination. Clonality testing has evolved from Southern blot techniques to PCR-based assays and will likely move to high-throughput sequencing analyses (next-gen sequencing) in the near future. Regardless of methodology, clonality assays can be challenging to interpret. Clonal expansion occurs with malignancies following molecular oncogenic-driving events, but a controlled form of clonal expansion is also a normal process that occurs when nonmalignant B cells and T cells respond to antigen. Therefore, clones can be detected in reactive/inflammatory dermatoses, and due to limitations of the molecular assays, malignant processes may fail to have a detectable clone, emphasizing the importance of the multipronged diagnostic approach.

The differential diagnosis of cutaneous lymphoma includes reactive processes and other malignancies that secondarily involve the skin. Many of the entities in this latter group, specifically leukemias and nodal lymphomas, carry recurrent specific genetic abnormalities such as translocations and, less commonly, point mutations. These molecular events can be utilized for diagnostic purposes or for monitoring disease, such as determining remission or recurrence/relapse. Similar to evaluating soft tissue tumors, evaluating hematopoietic tumors via their macrochromosomal abnormalities is best achieved by using fluorescence in situ hybridization (FISH)-and PCR (or RT-PCR)-based methods.

As our understanding of the molecular underpinnings of hematopoietic tumors continues to evolve, molecular diagnostics will undoubtedly play an increasingly important role in the management of these patients. Diagnosis continues to be the most widely used application for these molecular tests, but new applications (prognostic, theranostic, etc.) will undoubtedly surface as they have in other fields.

The previous chapter gave an in-depth look at molecular diagnostic applications for the diagnosis and management of the MF/SS patient, with a detailed discussion of the T-cell receptor gene rearrangement assay. In this chapter, the discussion on cutaneous hematopoietic tumors

## 7.1 Introduction

Diagnosis of cutaneous hematopoietic tumors remains one of the most challenging arenas in dermatopathology. Accurate diagnosis is important not only for planning appropriate therapy for the individual but also for interpreting therapeutic efficacy, qualifying patients for clinical trials, and providing meaningful epidemiologic and prognostic data. As evident from the previous chapter, molecular testing plays an integral role in the diagnosis and management of mycosis fungoides (MF) and Sézary syndrome (SS) patients. While the literature is not as robust for the less common non-MF/SS T-cell and B-cell cutaneous neoplasms, molecular diagnostics plays a similar role for these tumors and the limitations of these tests still apply.

Diagnosis of cutaneous hematopoietic tumors requires a multipronged approach. Integration of clinical findings, histomorphology, immunohistochemistry, and now molecular testing is considered standard practice. Regarding molecular testing, some authors estimate that 10–15 % of cases rely upon the molecular test result to firmly establish the correct diagnosis. With that said, too much emphasis on the molecular result or any one of these parameters will lead to misdiagnosis in some cases. Clonality testing is the most common molec-

continues, with emphasis on using molecular tools for diagnosing primary cutaneous B-cell tumors, non-MF/SS T-cell tumors, and tumors that secondarily involve the skin. The corollary B-cell receptor (immunoglobulin) gene rearrangement assay is highlighted. Other molecular tests used in diagnosis and other aspects of management of these patients are also discussed.

## 7.2 Determination of Clonality in B-Cell Infiltrates

### 7.2.1 Immunohistochemistry and Flow Cytometry

Unlike with T-cell infiltrates, B-cell infiltrates can be effectively screened for clonality by analyzing intracellular and surface protein expression. These B-cell “antigens” can be detected by targeted antibodies, either by immunohistochemistry or by flow cytometry. Even though most of these antigens are expressed on normal, nonmalignant counterparts, if there are unusual patterns or levels of expression within infiltrates, these antigens can act as surrogates for clonality. For example, Bcl-6 (B-cell lymphoma-6) is a nuclear protein expressed by the normal follicle center cell. A high proportion of Bcl-6+ cells in an infiltrate is highly unusual, and in the appropriate context, this is suggestive of follicle center cell lymphoma. Similarly, a skewed kappa to lambda ratio (by immunohistochemistry or in situ hybridization) within an infiltrate would suggest clonality, as most benign/reactive infiltrates are polymorphous, or mixed. Because flow cytometry can analyze expression levels of multiple antigens on individual cells within large populations, it is a very powerful tool for detecting B-cell malignancies and is preferred over immunohistochemistry if testing is practical. T-cell infiltrates do not express kappa or lambda light chains. Their closest counterpart is the restricted expression of either CD4 or CD8, but because there is extreme overlap in CD4:CD8 ratios between benign and malignant infiltrates, antigen expression assays are less powerful for clonality determination in T-cell processes.

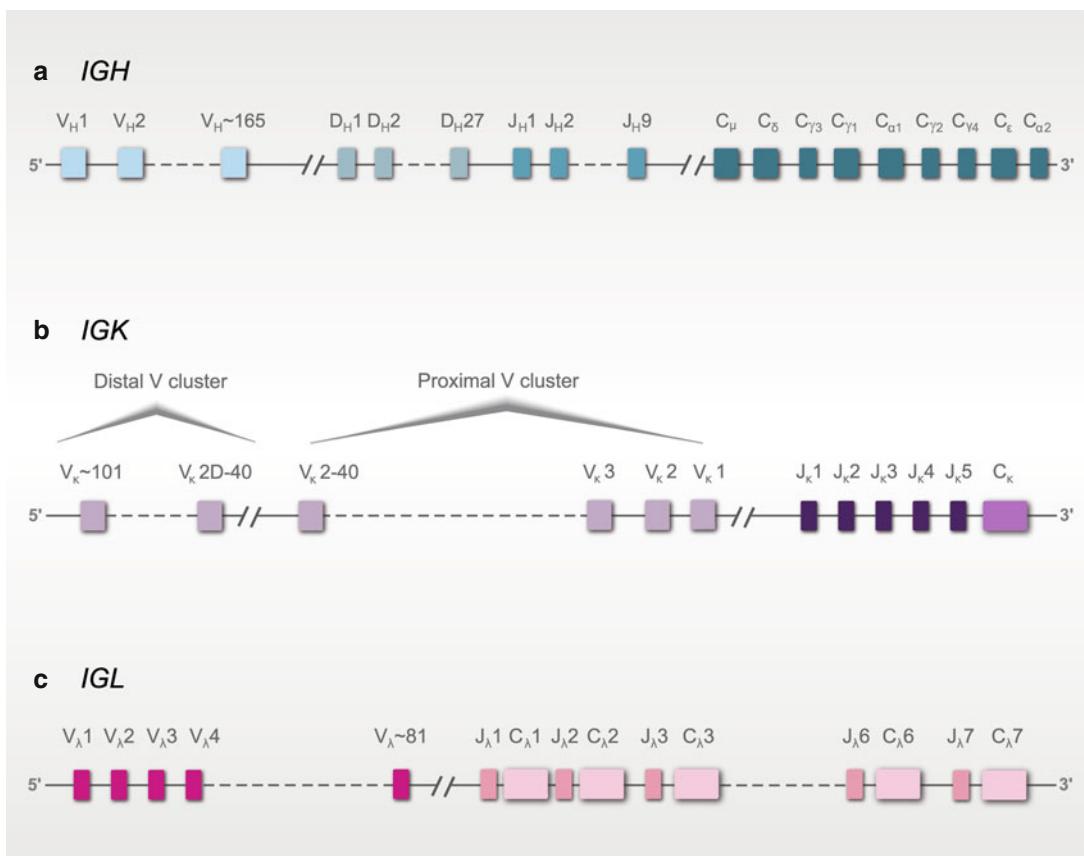
### 7.2.2 Molecular Studies

The above antigen expression assays can be surrogates for clonality, but molecular assays can test for true clonality. True clonality refers to a cell population’s derivation from a single cell, but is not synonymous with malignancy. During development, B cells and T cells undergo somatic recombination of their antigen receptor genes, generate surface receptors (immunoglobulin for B cells and TCR for T cells), and respond to antigen with clonal expansion. Molecular analysis of these loci within a population of cells can identify overrepresentation of unique gene recombinations, providing evidence for true clonality. The generation of receptor diversity and molecular clonality testing is very similar among B cells and T cells. The reader is referred to Chap. 6 for a more in-depth discussion on these biologic processes and limitations of clonality testing. Issues specific to B cells are discussed below.

#### 7.2.2.1 Recombination of the Heavy and Light Chain Genes

During normal B-cell development, within the bone marrow, for any given B cell, heavy and light chain gene segments rearrange with the purpose of generating a unique functional surface immunoglobulin, capable of recognizing a foreign antigen but not a self-antigen [1]. This “cut-and-paste” somatic recombination of the variable, diversity, and joining gene segments at heavy and light chain gene loci partially explains how immense antigen-recognition diversity—up to  $10^{18}$  unique amino acid sequences—can be generated using only 25,000 total genes on the human genome [2].

There are three loci—*IGH*, *IGK*, and *IGL*—that encode the proteins of the human immunoglobulin (Ig). *IGH* encodes the IgH heavy chain. *IGK* and *IGL* encode the kappa and lambda light chains, respectively. These loci vary in their numbers and orientation of V, D, and J gene segments (Fig. 7.1). Nomenclature of gene segments evolves, and new variations and haplotypes are continually discovered. Up-to-date maps, official gene designations, and sequencing of the immunoglobulin loci can be



**Fig. 7.1** Immunoglobulin gene loci. *IGH* (heavy chain), *IGK* (kappa light chain), and *IGL* (lambda light chain) loci are shown. *IGH* has many V, D, and J gene segments along with many constant (C) region gene segments (**a**). The C gene segments play a role in isotype switching. *IGK* has V, J, and C gene segments (**b**). The V gene segments are arranged in two large duplicated clusters designated proximal and distal (current official V gene designations, 2-40 and 2D-40, are depicted to delineate these regions, all distal segments have a “D” in their official designation). A rare human haplotype has only half the number of V genes

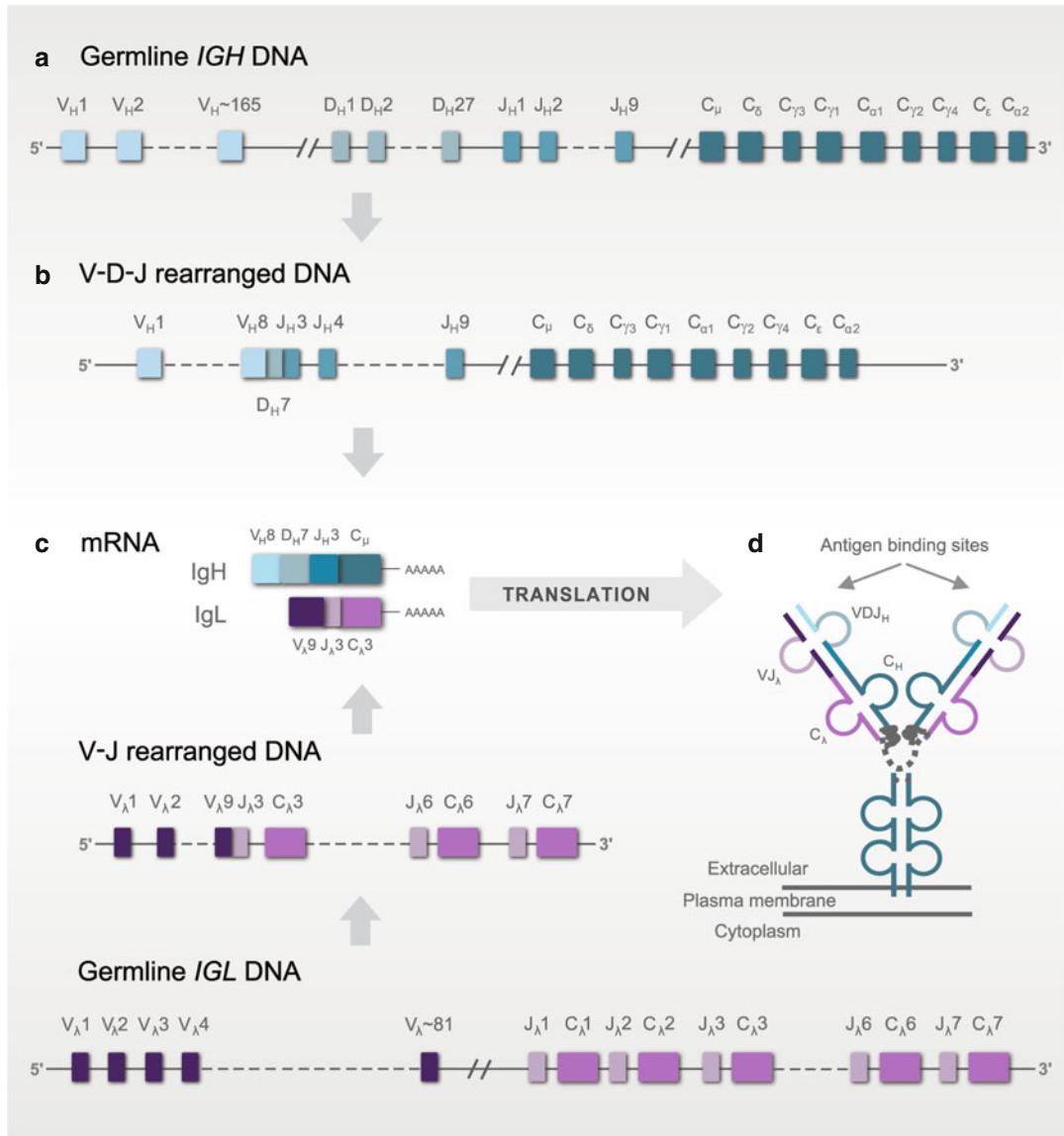
(not shown). The *IGK* distal V region has opposite (3' → 5') orientation. The *IGL* locus has V, J, and C gene segments (**c**). The *IGL* locus has a J-C cluster with approximately 11 total genes each (only functional J-C genes are shown for clarity). With the exception of the distal *IGK* V region, the orientation of these loci is forward with transcription in the 5' → 3' direction. *IGH* V, D, J, *IGK* V, and *IGL* V gene segments are numbered sequentially for clarity but include pseudogenes and orphans. For up-to-date loci maps and gene designations, refer to the international ImMunoGeneTics database at [www.imgt.org](http://www.imgt.org) [3].

found in the international ImMunoGeneTics database at [www.imgt.org](http://www.imgt.org) [3].

The *immunoglobulin heavy chain* locus, *IGH*, located on 14q32.33, is the first to rearrange (Fig. 7.2). It has V, D, and J gene segments, similar to the *TRB* (β) and *TRD* (δ) TCR loci. A randomly selected D gene segment recombines with a randomly selected J gene segment, forming a complex rolling circle formation with a D-J union. The interval DNA (the rolling circle) is spliced out and removed. D-J then recombines with a randomly selected V gene segment to form V-D-J. A primary

RNA transcript is formed. Via more splicing mechanisms, removing all the interval RNA including other gene segments, V-D-J joins C (constant region) to form mRNA, which gets translated into the IgH protein. If a functional IgH is not produced, the other allele begins rearrangement.

A similar process occurs with the immunoglobulin light chains. The *kappa light chain* locus, *IGK*, is located on 2p11.2 and is the first of the light chains to rearrange. It contains V and J gene segments, similar to the *TRA* (α) and *TRG* (γ) TCR loci. Unlike *IGH*, there are no D gene



**Fig. 7.2** Immunoglobulin somatic recombination. This schematic highlights the steps involved in rearrangement of the *IGH* and *IGK* loci to form a surface immunoglobulin protein. *IGH* contains V, D, J, and C gene segments (**a**). During somatic recombination, a randomly selected *D<sub>H</sub>* gene segment first recombines with a *J<sub>H</sub>* gene segment, then a *V<sub>H</sub>* gene segment, deleting the interval genetic material (**b**). The newly recombined DNA is transcribed into primary RNA transcripts (not shown). These transcripts are modified to mRNA, splicing out all interval

genetic material to achieve the V-D-J-C union (with a poly-A tail) (**c**). A similar process is taking place at the *IGK* locus. *IGK* is slightly different from *IGH* in that it has no D gene segments (*bottom half* of figure). Once mRNA transcripts for the heavy and light chains are generated, they are translated into the heavy and light chain proteins and expressed as an immunoglobulin on the surface of the B cell (**d**). If both alleles of *IGK* fail at recombination, the *IGL* light chain recombines in a similar fashion (not shown)

segments. Randomly selected V and J segments recombine at the DNA level. These are transcribed into primary RNA transcripts, which are modified/spliced into V-J-C mRNA, removing

the interval sequence. The mRNA is translated into the kappa light chain and organizes with the heavy chains to form a surface immunoglobulin. If a functional kappa light chain protein is not

**Table 7.1** Number of gene segments and potential rearrangements from different immunoglobulin loci

Ig locus		Genes	Rearrangeable genes <sup>a</sup>	Functional rearrangeable genes	Combinatorial diversity (max) V × D × J
<i>IGH</i>	V	165	66	46	6,348
	D	27	27	23	
	J	9	6	6	
	C	11	9	9	
<i>IGK</i>	V	101	76	38	190
	J	5	5	5	
	C	1	1	1	
<i>IGL</i>	V	81	56	33	165
	J	11	5	5	
	C	11	5	5	

Numbers of genes are approximate and include pseudogenes and orphans, data compiled from several sources [1, 3, 4]

<sup>a</sup>Rearrangeable genes have the appropriate flanking recombination signal sequences (RSS)

formed, the other allele begins rearrangement. If that also fails, rearrangement of the lambda locus proceeds.

The *lambda light chain* locus, *IGL*, is located on 22q11.2. V and J segments recombine and ultimately are transcribed into V-J-C mRNA, similar to the *IGK* locus. The mRNA is translated into the lambda light chain protein, which organizes with the heavy chain protein to form surface immunoglobulin. Again, if a functional lambda light chain is not generated with rearrangement of the first allele, the other allele attempts recombination.

Not all gene segments are functional. Some segments lack a recombination signal sequence (RSS), and therefore cannot recombine, and there are also admixed pseudogenes and orphans (non-functional genes located outside the main chromosomal locus). The total number of functional genes, which participate in the random coupling of V-D-J, is shown in Table 7.1. In addition to this *combinatorial* diversity of the heavy and light chains, there is random pairing of the heavy chain with a light chain, and there is junctional imprecision during recombination (“N” nucleotide addition, etc.), all contributing to the *total* diversity ( $10^{18}$  unique amino acid sequence permutations) of the B-cell repertoire. Another event, somatic hypermutation, occurs over the life of B cells but not T cells and also contributes to diversity. In this process, there are primarily point mutations in the V-D-J region, allowing minor

alterations over time at the antigen-recognition site within clonal populations in order to “fine-tune” antigen recognition.

Each naïve B cell has its own unique V-J (light chain) and V-D-J (heavy chain) recombination sequences. Due to allelic exclusion (only one but not both alleles of each chain are expressed), these sequences correspond to the expression of numerous identical surface immunoglobulins, with specificity for a single antigen/epitope. These surface immunoglobulins contain two heavy chains and either two kappa light chains or two lambda light chains, but not both, resulting in a Y-shaped tetramer. When this surface immunoglobulin becomes engaged with its targeted antigen (along with cytokine and/or direct cell-to-cell signaling), the B cell becomes activated and proliferates in a process termed *clonal expansion*. Because only the cells that recognize antigen are activated and expanded, discrete clonal populations of cells become highly represented among the entire B-cell population, a process termed *clonal selection*. B cells that recognize antigen may become terminally differentiated into plasma cells, which are immunoglobulin-generating (and immunoglobulin-secreting) machines.

### 7.2.2.2 Using Molecular Tools to Determine B-Cell Clonality

B-cell malignancies are also clonally expanded, but this is due to molecular oncogenic events, not

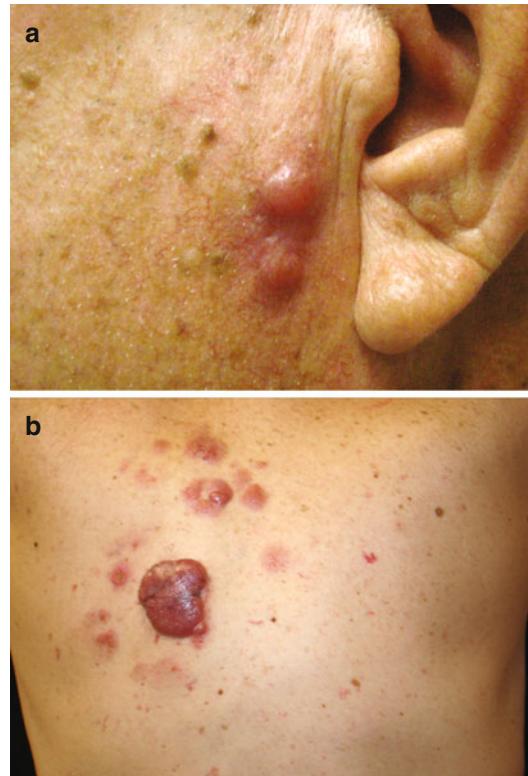
the normal controlled clonal expansion from antigen recognition. These malignant clones have unique heavy and light chain recombinations. Conversely, most benign B-cell infiltrates are responding to numerous different or complex antigens. Therefore, the multitude of single clonal expansions becomes, effectively, a polyclonal response.

Molecular assays, specifically Southern blot and PCR, take advantage of the normal recombination events of lymphocyte development. By amplification or probing of the recombination region, overrepresentation of a single recombination provides evidence for clonality (see Practical Considerations section for more on molecular techniques).

### 7.3 Diagnostic Applications for Molecular Testing

Outside of classic presentations of MF and SS (and perhaps lymphomatoid papulosis when included in this group), the clinical features of primary cutaneous lymphoma offer few diagnostic clues (Fig. 7.3). The B-cell and non-MF/SS T-cell lymphomas may slightly vary with respect to patient age, anatomic site prevalence, and disease course, but at initial presentation early in the disease process, there is too much overlap to make any bold statements regarding a specific diagnosis prior to biopsy. Patients are usually older adults and present with solitary or grouped papules and/or nodules. More deep-seated tumors are more ill defined. The color also varies with depth but usually is violaceous. Some tumors, like the low-grade B-cell lymphomas and subcutaneous panniculitis-like T-cell lymphomas, may actually wax and wane, thus confusing the clinical picture.

In theory, all malignancies, including primary cutaneous lymphomas, are clonal processes. In practice, likely due to limitations imposed by the clonality assays, most but not all tumors have a detectable clone (Table 7.2). The sensitivity of clonality assays varies by tumor type and study. With the exception of MF/SS, large studies are lacking to adequately determine the power of



**Fig. 7.3** Clinical features of cutaneous lymphoma. The clinical features of non-MF/SS cutaneous lymphoma offer few clues to a specific diagnosis. Many non-MF/SS cutaneous lymphomas are solitary or grouped papules, as in this case of primary cutaneous follicle center cell lymphoma from the preauricular region of a 68-year-old man (a). They may also be large nodules or tumors, as in this case of diffuse large B-cell lymphoma from the back of a 42-year-old man (b). Diagnoses were confirmed by biopsy (Images courtesy of Dr. Stephen Weis, Ft. Worth, TX)

these assays on skin-specific tumors. Practically, for the non-MF/SS tumors, molecular clonality testing is most helpful for distinguishing low-grade B-cell malignancies from reactive processes since these entities can have significant histomorphologic and immunohistochemical overlap. Less commonly, testing is used to distinguish reactive infiltrates from low-grade T-cell tumors. And rarely, there may be a need to distinguish high-grade tumors from reactive processes. This latter scenario is less common because high-grade tumors are often clearly histologically malignant, abrogating any added benefit of clonality testing. In ambiguous cases, however,

**Table 7.2** TCR and immunoglobulin rearrangement in primary cutaneous lymphomas

Tumor			% of tumor cases with Ig/TCR rearrangement <sup>a</sup>
T cell	Indolent	Reactive/pseudolymphoma	10–30
		Mycosis fungoides and variants	80–90
		Anaplastic large cell lymphoma	Most <sup>b</sup>
		Lymphomatoid papulosis	60–70
		Subcutaneous panniculitis-like T-cell lymphoma	Most <sup>b</sup>
		CD4+ small/medium pleomorphic T-cell lymphoma <sup>c</sup>	Most <sup>b</sup>
	Indolent/Aggressive	Adult T-cell leukemia/lymphoma	40–60 <sup>b</sup>
		NK-/T-cell lymphoma, nasal type	Low, but up to 30 %
		Aggressive CD8+ T-cell lymphoma <sup>c</sup>	Most <sup>b</sup>
		γ/δ T-cell lymphoma <sup>c</sup>	Most <sup>b</sup>
B cell	Indolent	Sézary syndrome	>95
		Peripheral T-cell lymphoma, unspecified	Most <sup>b</sup>
	Intermediate	Reactive/pseudolymphoma	10–25
		Marginal zone lymphoma	50–80
		Follicle center cell lymphoma	60–90 <sup>b</sup>

<sup>a</sup>Data compiled from several sources [5–9]

<sup>b</sup>Large studies are lacking

<sup>c</sup>Provisional entities

<sup>d</sup>Currently lumped under diffuse large B-cell, other

distinction between a benign infiltrate and high-grade malignant process is crucial, and clonality testing is warranted.

Hematopoietic tumors that secondarily involve the skin usually do not require molecular testing for diagnosis. Assuming the patient history is adequately transferred to the dermatologist and dermatopathologist, cutaneous involvement of the patient's known tumor can be diagnosed by histology or with few confirmatory immunohistochemical stains. In rare scenarios, these patients with secondary cutaneous infiltrates may benefit from molecular testing: (1) The patient carries a previous diagnosis but only has a sparse infiltrate, requiring clonality testing to determine skin involvement. (2) The patient has a previous diagnosis of leukemia or lymphoma with a known translocation, now with subtle cutaneous infiltrates, requiring FISH or PCR (or RT-PCR) to determine skin involvement. (3) The patient's systemic tumor initially presents in the skin, requiring molecular testing for diagnosis.

## 7.3.1 Primary Cutaneous B-Cell Lymphomas

Primary cutaneous B-cell lymphomas account for approximately 25 % of cutaneous lymphomas. These tumors are considered primary if there is no evidence of extracutaneous or systemic disease at presentation. In the most recent World Health Organization-European Organization for the Research and Treatment of Cancer (WHO-EORTC) classification system, these tumors are divided into indolent and intermediate behavior groups [5]. Molecular clonality testing is more commonly performed with the indolent groups to help distinguish lymphoma from pseudolymphoma.

### 7.3.1.1 Pseudolymphoma Versus Low-Grade B-Cell Lymphoma

Distinguishing low-grade B-cell lymphomas from reactive infiltrates (pseudolymphomas) is one of the more common challenges in dermatopathology. These entities can be identical on clinical presentation, and on biopsy, there is

significant histologic overlap (Fig. 7.4). Combining the histologic features with immunohistochemistry and molecular studies can often lead to the correct diagnosis (Table 7.3).

### Pseudolymphoma

The term “pseudolymphoma” refers to a reactive lymphocytic process and is preferred over the antiquated and confusing designation lymphocytoma cutis. Technically, the term “pseudolymphoma” is appropriate for any benign/reactive process that mimics lymphoma, but, in practice, it is more commonly used for B-cell-rich processes (as opposed to T-cell processes mimicking mycosis fungoides). There are many causes of pseudolymphoma, and because of this, all ages and demographics can be affected. Penetrating injury/trauma, arthropod bites, and remote ruptured cysts are commonly implicated. Any persistent antigen-presenting state can lead to localized chronic overstimulation of lymphocytes and the development of a mass. Additionally, other ill-defined inflammatory processes, such as tumid lupus erythematosus and lymphocytic infiltrate of Jessner, can present as a single lesion, mimicking lymphoma.

Because pseudolymphoma, by definition, is a reactive process, the infiltrate is usually mixed. The infiltrate can range from light to heavy and virtually fill the dermis (see Fig. 7.4). Usually, there is a grenz zone, separating the dermal infiltrate from the uninvolved epidermis. Additionally, the infiltrate usually spares adnexal units, and when the adnexa are involved, they have significant reactive changes (spongiosis, dyskeratosis, etc.). Large germinal centers may be present. The germinal centers are reactive with polarization (appear zonal instead of uniform throughout) and have numerous tingible-body macrophages, which are cleaning up the cellular debris from high cellular turnover. The infiltrate is made up of a mixture of cell types with a range of sizes. The cells are not atypical. Usually, mature lymphocytes are present, admixed with histiocytes and variable degrees of plasma cells and granulocytes (neutrophils/eosinophils).

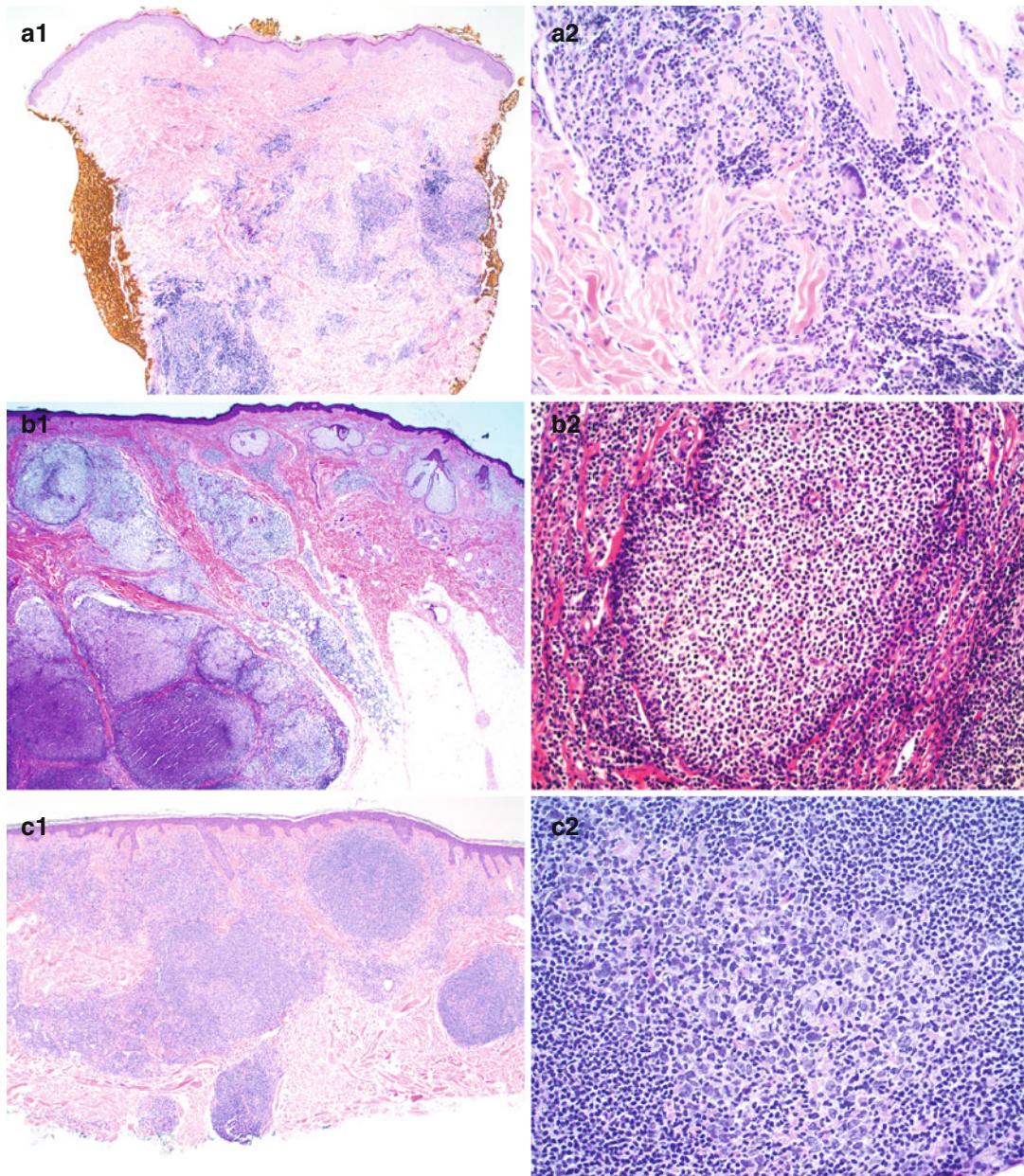
The mixed nature of the infiltrate is also evident by immunohistochemistry. Most cases of

pseudolymphoma have a mixture of CD3+ T cells and CD20+ B cells. With a “bottom-heavy” deep dermal infiltrate (as opposed to the “top-heavy” epidermotropic infiltrate of mycosis fungoides), a preponderance of CD3+ T cells is a reassuring finding, as T cells are the most commonly observed lymphocytes in cutaneous inflammatory reactions. Occasionally, CD20+ B-cell-rich cases are observed and can be striking when there is prominent germinal center formation. Overall, however, CD3/CD20 immunohistochemical analysis provides insight into the architecture of the infiltrate, and with pseudolymphomas, this architecture consists of a background T-cell scaffold admixed with few, small B-cell aggregates and/or occasional reactive germinal centers. Kappa and lambda are useful markers (by immunohistochemistry or *in situ* hybridization), especially in cases with visible plasma or plasmacytoid cells. Reactive processes will have appropriate kappa to lambda ratios, in the 2:1 range, among these populations (ratios >5:1 or inverted ratios are concerning for clonality as are unusual distributions of kappa and lambda expression). Other immunohistochemical markers—CD10, CD21, CD23, CD43, bcl-2, and bcl-6—may be useful to further characterize the architecture of the infiltrate and its cellular constituents.

For cases which deviate from these typical histologic and immunophenotypic findings, molecular analysis is appropriate. B-cell receptor (*IGH*, *IGK*, *IGL*) gene rearrangement studies may help prove polyclonality. As discussed, however, lack of a detectable clone does not exclude malignancy. Additionally, because many pseudolymphomas are due to chronic antigen stimulation in a focal area, the occasional detection of a clone should not be surprising. If the molecular test sample contains a single germinal center, for example, a clonal result is likely, emphasizing the importance of pre-analytical sample selection prior to molecular analysis.

### Primary Cutaneous Marginal Zone Lymphoma

Primary cutaneous marginal zone lymphoma (PCMZL) is also common, accounting for 25 % of all primary cutaneous B-cell lymphomas [12].



**Fig. 7.4** Histologic features of pseudolymphoma and low-grade B-cell lymphomas. In pseudolymphoma (**a**), there is usually a dense infiltrate of inflammatory cells occupying the dermis, as in this case of a 64-year-old female with tumid lupus erythematosus (**a1**, H&E, 40 $\times$  original magnification). Pseudolymphomas usually have a mixed infiltrate with cells of different sizes and without overt atypia (**a2**, H&E, 200 $\times$ ). This case of primary cutaneous follicle center cell lymphoma from the cheek of a 91-year-old man (**b**) has a nice follicular pattern (**b1**, H&E, 20 $\times$ ). The germinal centers are monotonous without

polarization or tingible-body macrophages (**b2**, H&E, 200 $\times$ ). Primary cutaneous marginal zone B-cell lymphoma (**c**) also usually has a nodular and diffuse pattern with an overlying grenz zone and an expanded population of cells around germinal centers, as in this case from the back of a 62-year-old woman (**c1**, H&E, 40 $\times$ ). Many times, there is a prominent population of monocytoid cells encasing or even colonizing reactive germinal centers (**c2**, H&E, 200 $\times$ ). The germinal centers are polarized with tingible-body macrophages

**Table 7.3** Pseudolymphoma versus low-grade B-cell lymphoma

	Pseudolymphoma	PCMZL	PCFCL	CLL/SLL <sup>a</sup>
Clinical	Wide age range and presentations	Wide age range, usually older adults; trunk and extremities, head/neck; usually solitary; can be grouped papules, plaques, and nodules	Elderly, head/neck, solitary, or grouped papules, plaques, and nodules	Elderly, localized papules/nodule or generalized
Histology	Dense infiltrate, usually mixed, reactive germinal centers common	Nodular and diffuse, can have plasmacytoid and/or monocytoid morph, germ centers common	Nodular and diffuse, may have large irregular germinal centers, variable proportions of centroblasts and centrocytes	Diffuse, interstitial, monomorphic small mature-appearing lymphocytes
IHC <sup>b</sup>	Mixture of CD20+ and CD3+ cells, Bcl-6-, Bcl-2-, CD43-, CD5-, κ/λ mixed, ↑Ki67	κ/λ restricted <sup>c</sup> , CD43var, CD23var, Bcl-2var, Bcl-6-, CD10-, CD5-	Bcl-6+, Bcl-2var, CD10var, CD43var, CD5-, ↑Ki67, CD21/CD23 dendritic cell lysis	CD5+, CD23+, κ/λ restricted, CD43+, Bcl-2var, Bcl-6-
Cytogenetics	Normal	t(14;18) <i>IGH-MALT1</i> rare t(11;18) <i>API2-MALT1</i> rare t(3;14) rare	t(14;18) <i>IGH-BCL2</i> rare	Trisomy 12 13q abnormalities
Molecular	Polyclonal	Clonal	Clonal	Clonal

Data compiled from several sources [10, 11]

<sup>a</sup>Not a primary cutaneous lymphoma but is low-grade B-cell leukemia/lymphoma in differential diagnosis

<sup>b</sup>All B-cell lymphomas have prominent CD20+/PAX-5+ B-cell component and may be CD20- if patient is receiving rituximab

<sup>c</sup>Useful only when there is plasmacytoid differentiation

This tumor is the cutaneous counterpart of the mucosa-associated lymphoid tissue (MALT) lymphoma (or MALToma), which is most commonly observed in the gastrointestinal tract. When cutaneous, PCMZL carries the alternative designation of skin-associated lymphoid tissue (SALT) lymphoma, or SALToma. Primary nodal marginal zone lymphomas also occur. Tumors with prominent plasmacytoid differentiation were previously called immunocytomas but are now considered histologic variants of PCMZL. As with MALTomas and *Helicobacter pylori*, PCMZL are likely caused by chronic stimulation, and therefore, it is not surprising that many cases arise in a background of cutaneous lymphoid hyperplasia. *Borrelia burgdorferi* has been implicated in some cases, but a strong correlation between PCMZL and a specific infectious agent has not yet been realized. PCMZL usually affects middle-aged adults, but all ages can be affected. The clinical presentation is not specific, with one

or more papules or nodules. Localized tumors can be treated by injection, excision, or radiation, but recurrences are common. Overall, PCMZL is an indolent disease with a very good prognosis. The 5-year (and 10-year) survival was 93 % in a recent retrospective analysis of 137 PCMZL patients [6], and others have reported the 5-year survival at >99 % [5].

On histology, the first clue to a diagnosis of PCMZL is the presence of a dense nodular or diffuse infiltrate in the dermis (see Fig. 7.4). As is typical for B-cell tumors, the infiltrate is usually bottom heavy with an overlying grenz zone. Germinal centers are common but usually appear restricted or “choked” by the tumor or colonized by the tumor as opposed to the large irregular germinal centers observed in follicle center cell lymphoma. There may be so-called lymphoepithelial lesions, which were originally described in the gastrointestinal tract but can occur in the skin as tumor cells invade adnexal units. PCMZL

can have different cell types, with varying degrees of representation. Most cells are small, mature-appearing lymphocytes, but there are often admixed larger cleaved cells, plasmacytoid cells (eccentric nucleus), and monocytoid cells (fried-egg appearance). Transformation into a large B-cell lymphoma can (rarely) occur [13].

Immunohistochemistry can be helpful in the diagnosis of PCMZL in certain circumstances. In general, the infiltrates are B-cell rich, with CD20 expression on a preponderance of cells. Plasmacytoid differentiation can be very helpful because these cells will express immunoglobulin, and infiltrates restricted to kappa or lambda point to malignancy. Light chain restriction is observed in approximately two-thirds of cases, and of those cases, kappa restriction is twice as frequent [6]. Aberrant expression of CD43, a normal T-cell marker, is also suggestive of malignancy, but this only occurs in about half of PCMZL cases. Because PCMZL may only minimally deviate from pseudolymphoma using histologic and immunophenotypic criteria, molecular testing for clonality can be very helpful for distinguishing these two entities.

Molecular analysis identifies a clone, by *IGH* rearrangement, in approximately one-half of cases [6]. This sensitivity can be pushed higher by also testing for *IGK* and *IGL* rearrangements. At times, multiple biopsies over time are required to detect a clone, suggesting earlier samples can be false negatives due to a low percentage of clonal cells in the infiltrate. Chromosomal abnormalities have been observed in subsets of extranodal marginal zone lymphomas, specifically trisomy 3 in approximately 60 % of cases and the t(11;18) (q21;q21) translocation in up to 50 % of cases. The latter translocation creates the *API2-MALT1* fusion gene, which encodes for an anti-apoptosis chimeric protein, likely acting as an oncogenic driver. Other translocations—t(14;18)(q32;q21), t(3;14)(p14.1;q32), and t(1;14)(p22;q32)—occur in a smaller subset of cases. Interestingly, chromosomal abnormalities are only rarely observed in the cutaneous forms of marginal zone lymphoma [14], providing molecular evidence that PCMZL is not the exact same tumor as its gastrointestinal and nodal counterparts.

## Primary Cutaneous Follicle Center Cell Lymphoma

Primary cutaneous follicle center cell lymphoma (PCFCL) is the most common primary cutaneous B-cell lymphoma, accounting for approximately 40–50 % of cases [15]. It has a nodal counterpart, follicular lymphoma, but these are now separated into two distinct diseases in current classification systems based on molecular and clinical differences. The typical PCFCL patient is a white, older adult (median age 60) with solitary or grouped papules/nodules on the head and neck region. Variations on these demographics and presentations occur. PCFCL is also an indolent disease, but progression/transformation to a more aggressive large B-cell lymphoma can occur. While recurrences are common, with treatment, the 5-year survival of PCFCL is approximately 95 %. Treatment usually consists of local injection, excision, and/or radiation, with systemic chemotherapy for multifocal disease.

On histology, PCFCL is a nodular and/or diffuse infiltrate, involving the dermis and subcutis, with an overlying grenz zone (see Fig. 7.4). In classic cases, there is a so-called follicular pattern, with large irregular and sometimes serpiginous germinal centers, surrounded by lymphoid cuffs. Unlike reactive germinal centers, the malignant germinal centers are not polarized, or zonal, and are devoid of tingible-body macrophages. The tumor cells can range in size from small, cleaved cells (centrocytes) to larger cells with open chromatin (centroblasts). The relative proportion of these cells impacts the tumor grade, with higher-grade tumors exhibiting greater centroblast populations.

Immunohistochemistry reveals a follicle center cell immunophenotype, specifically CD20+ and Bcl-6+ (CD20 may be absent if patient has received anti-CD20 therapy such as rituximab). Counterintuitively, the germinal centers in low-grade PCFCL have a low-to-moderate proliferative index (measured by Ki-67) as opposed to the high proliferative rate of pseudolymphoma. CD10, often observed in nodal follicular lymphoma, is variably present in the cutaneous tumors. Also, Bcl-2, which is commonly observed in the nodal tumors, is positive in less

than half of PCFCL. In Bcl-2+/Bcl-6+/CD10+ tumors, there should be high suspicion for nodal follicular lymphoma secondarily involving the skin.

Many pseudolymphomas have striking germinal center formation, which will also contain numerous Bcl-6+ cells. Also, some PCFCLs do not exhibit the follicular pattern and do not have recognizable germinal centers. Molecular clonality studies may be useful in these ambiguous cases. The t(14;18)(q32;q21) translocation that results in the *IGH-BCL2* fusion, which promotes a cellular anti-apoptosis signal, is observed in up to 90 % of nodal follicular lymphomas but is present in only a minority of PCFCL cases [16–18]. These immunophenotypic and molecular differences suggest only a loose relationship between nodal follicular lymphoma and PCFCL.

### 7.3.1.2 Other Primary Cutaneous B-Cell Lymphomas

The other primary cutaneous B-cell lymphomas in the most recent classification are diffuse large B-cell lymphoma, leg type, and diffuse large B-cell lymphoma, other (which includes intravascular large B-cell lymphoma) [5]. These entities are often clonal by molecular studies (see Table 7.2), but since these tumors are intermediate to high grade, their malignant status is usually not in question, and therefore, molecular studies for diagnosis have only limited utility. Large B-cell lymphomas can have a variety of chromosomal abnormalities, including gains of chromosomes 12, 7, 3, 18q, 11, X. Because of the heterogeneity of these findings among tumors, there is no clear current practical application for this karyotypic information.

### 7.3.2 Non-MF/SS Primary Cutaneous T-Cell Lymphomas

Besides MF and SS (discussed in Chap. 6), there are other primary cutaneous T-cell malignancies, categorized by clinical behavior in the recent classification systems (see Table 7.2). The indolent tumors are MF and its variants, the CD30+ lymphoproliferative disorders (primary cutaneous

anaplastic large cell lymphoma and lymphomatoid papulosis), subcutaneous panniculitis-like T-cell lymphoma, and primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma (provisional entity). The aggressive tumors are primary cutaneous NK/T-cell lymphoma, nasal type, primary cutaneous aggressive CD8+ T-cell lymphoma (provisional entity), primary cutaneous γ/δ T-cell lymphoma (provisional entity), and primary cutaneous peripheral T-cell lymphoma, unspecified [5]. Adult T-cell leukemia/lymphoma can be indolent or have an acute progressive course.

#### 7.3.2.1 Indolent Primary Cutaneous Non-MF/SS T-Cell Tumors

##### CD30+ Lymphoproliferative Disorders

In the most recent classification system, the CD30+ lymphoproliferative disorders include primary cutaneous anaplastic large cell lymphoma (ALCL) and lymphomatoid papulosis (LyP). Systemic ALCL involving the skin, transformed mycosis fungoides, and other tumors may express CD30 but are categorized elsewhere [19].

LyP is a classic example of a condition with malignant cytology but a benign clinical course. The 5-year survival approaches 100 %. Patients with LyP develop papules or nodules that spontaneously regress. The lesions do not exceed 3 cm in diameter, and the patients do not develop lymphadenopathy. If either of these occurs, the process is better classified as lymphoma. There are now four (A–D) histologic variants of LyP. Type A contains large atypical cells admixed with lymphocytes and neutrophils in a wedge-shaped infiltrate. Type B resembles MF but with a clinical course more in line with LyP. Type C has sheets of atypical cells. Type D, unlike types A–C, is CD8+.

Primary cutaneous ALCL, despite its high-grade morphology, has a fairly good prognosis with a 5-year survival of 90–95 %. On histology, there are sheets of atypical cells, similar to type C LyP. The cells are CD4+/CD2+/CD30+. CD3 is usually negative and there are rare CD8+ variants. Nodal ALCL often has a t(2;5) translocation and is ALK+ by immunohistochemistry. The ALK- variants have a worse prognosis. Because

of this, it is important to distinguish primary cutaneous ALCL, which is also ALK-, from nodal ALCL secondarily involving the skin.

Both LyP and ALCL have rearranged TCR genes. Testing is not routinely performed since the clinical, morphologic, and immunophenotypic features often point to the diagnosis. In rare cases, however, the differential diagnosis includes reactive processes (scabies, arthropod bite, etc.), and T-cell gene rearrangement studies may have some value.

### **Subcutaneous Panniculitis-Like T-Cell Lymphoma (SCPLTCL)**

SCPLTCL is an indolent lymphoma usually affecting adults. Patients present with nodules of the proximal extremities and buttocks. In some cases, the lesions regress, raising the possibility of a reactive process. The more aggressive  $\gamma/\delta$  variant is now classified under  $\gamma/\delta$  lymphoma. On histology, SCPLTCL has a dense infiltrate in the subcutis. The lymphocytes are mildly enlarged, at times pleomorphic, and in many cases rim individual adipocytes. Cytophagocytosis is variably present. Most cases are CD8+ (minority are CD4+) with a cytotoxic phenotype (TIA-1+/granzyme+/perforin+). T-cell gene rearrangement studies with a strong monoclonal spike may be useful in cases that overlap with a persistent lobular panniculitis, such as lupus panniculitis. Of note, however, a subset of lupus cases will be clonal or oligoclonal, emphasizing the importance of interpreting results in the context of the whole patient. Some cases histologically and molecularly fall somewhere in between reactive and malignant and have been given terms such as “atypical lobular panniculitis” and “T-cell dyscrasia”.

### **Primary Cutaneous CD4+ Small/Medium Pleomorphic T-Cell Lymphoma**

After MF and the CD30+ lymphoproliferative disorders (ALCL and LyP), primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma is the 3rd most common primary cutaneous T-cell lymphoma, accounting for approximately 10 % of cases. This tumor is a provisional entity, currently lumped under the header primary cutaneous peripheral T-cell lymphoma, unspecified. Primary cutaneous CD4+ small/medium pleomorphic

T-cell lymphomas are heterogeneous in their clinical presentation and their histologic features, likely because this diagnosis encompasses a spectrum of T-cell tumors that fail to meet criteria for the other more common cutaneous T-cell tumors (and if the tumor does not meet criteria for primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma, it is classified under the umbrella peripheral T-cell lymphoma, unspecified).

Primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma usually presents in adults as solitary or grouped nodules or tumors. The 5-year survival is 60–80 %. As the name implies, the histology is notable for the presence of small- to medium-sized pleomorphic lymphocytes. The cells are considered pleomorphic due to nuclear features, which include polylobation, folding, and hyperchromasia. There may be admixed large cells, but these should represent <30 % of the infiltrate. The infiltrate is most heavy in the dermis with epidermotropism minimal to absent. The tumor cells usually carry a helper T-cell immunophenotype (CD3+/CD4+). CD2, CD5, and CD7 have variable expression. Histomorphologic and immunophenotypic features usually allow distinction between primary cutaneous CD4+ small/medium pleomorphic T-cell lymphoma and other cutaneous lymphomas. TCR gene rearrangement analysis can help distinguish this tumor from reactive processes in challenging cases.

### **Adult T-Cell Leukemia/Lymphoma**

Adult T-cell leukemia/lymphoma is most common in Japan, the Caribbean, and Central Africa. It is rare in the United States. The etiologic agent is human T-cell leukemia virus type 1 (HTLV-1). Adult T-cell leukemia/lymphoma can be an acute, progressive tumor or take a chronic, indolent course. The acute form is associated with hypercalcemia, lymphadenopathy, hepatosplenomegaly, and blood involvement. It has a high mortality. There is cutaneous involvement in both forms.

There is significant histologic overlap between adult T-cell leukemia/lymphoma and mycosis fungoides. Both tumors are epidermotropic and have lymphocytes with cerebriform, polylobated nuclei. The immunophenotype, CD3+/CD4+/CD7−, is also similar. Adult T-cell leukemia/lymphoma has

rearranged TCR loci, and the diagnosis can be confirmed by identifying the presence of HTLV-1. HTLV-1 can be detected by serologic studies and RT-PCR or by *in situ* hybridization of viral nucleic acids integrated into the tumor genome [20]. This latter method is not routinely performed on clinical samples.

### 7.3.2.2 Aggressive Primary Cutaneous T-Cell Tumors

The aggressive primary cutaneous T-cell tumors include primary cutaneous NK-/T-cell lymphoma, nasal type, primary cutaneous aggressive CD8+ T-cell lymphoma (provisional), primary cutaneous  $\gamma/\delta$  T-cell lymphoma (provisional), and the umbrella category primary cutaneous peripheral T-cell lymphoma, unspecified. These tumors are clonal by TCR gene rearrangement studies, but molecular testing has a limited role in their diagnosis since the clinical, histomorphologic, and/or immunophenotypic findings usually point to malignancy [21].

Cutaneous  $\gamma/\delta$  T-cell lymphomas deserve special mention. These tumors express TCR $^{\gamma/\delta}$  on their surface and therefore have rearranged *TRG* ( $\gamma$ ) and *TRD* ( $\delta$ ) loci. Cutaneous  $\gamma/\delta$  T-cell lymphomas are more commonly diagnosed by identifying TCR $^{\gamma/\delta}$  protein expression by flow cytometry (or by lack of TCR $^{\alpha/\beta}$  by immunohistochemistry) but can potentially be diagnosed by evaluation of the rearranged *TRD* ( $\delta$ ) locus. Recall that the *TRD* ( $\delta$ ) locus is completely removed by splicing following *TRA* ( $\alpha$ ) rearrangement at the same locus (see Chap. 6), and therefore, most T-cell lymphomas and reactive processes lack *TRD* ( $\delta$ ) VDJ genes and will be negative for clonality by *TRD* ( $\delta$ ) gene rearrangement analysis. Analysis of the *TRD* ( $\delta$ ) locus can be useful in select settings, such as the diagnosis of cutaneous  $\gamma/\delta$  T-cell lymphomas and immature lymphoblastic T-cell tumors.

### 7.3.3 B-Cell Versus T-Cell Lymphoma

On occasion, the dermatopathologist is presented with a case of uncertain lineage (Fig. 7.5). There

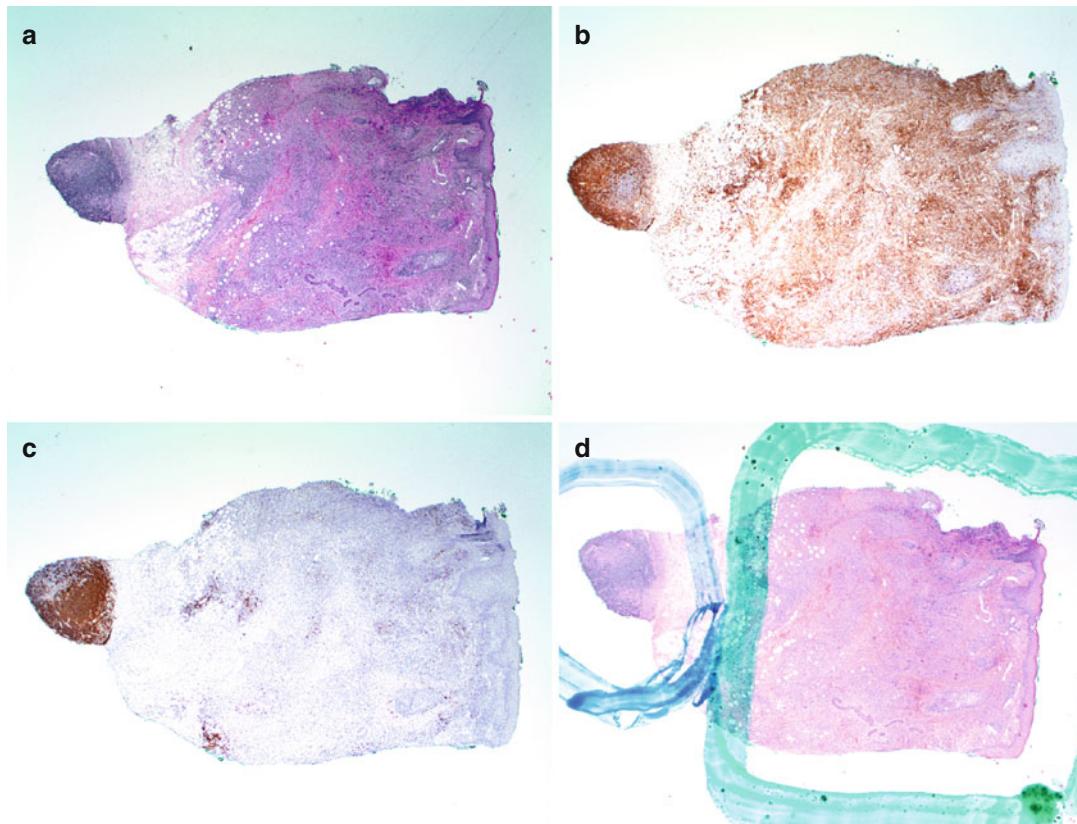
may be a persistent and dense infiltrate, both clinically and histologically concerning for lymphoma, but the tumor cell of origin may not be clearly defined by immunohistochemistry. Some T-cell neoplasms have a background infiltrate of neutrophils, eosinophils, and B-cell aggregates. Conversely, some B-cell tumors, like Hodgkin lymphoma, have a significant non-B-cell component. In these scenarios, T-cell and B-cell clonality testing may be useful for distinction, and the utility of these tests can be enhanced by purifying or microdissecting DNA from populations of interest.

It must be noted, however, that B-cell neoplasms can have rearranged TCR and vice versa [22]. In B-ALL, virtually all cases have clonal D-J at the *IGH* locus, but TCR is also rearranged in a high number of cases [23]. Likewise, virtually all T-ALL cases have rearranged TCR, but up to 20 % will have rearrangement of *IGH* [24, 25]. While this is common with primitive tumors, it is less common with mature cutaneous lymphomas. Therefore, use of TCR and Ig rearrangement assays for determination of lineage should be restricted to more mature post-thymic and post-bone marrow leukemias/lymphomas.

### 7.3.4 Other Hematopoietic Tumors Primarily and Secondarily Involving the Skin

#### 7.3.4.1 Non-B-/Non-T-Cell Hematopoietic Tumors

The most recent classification of primary cutaneous lymphomas includes blastic plasmacytoid dendritic cell neoplasm. This tumor as well as tumors of natural killer (NK) cells and Langerhans cells (and others of monocytic/dendritic lineage) do not undergo gene rearrangement. There are reported cases of non-lymphocyte-derived tumors (Merkel cell carcinoma and Langerhans cell sarcoma, as examples [26, 27]) with rearranged Ig or TCR loci, but as a rule, in tumors derived from a non-lymphocyte lineage, the TCR and Ig loci are in germline configuration and clonality studies will be negative. Other molecular testing (FISH, mutational analysis, etc.) may be useful for diagnosis in some settings.



**Fig. 7.5** T-cell versus B-cell lymphoma. At times, there are cases that are clinically and histologically suspicious for lymphoma but immunohistochemistry fails to point to a specific cell of origin. In this case, a 65-year-old female presented with a persistent large forehead plaque. She was biopsied multiple times without conclusive results. Histologically, there was a diffuse mononuclear infiltrate with a grenz zone (**a**, H&E, 20 $\times$  original magnification). Overt cytologic atypia was not present. Immunohistochemistry revealed a

predominantly CD3+ T-cell population in the superficial dermis (**b**, CD3, 20 $\times$ ) and nodules of CD20+ B cells deeper in the biopsy (**c**, CD20, 20 $\times$ ). Manual microdissection of the B-cell (blue) and T-cell (green) populations was performed to enrich the DNA (**d**, H&E, 20 $\times$ ), which was used for Ig and TCR gene rearrangement analysis, respectively. A clone was identified by *TRG* ( $\gamma$ ) rearrangement (*IGH* was polyclonal), supporting a diagnosis of a peripheral T-cell lymphoma (not shown)

### 7.3.4.2 Hematopoietic Tumors Secondarily Involving the Skin

Outside of the primary cutaneous lymphomas, there are many other hematopoietic tumors that can involve the skin. In fact, virtually any systemic tumor can potentially migrate to the skin. Many of these leukemias and lymphomas have recurrent molecular events. These events include Ig and TCR gene rearrangements as well as oncogenic-driving point mutations and macrochromosomal abnormalities such as translocations. As mentioned above, there may be rare circumstances when detection of these events may help for a primary diagnosis or for determining whether a subtle

cutaneous eruption is due to the patient's known hematopoietic tumor. Examples of tumors with recurrent genetic abnormalities secondarily involving the skin are discussed below. For a discussion of the diagnostic criteria for these entities and tumors without recurrent genetic abnormalities that may secondarily involve the skin, the reader is directed elsewhere [19].

#### Recurrent Genetic Abnormalities in Leukemias Secondarily Involving the Skin

Many acute and chronic leukemias secondarily involve the skin. Some, such as chronic myelomonocytic leukemia (CMML) and juvenile

**Table 7.4** Recurrent genetic abnormalities in leukemias secondarily involving the skin

Tumor	Ig/ TCR <sup>a</sup>	Abnormality	Fusion gene	% of tumor cases with abnormality
B-cell lymphoblastic leukemia/lymphoma (B-ALL)	Y	t(9;22)(q34;q11.2) t(var;11q23) t(12;21)(p13;q22) t(5;14)(q31;32) t(1;19)(q23;p13.3) Hyperdiploidy Hypodiploidy	<i>BCR-ABL1</i> <i>MLL</i> rearranged <i>TEL-AML1</i> ( <i>ETV6-RUNX1</i> ) <i>IL3-IGH</i> <i>E2A-PBX1</i> – –	25 (adults) High (infants) 25 <1 6 25 5
Acute myeloid leukemia (AML)	N	t(8;21)(q22;q22) t(16;16)(p13.1;q22) inv(16)(p13.1;q22) t(9;11)(p22;q23) t(6;9)(p23;q34) t(3;3)(q21;q26.2) inv(3)(q21;q26.2) t(1;22)(p13;q13) <i>NPM1</i> mutation <i>CEBPA</i> mutation <sup>b</sup> <i>FLT3</i> mutations	<i>RUNX1-RUNX1T1</i> <i>CBFB-MYH11</i> <i>MLLT3-MLL</i> <i>DEK-NUP214</i> <i>RPN1-EVII</i> <i>RBM15-MKL1</i> – – –	5 5–8 12 (pediatric) 2 2 <1 30 15 20–40
Acute promyelocytic leukemia (APL)	N	t(15;17)(q22;q12)	<i>PML-RARA</i>	>95
T-cell lymphoblastic leukemia/lymphoma (T-ALL)	Y	Translocations involving TCR loci	Var TCR loci and partners	50–70
T-cell prolymphocytic leukemia	Y	inv(14)(q11;q32) t(14;14)(q11;q32)	<i>TRA-TCL1A</i> or <i>TRA-TCL1B</i>	80 10
T-cell large granular lymphocytic leukemia	Y	–	–	Var
Chronic lymphocytic leukemia (CLL)	Y	<sup>b</sup> <i>IGHV</i> mutation	–	Var
Chronic myelogenous leukemia (CML)	N	t(9;22)(q34;q11.2)	<i>BCR-ABL1</i>	>95
Essential thrombocythemia <sup>c</sup>	N	<i>JAK2</i> mutation <i>MPL</i> mutation	–	50 5
Polycythemia vera <sup>c</sup>	N	<i>JAK2</i> mutation <i>MPL</i> mutation	–	95 Rare
Primary myelofibrosis <sup>c</sup>	N	<i>JAK2</i> mutation <i>MPL</i> mutation	–	50 10
Mastocytosis	N	<i>KIT</i> (D816V) mutation	–	Var
Multiple various myeloid and lymphoid neoplasms with eosinophilia	Var	t(v;4q12) t(v;5q33) t(v;8p11)	<i>PDGFRA</i> rearranged <i>PDGFRB</i> rearranged <i>FGFR1</i> rearranged	Var Var Var

<sup>a</sup>Ig or TCR is rearranged and clonal by molecular analysis<sup>b</sup>Prognostic utility only<sup>c</sup>The chronic myeloproliferative diseases may rarely present in the skin as extramedullary hematopoiesis (essential thrombocythemia is included for completeness, but would not present in the skin)

myelomonocytic leukemia (JMML), fairly commonly cause cutaneous eruptions but do not have recurrent molecular abnormalities amenable for a

molecular assay. Others do have recurrent molecular events that can be used in select settings (Table 7.4). There is also a group of tumors, such

as T-cell large granular lymphocytic leukemia and chronic lymphocytic leukemia, which have rearranged TCR/Ig loci capable of detection by molecular analysis.

Many of the leukemias, particularly the acute lymphoblastic leukemias (ALLs) and acute myeloid leukemias (AMLs), have distinct molecular abnormalities that have impacted recent classification systems [19], due to biologic considerations, therapeutic considerations, or both. Many of these genetic events are translocations creating oncogenic-driving fusion genes, similar to those observed in soft tissue tumors. For example, the t(9;22)(q34;q11.2) translocation causing the *BCR-ABL1* fusion gene is observed in a subset of B-ALL and virtually all chronic myelogenous leukemias (CMLs). Acute promyelocytic leukemia (APL) has a characteristic t(15;17)(q22;q12) translocation creating the *PML-RARA* fusion gene, which can be shut down using all-trans retinoic acid (ATRA) therapy. Detection of these translocations by FISH or RT-PCR can be used as a diagnostic tool, for theranostic applications (qualifying the patient for imatinib mesylate or ATRA, respectively) or for monitoring tumor burden during therapy.

Other molecular abnormalities in this group include aneuploidy (AML) and oncogenic-driving point mutations such as *NPM1* and *CEBPA* mutations (AML). The chronic myeloproliferative diseases have *JAK2* and *MPL* mutations. These conditions, particularly idiopathic myelofibrosis and polycythemia vera, may (rarely) present in the skin as extramedullary hematopoiesis. *FLT3* mutations also occur, but this is used more for prognostic, not diagnostic, purposes.

The immunoglobulin and TCR loci are rearranged in the tumors of lymphocytic lineage. Of these, chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL) deserves special consideration. CLL/SLL is not included in the primary cutaneous lymphomas under current classification systems. It is very common. Because of its indolence, CLL/SLL is often present in biopsies of elderly individuals as an incidental finding (biopsies of basal cell carcinomas, etc.). Sometimes, infiltrates may mimic the primary cutaneous B-cell lymphomas or

pseudolymphoma (see Table 7.2). Diagnosis can usually be made by clinical history, morphology, and immunohistochemistry, but in ambiguous cases, molecular testing may play a role.

## Recurrent Genetic Abnormalities in Lymphomas Secondarily Involving the Skin

Similar to leukemias, lymphomas can secondarily involve the skin and some carry recurrent molecular abnormalities (Table 7.5). These abnormalities are primarily translocations with fusion genes. As evident from the list, many of the translocations involve Ig/TCR loci, juxtaposing actively transcribed genes (and promoters) with oncogenes. Some of these lymphomas have primary cutaneous counterparts but do not share the same molecular events, arguing these tumors are different biologic processes. Because the lymphomas are lymphocyte derived, they will have detectable clones by immunoglobulin and TCR gene rearrangement. Hodgkin lymphoma, now considered a B-cell process (and many cutaneous cases reclassified as EBV mucocutaneous ulcers), will also have a rearranged *IGH* locus.

## 7.4 Other Applications for Molecular Testing

### 7.4.1 Prognosis

#### 7.4.1.1 Limited Role for Molecular Testing in the TNM Staging System

The prognosis of the lymphoma patient is largely determined by the extent of disease as defined by the tumor-node-metastasis (TNM) staging system [28]. Because cutaneous lymphomas behave differently from systemic lymphomas and lymphomas from other sites, the International Society for Cutaneous Lymphoma (ISCL) and the European Organization for Research and Treatment of Cancer (EORTC) proposed a revised TNM system for primary cutaneous lymphomas (Table 7.6). This proposed TNM system applies to all primary cutaneous lymphomas except MF and SS. It is separate from the TNMB

**Table 7.5** Recurrent genetic abnormalities in lymphomas secondarily involving the skin

Tumor	Ig/TCR <sup>a</sup>	Abnormality	Fusion gene	% of tumor cases with abnormality
Anaplastic large cell lymphoma	Y	t(2;5)(p23;q35)	<i>ALK-NPM</i>	84
		t(1;2)(q25;p23)	<i>ALK-TPM3</i>	13
		inv(2)(p23q35)	<i>ALK-ATIC</i>	1
		t(v;2p23)	Other <i>ALK</i> rearranged	<1
Angioimmunoblastic T-cell lymphoma	Y	—	—	75–90 (TCR) 25–30 (Ig)
Burkitt lymphoma	Y	t(8;14)(q24;q32)	<i>MYC-IGH</i>	Most
		t(8;22)(q24;q11)	<i>MYC-IGL</i>	Low
		t(2;8)(p12;q24)	<i>MYC-IGK</i>	Low
Diffuse large B-cell lymphoma, NOS	Y	t(v;3q27)	<i>BCL6</i> rearranged	30
		t(14;18)(q32;q21)	<i>IGH-BCL2</i>	30
Follicular lymphoma	Y	t(14;18)(q32;q21)	<i>IGH-BCL2</i>	80–90
Hodgkin lymphoma <sup>b</sup>	Y	—	—	High (Ig)
Mantle cell lymphoma	Y	t(11;14)(q13;q32)	<i>CCND1-IGH</i>	>95
Marginal zone (nodal) and MALT lymphoma	Y	t(11;18)(q21;q21)	<i>API2-MALT1</i>	50 (pulmonary/gastric)
		t(14;18)(q32;q21)	<i>IGH-BCL2</i>	20 (head/neck)
		t(3;14)(p14.1;q32)	<i>IGH-FOXP1</i>	10 (thyroid/skin)

<sup>a</sup>Ig or TCR is rearranged and clonal by molecular analysis

<sup>b</sup>Many cutaneous Hodgkin lymphomas are now considered EBV mucocutaneous ulcers. These tumor cells have rearranged Ig, but the overall paucity of tumor cells usually requires sample manipulation for a positive result

**Table 7.6** The tumor-node-metastasis (TNM) staging of primary cutaneous lymphoma

ISCL/EORTC proposal on TNM classification of cutaneous lymphoma other than MF/SS [28]	
T	
T1	Solitary skin involvement
T1a	A solitary lesion <5-cm diameter
T1b	A solitary >5-cm diameter
T2	Regional skin involvement: multiple lesions limited to 1 body region or two continuous body regions
T2a	All-disease-encompassing in a <15-cm-diameter circular area
T2b	All-disease-encompassing in a >15- and <30-cm-diameter circular area
T2c	All-disease-encompassing in a >30-cm-diameter circular area
T3	Generalized skin involvement
T3a	Multiple lesions involving two noncontiguous body regions
T3b	Multiple lesions involving ≥3 body regions
N	
N0	No clinical or pathologic lymph node involvement
N1	Involvement of one peripheral lymph node region that drains an area of current or prior skin involvement
N2	Involvement of two or more peripheral lymph node regions or involvement of any lymph node region that does not drain an area of current or prior skin involvement
N3	Involvement of central lymph nodes
M	
M0	No evidence of extracutaneous non-lymph node disease
M1	Extracutaneous non-lymph node disease present

classification of MF and SS since the definitions and descriptions of skin lesions cannot be interchanged (see Table 6.3). Notably absent in the

non-MF/SS lymphoma proposed TNM staging are molecular determinations of blood and bone marrow involvement. This is partially due to the

definition of primary cutaneous non-MF/SS lymphoma, which states the tumor is confined to the skin at diagnosis. Therefore, all of these patients at diagnosis are N0 and M0. The N1, N2, N3, and M1 categories are only included to aid in classifying tumor progression and/or relapse.

Although molecular analysis is not included in the current proposed staging criteria, this may change as larger studies identify new independent prognostic parameters. Smaller studies have suggested molecular detection of a clone in lymph nodes, blood, and/or bone marrow, identical to the skin clone, portends a worse prognosis [7].

#### 7.4.1.2 Other Prognostic Tests and Applications

Identifying biomarkers of lymphomas that will predict tumor behavior is an active area of research. Candidates constantly surface by using powerful molecular tools such as aCGH, gene expression profiles, and high-throughput sequencing. Some prognostic markers have been identified and are currently used to evaluate tumors from leukemia/lymphoma patients. Examples indicating an aggressive tumor biology and/or worse prognosis include (but are not limited to) the following: (1) translocations involving Ig loci and *BCL2*, *MYC* translocations, and/or *BCL6* translocations in so-called “double-hit” and “triple-hit” large B-cell lymphoma and Burkitt lymphoma (evaluated by FISH); (2) del(17p), del(11q), and trisomy 12 and/or lack of del(13q) or normal karyotype in CLL (evaluated by FISH or aCGH); (3) normal (un-mutated) *IGHV* and/or use of *VH3-21* in CLL (evaluated by mutational analysis); and (4) *FLT3* internal tandem duplications and point mutations in AML (evaluated by mutational analysis). All of these assays are available commercially. For primary cutaneous lymphomas, the identification of biomarkers and development of corresponding molecular detection assays are still in the investigative stage.

#### 7.4.2 Therapy

A comprehensive discussion on the treatment for non-MF/SS hematopoietic malignancies is beyond the scope of this text. Regarding molecular diagno-

tic applications for guiding therapy (theranostic applications) in hematopoietic tumors, several exist but this list contains only tumors that secondarily involve the skin. Examples include the previously mentioned t(9;22)(q34;q11.2) translocation in CML and the t(15;17)(q22;q12) translocation in APL qualifying patients for tyrosine kinase inhibitors and ATRA, respectively. *FLT3* inhibitors are in clinical trials. Immunotherapies are becoming a more common option for leukemia/lymphoma patients and require (nonmolecular) immunohistochemical proof of tumor expression before initiation. These include but are not limited to antibodies to CD20 (rituximab, veltuzumab), IL-2R/CD25 (denileukin ditox), CD4 (zanolimumab), CD30 (brentuximab vedotin), CD52 (alemtuzumab), and CD74 (milatuzumab). There are no molecular theranostic applications for primary cutaneous lymphoma currently being used. A current list of ongoing clinical trials for cutaneous leukemia and lymphoma can be found at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) [29].

### 7.5 Practical Considerations for Ordering, Performing, and Interpreting Molecular Tests

By far, the most common currently employed molecular assay for the evaluation of cutaneous hematopoietic tumors on clinical samples is the PCR-based gene rearrangement assay. In rare circumstances, there may be a need for FISH- or PCR-based mutational analysis to aid in diagnosis or predict prognosis and/or optimize therapy, specifically in tumors secondarily involving the skin.

#### 7.5.1 Gene Rearrangement Assays

There are many issues to consider when ordering, performing, and/or interpreting a gene rearrangement assay. Among them are the following:

- Limits of detection and the biologic meaning of a clone
- Types of assays—Southern blot versus PCR-based assays
- Pre-analytical variables such as sample preparation, targeted locus, and primer design
- Detection method

- Interpretation of data
- Incorporation of molecular test into diagnosis in the context of the individual patient

The philosophy of molecular gene rearrangement analysis along with practical issues regarding test limitations, selection, and design is discussed in detail in Chap. 6 in the context of T-cell TCR gene rearrangement in MF/SS. In general, the same principles apply for T-cell (TCR) and B-cell (Ig) analysis: There are variations among rearrangement assays, each assay has its limitations, and assay interpretation must be performed in the context of the individual patient. The following discussion highlights issues specific to B-cell gene rearrangement assays as well as T-cell gene rearrangement assays for non-MF/SS tumors.

### 7.5.1.1 Variations in Gene Rearrangement Assays

#### Targeted Locus

All gene rearrangement assays are predicated on the events of somatic recombination of B-cell receptor (Ig) and T-cell receptor (TCR) genes during normal lymphocyte development (refer to Figs. 6.5 and 7.2). These assays amplify DNA by using primers to conserved sequences in the V, D, and/or J regions that flank the recombined DNA (or probe the recombined DNA region in the case of Southern blot).

In the context of immunoglobulin formation, there are three loci—*IGH* (Ig heavy chain), *IGK* (Ig kappa light chain), and *IGL* (Ig lambda light chain)—that are potentially rearranged in the neoplastic B cell and are candidates for assay design. To produce a functional immunoglobulin, the B cell must have at least one allele successfully rearrange at the *IGH* locus and at least one allele successfully rearrange at the *IGK* or *IGL* locus. It is logical that the *IGH* locus is the most common target as it is the first of the loci to rearrange and rearrangement is required for the survival and propagation of B-cell neoplasms. *IGK* and *IGL* loci are also often assayed, either in parallel or as a reflex to a negative *IGH* assay, to increase the overall sensitivity.

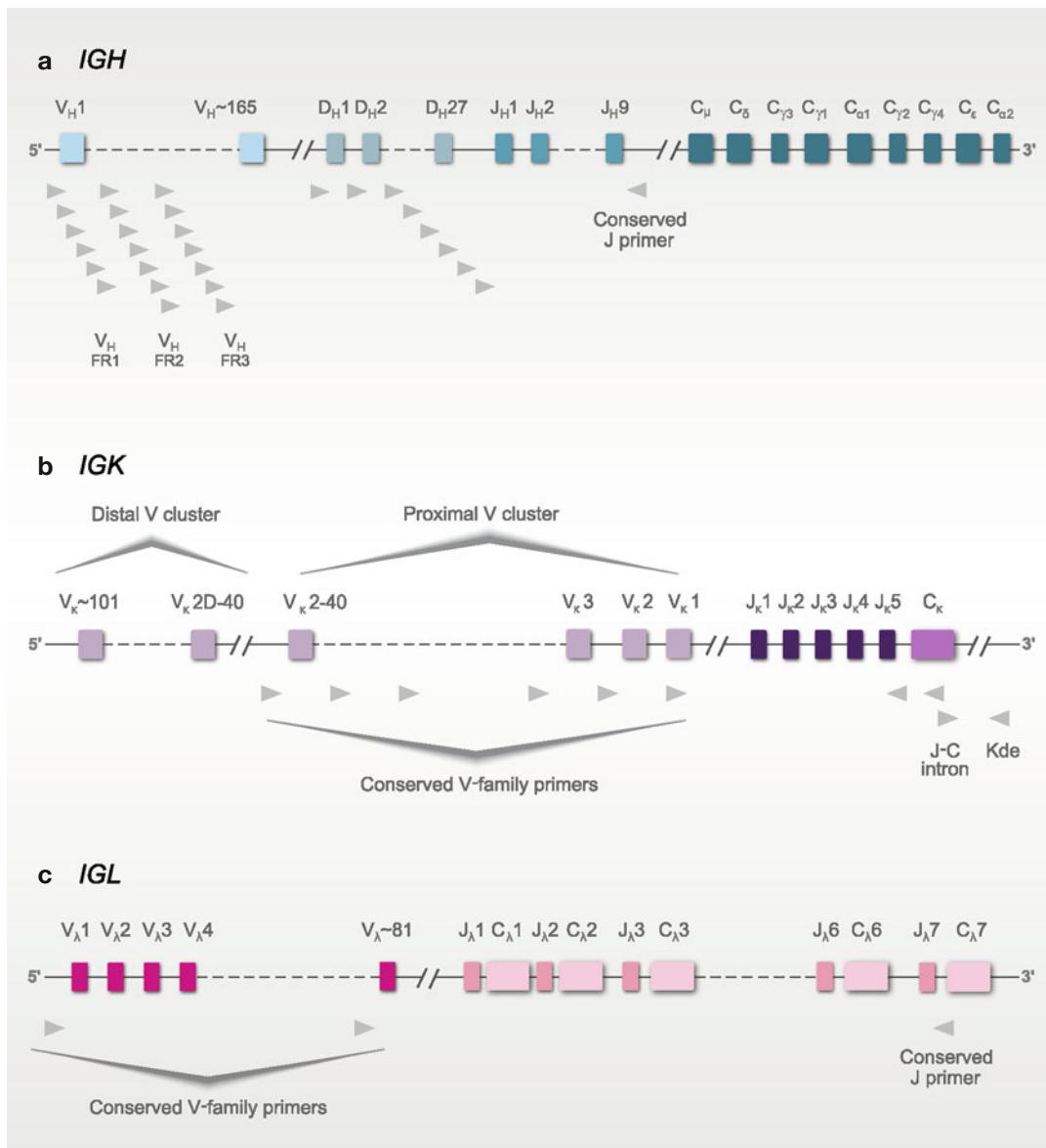
For T-cell neoplasms, the loci that are potentially rearranged and therefore targets for assay design are *TRA* ( $\alpha$ ), *TRB* ( $\beta$ ), *TRG* ( $\gamma$ ), and *TRD*

( $\delta$ ). For the reasons discussed in Chap. 6, *TRB* ( $\beta$ ) and *TRG* ( $\gamma$ ) are the most common targets for clonality assays. *TRD* ( $\delta$ ) can be used as a target in select scenarios, such as with  $\gamma/\delta$  and primitive (lymphoblastic) T-cell tumors. *TRD* ( $\delta$ ) is not a good choice as a target for other T-cell tumors since it is spliced/removed during the rearrangement of *TRA* ( $\alpha$ ).

#### PCR Primer Selection

Selection of primers for a rearrangement assay is a balancing act. On the one hand, enough primers must be included in the assay to detect as many possible V-D-J recombination events (one primer may be used for more than one gene segment if there is sequence homology). If the clone recombined its DNA using a gene segment unrecognized by the primer cocktail, the result will be a false negative. On the other hand, if too many primers are included in the assay, it may not be possible to achieve reaction conditions for all primers to optimally amplify the interval DNA, also resulting in a false-negative result. One strategy to tackle this problem is to use multiple reaction mixes/tubes. Of course, this is a balancing act as well since the more reaction mixes employed relieves the strain of complexity but adds to time, labor, and cost.

The Ig loci are particularly challenging for primer design due to their relatively large number of V-D-J gene segments and lack of complete sequence homology flanking those gene segments (Fig. 7.6). For the *IGH* locus, there are three V framework regions (FR1, FR2, FR3), each requiring multiple primers to “cover” all possible rearrangements. For example, in the commercially available EuroClonality/BIOMED-2 assay (InVivoScribe Technologies, San Diego, CA), five different reaction tubes are used (tubes A–E) to reduce the complexity of any single reaction tube [30]. In addition to a  $J_H$  consensus primer required in each tube, there are 6  $V_H$ -FR1 primers (tube A), 7  $V_H$ -FR2 primers (tube B), 7  $V_H$ -FR3 primers (tube C), 6  $D_H$ 1-6 primers (tube D), and 1  $D_H$ 7 primer (tube E), for a total of 28 different primers. *IGK* and *IGL* are less complex with more sequence homology, requiring 10 total primers in two reaction tubes for *IGK* and 3 total primers in a single reaction tube for *IGL*.



**Fig. 7.6** Primer design strategy at the *IGH*, *IGK*, and *IGL* loci. For *IGH*, the  $V_H$  gene segments are arranged into three groups—framework regions 1, 2, and 3 (FR1, FR2, FR3). Multiple primers are required to cover most-to-all possible V-D-J rearrangements (a). The EuroClonality/BIOMED-2 protocol uses 5 reaction tubes and a total of 28 primers. For the *IGK* locus, most assays require up to six  $V_{\kappa}$  primers and two  $J_{\kappa}$  consensus primers to “cover” the possible V-J rearrangements (b). The distal region is an inverted duplication.

In addition to the above primer set (which would correspond to tube A), the EuroClonality/BIOMED-2 protocol uses the same V primers along with a distal Kde primer and a primer to the  $J_{\kappa}$ -C $_{\kappa}$  intron for their tube B reaction, for a total of ten primers. For the *IGL* locus, most-to-all V-J rearrangements can be covered by two  $V_{\lambda}$  primers and one  $J_{\lambda}$  primer (c). For up-to-date maps, gene designations, and sequence information of these loci, refer to the international ImMunoGeneTics database at [www.imgt.org](http://www.imgt.org) [3].

### 7.5.1.2 Selection of a PCR-Based Clonality Assay

There are many different permutations of PCR-based assays currently used by laboratories to determine clonality. These assays vary by any or

all the variables described above as well as ease of use, cost, and interpretation.

Deserving special mention is the EuroClonality/BIOMED-2 assays. These assays stand out among the others due to the extensive

multinational and multi-institutional effort put forth to help standardize the field [4]. Standardization allows for better data comparison between laboratories and studies as well as following individual patients over time. The EuroClonality consortium has optimized these highly sensitive and specific PCR-based assays for B-cell and T-cell neoplasms. Moreover, they have put together guidelines on performance and interpretation of results, and the assays are available commercially through InVivoScribe Technologies, Inc. (San Diego, CA) [31]. Commercially available kits are available for both B-cell tumors (*IGH*, *IGK*, *IGH+IGK*, and *IGL* assays) and T-cell tumors (*TRG* ( $\gamma$ ), *TRB* ( $\beta$ ), *TRG* ( $\gamma$ ) + *TRB* ( $\beta$ ), and *TRD* ( $\delta$ ) assays). For laboratories interested in performing clonality assays, the commercially available kits may be a good place to start. The following should be noted, however:

- (1) These assays have been optimized on fresh or cryopreserved material and non-cutaneous leukemia/lymphoma samples (formalin-fixed and paraffin-embedded material, by far the most common material for cutaneous lymphoma, has also been tested, as have cutaneous lymphomas, but not to the extent of non-cutaneous fresh or cryopreserved material [32], and therefore, the assays' performance parameters may not be quite as good as advertised for the dermatology patient).
- (2) With commercially available kits, there is a trade-off between the ease of using cookbook-like protocols and the elevated costs.
- (3) Even though these assays are commercially available, there are no FDA-approved clonality tests.
- (4) InVivoScribe Technologies has intellectual property surrounding clonality testing [33].
- (5) These assays may be replaced by newer methods, such as high-throughput (next-gen) sequencing, within a few years.

According to a recent College of American Pathologists (CAP) survey, there remains a pretty wide spectrum of Ig gene rearrangement assays being offered by laboratories (see Chap. 6 for a similar comparison of TCR assays) [34]. *IGH* is the most commonly used target (twice as common as *IGK*, and when *IGK* is used, it is almost always in conjunction with *IGH*). Of 132 laboratories responding to this recent survey using *IGH* as the target in their PCR assay, 61 (46.2 %) used InVivoScribe's EuroClonality/BIOMED-2 proto-

col, 26 (19.7 %) used InVivoScribe's alternate *IGH* assay, 37 (28.0 %) used their own laboratory developed assay, and 8 (6.1 %) used a different method. Southern blot was still used by 9 (6.8 %) laboratories. Results between *IGH* and *IGK* were comparable, both with about a 90 % positivity rate (114/131 and 52/56). Interestingly, only 57/136 of laboratories reported performing testing in duplicate, which should be standard practice.

It is clear that despite efforts toward standardization, there remain many permutations of clonality assays being offered by laboratories. This may be due to a variety of reasons but most often comes down to experience and comfort with a particular test, the performance characteristics of the test in the particular laboratory's hands, and/or cost. Because different assays (and different approaches to pre-analytical variables) are used in different laboratories and post-analytical interpretation criteria may slightly vary, results from the same patient and even the same biopsy may not correlate.

#### <sup>1</sup>CPT coding

81261 (*IGH*), 81264 (*IGK*), 81340 (*TRB*  $\beta$ ), 81342 (*TRG*  $\gamma$ ), and 81402 (*TRD*  $\delta$ ) for PCR-based assays; 81262 (*IGH*) and 81341 (*TRB*  $\beta$ ) for direct probe assays (e.g., Southern blot); 81479 (unlisted procedure code) for all others, including *IGL* analysis.

### 7.5.1.3 Interpretation of the PCR-Based B-Cell Ig Gene Rearrangement Assay

Interpretation of the gene rearrangement assay has three tiers: interpretation of data from each reaction (tier 1), an overall interpretation of the molecular assay encompassing all reactions (tier 2), and incorporation of the molecular result into the context of clinical, histologic, and immunohistochemical data (tier 3). Issues surrounding

<sup>1</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [35]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

interpretation of the Ig gene rearrangement assays mirror those of the TCR assays, and the reader is directed to Chap. 6 for an in-depth discussion. A few important points on interpretation are reemphasized, and other issues specific to Ig gene rearrangement assays are discussed.

### Reaction Data (Tiers 1 and 2)

Most *IGH* gene rearrangement assays use a PCR multiplex format and require multiple reaction tubes to detect most-to-all potential rearrangements. Each individual reaction must be interpreted as clonal, polyclonal, or something in between (“peak noted,” “suspicious,” or similar verbiage) (tier 1). Once these are evaluated, a final interpretation encompassing the multiple reactions is rendered as clonal, polyclonal, or something in between (e.g., “indeterminate”) (tier 2). Important points about Ig assay interpretation are the following:

- Failed reactions occasionally occur and are mostly due to poor quality DNA or variations in sample preparation (fixation, inhibitors, etc.).
- Reactions with larger amplicons (*IGH* FR1, *IGH* FR2, *IGK* Kde, and *TRB*) are more susceptible than reactions with smaller amplicons (*IGH* FR3, *IGK* V-J, and *TRG*) to failure secondary to DNA degradation.
- With polyclonal populations, the amplicons will vary in size, in a vague Gaussian distribution, due to the numerous and random V-J and V-D-J rearrangements represented in the mix (Fig. 7.7a). Interestingly, likely due to the predominantly in-frame recombination events (e.g., in *IGH* as opposed to out-of-frame in *TRG*), spikes within the Gaussian distribution are observed every three base pairs, giving the tracings a slightly different appearance.
- Clonal populations have an overrepresented *IGH* (and/or *IGK* or *IGL*) rearrangement, and when present, the PCR amplification of this clone dominates within the mix (see Fig. 7.7b). In these cases, there is little or no background polyclonal tracing. Neoplastic populations may have rearrangement of one allele, causing a single peak, or both alleles, resulting in two peaks. True biclonality resulting in two or more peaks may occur in up to 5 % of cases

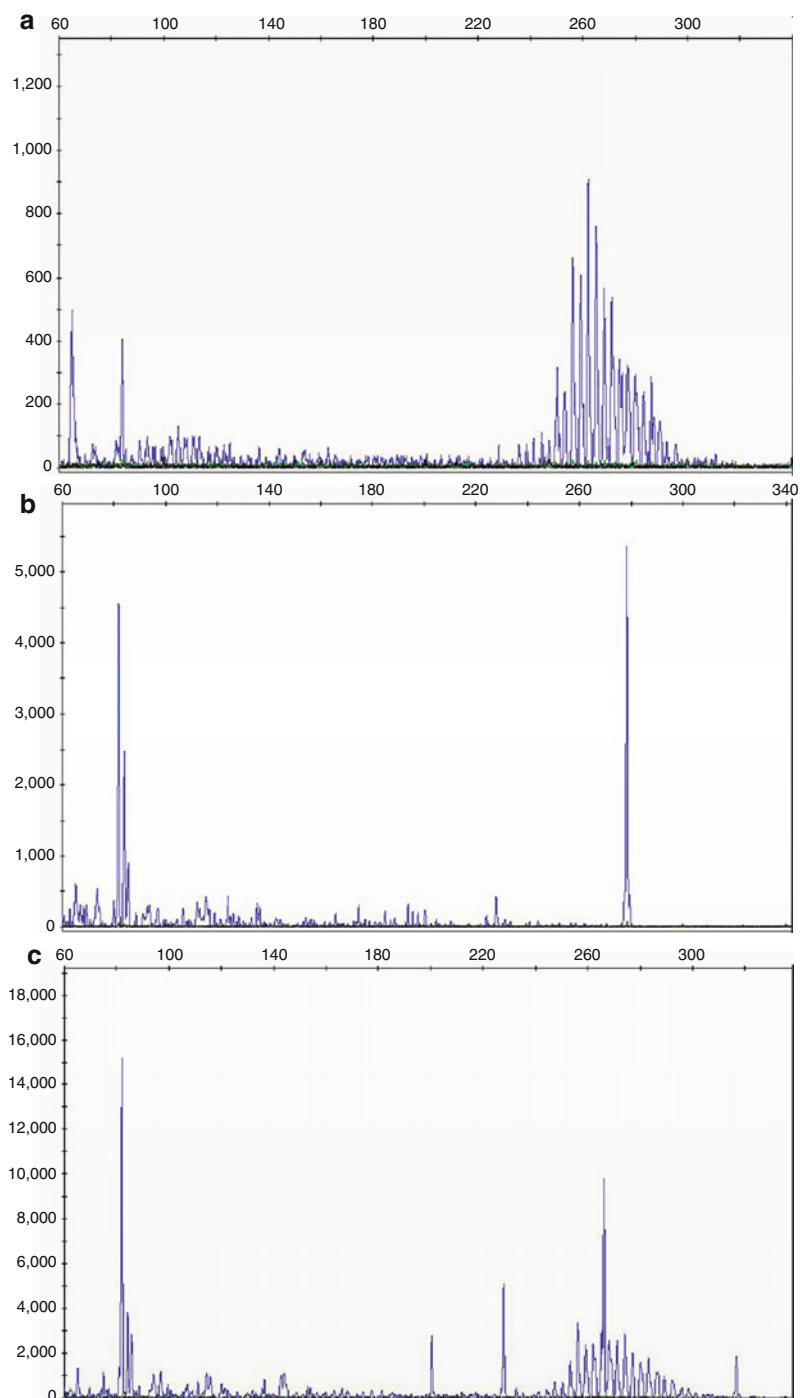
with B-cell infiltrates and can be an interpretive challenge [36].

- Some tracings fall in between typical tracings for clonal and polyclonal and can be interpretive challenges (see Fig. 7.7c).
- Patients with a known signature clone are more easily monitored for minimal residual disease or recurrence by following a specific rearrangement amplicon size (Fig. 7.8). Criteria for clonality may be loosened in this scenario.
- There is no standard definition for what constitutes a clone. There are some published guidelines on interpretation [37, 38], but these are not specific to skin FFPE samples and may not be completely transferable.
- Interpretation of the molecular data can be initially performed without other patient data, not unlike interpreting histology without clinical information, but using all available clinical and pathologic data prior to rendering a final molecular report is considered good practice.
- Different laboratories use different pre-analytical sample preparations, different assays, and different interpretive criteria. Therefore, results from the same patients and even the same samples from the same patients may vary between laboratories.

### Incorporation of Molecular Data in the Context of the Individual Patient (Tier 3)

It should be clear from the discussion that molecular analysis of rearranged Ig or TCR loci should never be a stand-alone test. There are technical and biologic reasons for false-positive and false-negative results (Table 7.7), requiring incorporation of all available data—clinical, histologic, immunophenotypic, and molecular—to reach an accurate diagnosis. The power of the molecular test can be enhanced by the following: (1) limitation of testing to cases with a high pretest probability; (2) communication between clinician, pathologist, and molecular diagnostician on the differential diagnosis and relative level of concern for each possibility; (3) preparation of the sample by enriching area of interest through manual microdissection; (4) assessment of DNA

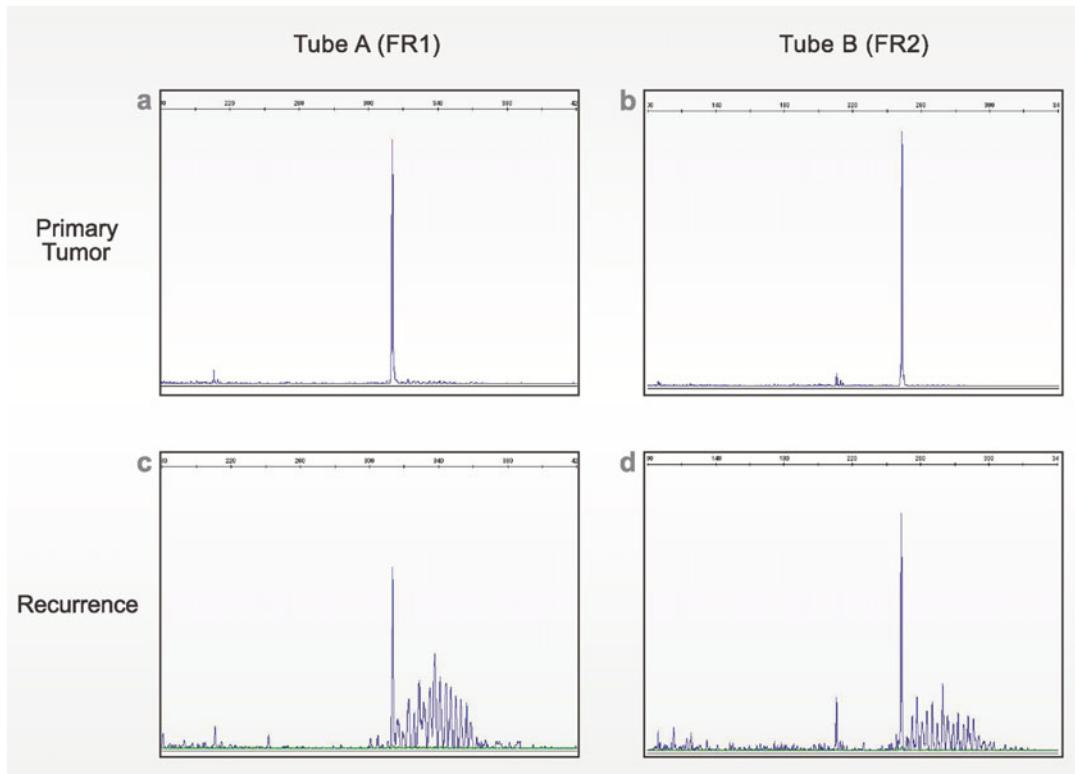
**Fig. 7.7** Interpretation of Ig gene rearrangement studies—polyclonal and clonal. In these examples, DNA is extracted from microdissected FFPE tissue from suspected primary cutaneous B-cell lymphoma patients. Fluorescently labeled primers are grouped into five separate PCR reactions (tube A–tube E, EuroClonality/BIOMED-2 protocol, InVivoScribe), and the amplicons are separated by capillary electrophoresis based on amplicon size (with GeneMapper technology). The x-axis corresponds to amplicon size and the y-axis to fluorescence intensity (quantity). Shown are three different cases (tube B only). A polyclonal population of B cells will have a Gaussian spread of amplicon sizes due to the numerous different rearrangements represented in the reaction (a). A clonal population of B cells will have an overrepresented rearrangement, indicated by a peak, or spike, with a low level or suppressed polyclonal background (b, right side of panel). Many cases fall somewhere in between. Often, a prominent spike or spikes are present with a background polyclonal component, requiring integration of all available clinical and pathologic data to render a final interpretation (c)



quality by quantifying extracted DNA and performing control amplifications; (5) performing the rearrangement assay with appropriate targets based on the sample characteristics; and (6) inter-

preting the results in the context of all available data for the individual patient.

Algorithms for clonality testing have been proposed, but few exist for the evaluation of the



**Fig. 7.8** Interpretation of Ig gene rearrangement studies—disease monitoring. Similar to Fig. 7.7, DNA is extracted from microdissected FFPE tissue from suspected primary cutaneous B-cell lymphoma patients. Fluorescently labeled primers are grouped into five separate PCR reactions (tube A–tube E, EuroClonality/BIOMED-2 protocol, InVivoScribe), and the amplicons are separated by capillary electrophoresis based on amplicon size (with GeneMapper technology). The x-axis corresponds to amplicon size and the y-axis to fluorescence

intensity (quantity). Spikes within a polyclonal background can be difficult to interpret. If the primary tumor's signature rearrangement is known (**a**, tube A, FR1; **b**, tube B, FR2), spikes with an identical size, even with a significant background polyclonal population (**c**, tube A, FR1; **d**, tube B, FR2), are likely significant. This may be helpful in the evaluation of occult tumor in lymph nodes or bone marrow, the evaluation of cutaneous recurrence (as in this case), and/or the evaluation of therapeutic efficacy

dermatology patient [31, 39, 40]. Dermatology patients are somewhat unique for several reasons. Systemic hematopoietic tumors with samples from blood, bone marrow, and lymph nodes are often tumor-rich. Fresh tissue is often available for flow cytometric and molecular analysis in these cases. Occasionally, a large juicy cutaneous tumor can be sent fresh for flow cytometric and molecular analysis, but usually only FFPE tissue is available with relatively sparse or mixed infiltrates. Due to issues with DNA integrity in FFPE samples, targets with smaller amplicons (*TRG*, *IGH* FR3, *IGK* V-J) may be preferred over targets with larger amplicons (*TRB*, *IGH* FR1 and FR2,

*IGK* Kde) [4, 31], especially in cases with limited sample. Moreover, with the growing concern of costs of medical care, assaying every rearrangement target for every sample in parallel may not be the best approach. Perhaps targets with a higher clonality hit rate should be performed first, only to reflex to other targets in cases with very high pretest probabilities [40]. For example, the *IGH* V-J (tubes A–C in the InVivoScribe assay) and *IGK* targets will detect >95 % of known B-cell neoplasms and may be sufficient in most cases [31, 41].

An example of a diagnostic algorithm for the suspected non-MF/SS cutaneous lymphoma

**Table 7.7** Potential technical and biologic sources of false-positive and false-negative gene rearrangement studies

	False positives	False negatives
Technical	Carryover contamination	Contaminated or poor quality DNA, poor amplification <sup>a</sup>
	Switched specimen	Inhibitors causing inefficient or failed PCR <sup>a</sup> Specific targeted gene rearrangements undetectable due to primer selection Tumor below limit of detection Switched specimen
Biologic	Limited DNA sample—pseudoclonaity and oligoclonality	Ig/TCR loci unarranged (germline)
	Clonal expansion of normal, nonmalignant inflammatory process	Ig/TCR locus with trans-rearrangement (e.g., V <sub>γ</sub> -J <sub>β</sub> )
	Oligoclonality/pseudoclonaity in patients with immunodeficiency (elderly, immunocompromised, etc.)	Targeted gene rearrangement and/or primer sites deleted or absent during lymphocyte development
	Premalignant but stable disease	Somatic hypermutation of IGH, IGK, and/or IGL loci
	Other biologically irrelevant clones	Translocation (e.g., with IGH) prohibiting amplification
	Incorrect clinical diagnosis (follow-up too short, patient actually has lymphoma)	Antitumor response resulting in oligoclonality Tumor with secondary rearrangement, resulting in oligoclonality Biclonality Incorrect clinical diagnosis (patient really doesn't have lymphoma)

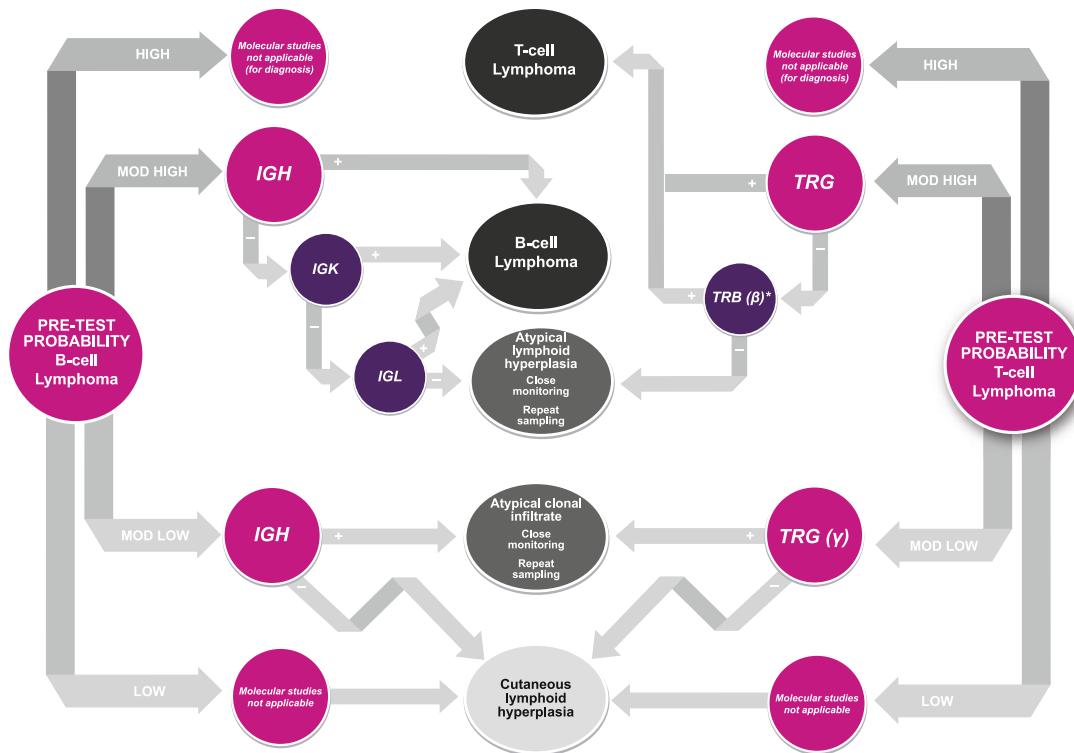
<sup>a</sup>Results in failed reaction, not false-negative result

patient is shown in Fig. 7.9 (see Fig. 6.9 for an example of an algorithm for the MF patient for reference). Following an initial assessment of the pre-test probability for lymphoma and the presumed cell of origin, molecular testing is performed. For B-cell tumors, testing would begin with *IGH* (could even start with just the *IGH* V-J reactions). If this is negative but there is a high index of suspicion for lymphoma, test *IGK*, and if that is negative, test *IGL*. Performing these tests in parallel may be appropriate when there are time constraints and less concern for cost. For suspected T-cell neoplasms, the testing algorithm should be similar for suspected MF/SS and non-MF/SS samples. An exception is the option to use *TRD* ( $\delta$ ) as a target for suspected  $\gamma/\delta$  and primitive (lymphoblastic) T-cell tumors, for the reasons described above. Again, the molecular result should not trump the other available data. If there are discordant results, there is no shame in diagnosing “atypical lymphoid hyperplasia” (high pretest probability but no clone) or “atypical clonal infiltrate” (low pretest probability with clone) and recommending close follow-up with repeat sampling, if necessary.

## 7.5.2 Other Molecular Methods for the Diagnosis and Management of the Cutaneous Leukemia/Lymphoma Patient

As mentioned throughout this chapter, other assays are used in very select settings for the diagnosis and management of the cutaneous leukemia/lymphoma patient. These are mentioned only briefly because they have virtually no current role for primary cutaneous lymphoma. Many applications exist for non-cutaneous hematopoietic tumors. With these tumors, the preferred samples are fresh bone marrow and blood. There may be rare instances when a cutaneous biopsy is the first or only available tissue for analysis, or there is a specific need for assessing cutaneous involvement, but these are not optimal samples and should be avoided if possible.

FISH and RT-PCR are commonly used to evaluate for translocations (see Tables 7.4 and 7.5). FISH may also be used to evaluate for amplifications, such as *MYC*. These assays are



**Fig. 7.9** Algorithm for incorporating molecular data. For B-cell and T-cell infiltrates, the molecular diagnostic strategy and interpretation of results will be dependent on the pretest probability for lymphoma. The algorithm uses tests in sequence, not in parallel, for cost considerations.

Assays can be performed in parallel if there are time constraints. For cases of ambiguous lineage, up front *IGH* and *TRG* testing are recommended. \* *TRD* testing may also be performed in suspected primitive (lymphoblastic) or  $\gamma/\delta$  T-cell tumors

widely available for fresh (blood, bone marrow) tissue. They can be adapted for paraffin-embedded tissue (PET-FISH), but some laboratories will reject FFPE samples. FISH may be performed by break-apart or dual-fusion methods. A more detailed discussion of the methods and applications of FISH and RT-PCR can be found in Chaps. 3 and 8.

Mutational analysis for *JAK2*, *MPL*, *KIT*, *FLT3*, *NPM1*, *CEBPA*, and *IGVH* can be performed to evaluate select tumors secondarily involving the skin (see Table 7.4). These assays are widely available on fresh (blood and bone marrow) tissue but can also technically be performed on FFPE cutaneous tissue. In general, these mutation-detection assays, specifically the ones evaluating point mutations, are fairly straightforward. Most *CEBPA* and *IGVH* assays are slightly more complex as larger stretches of

DNA must be sequenced. Many different methods can be used, including but not limited to Sanger sequencing, pyrosequencing, PCR with mass spectrometry (Sequenom MassARRAY), allele-specific or TaqMan real-time PCR, single nucleotide extension assays, and high-throughput (next-gen) sequencing. These methods are covered in detail in Chaps. 3 and 5.

#### <sup>2</sup>CPT coding

FISH probe codes include 88365 or 88367 (quantitative with computer assistance) or 88368 (quantitative, manual) (all are per probe)

<sup>2</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [35]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

or the combination of 88274 (per probe) and 88291, depending on how the test is performed and interpreted; for RT-PCR, 81206–81208 (*BCR-ABL1*), 81315–81316 (*PML-RARA*), 81401 for most other leukemia translocations; 81245 (*FLT3*); 81270 (*JAK2*); 81310 (*NPM1*); 81403 (*CEBPA*); 81402–81403 (*MPL*); 81479 (unlisted procedure code) for all others.

## 7.6 Summary

The diagnosis of cutaneous lymphoma remains one of the more challenging areas in dermatology and dermatopathology. The clinical and histopathologic features of lymphoma often mimic reactive inflammatory conditions, requiring integration of these data with immunohistochemistry and molecular analysis to achieve an accurate diagnosis. Molecular testing for clonality in lymphocytic infiltrates evaluates somatic recombination events at the TCR and Ig loci and has been used in various forms for several decades. The evolution of the gene rearrangement assay is proceeding as follows: Southern blot → PCR-based assay → high-throughput (next-gen) sequencing. Southern blot techniques have largely been replaced by PCR-based methods, and high-throughput sequencing will likely dominate by the next decade. Regardless of method, clonality testing will never be usable as a stand-alone test for malignancy since clonal expansion is a normal biologic response by lymphocytes to antigen. Until new methods are developed to assess clonality in terms of oncogenic transformation, this multipronged diagnostic approach will be required.

As with other tumors, FISH, aCGH, microRNA expression profile analysis, and high-throughput sequencing are emerging as powerful tools for the evaluation of cutaneous lymphomas. These are not routinely used on clinical samples for cutaneous lymphoma but are rapidly being added to molecular diagnostic menus. In the near future, expect more and more laboratories to offer these tests for diagnosis as well as for identifying biomarkers for predicting tumor behavior and predicting responses to newly developed targeted therapies.

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# Tumors of the Soft Tissue: Using Molecular Tools to Aid in the Diagnosis of Soft Tissue Tumors and the Management of the Sarcoma Patient

## Contents

8.1	<b>Introduction</b> .....	200
8.2	<b>Diagnosis</b> .....	200
8.2.1	Genetic Aberrations in Soft Tissue Pathology .....	202
8.2.2	Examples of Soft Tissue Tumors with Characteristic Molecular Defects.....	204
8.3	<b>Prognosis</b> .....	215
8.3.1	Translocations and Fusion Genes .....	216
8.3.2	Gene Amplification.....	216
8.4	<b>Therapy</b> .....	216
8.4.1	Fusion-Gene Targeted Therapy.....	217
8.4.2	Mutation-Specific and Other Signaling Pathway-Directed Therapies.....	217
8.5	<b>Molecular Tests Performed on Soft Tissue Tumors and Practical Considerations</b> .....	218
8.5.1	FISH.....	218
8.5.2	RT-PCR .....	220
8.5.3	Others.....	222
8.6	<b>Summary</b> .....	224
	<b>References</b> .....	224

### Key Points

- Soft tissue tumors are rare, and current molecular diagnostic applications for these tumors are limited.
- Soft tissue tumors are uncommonly encountered in dermatopathology, but generate significant diagnostic angst. Conventional diagnostic techniques can yield variable and ambiguous results.
- Many sarcomas have specific molecular events. Most of these are recurrent translocations creating fusion genes, which act as oncogenic drivers. Other examples include amplifications, complex karyotypes, and specific gene mutations.
- Our understanding of the role of translocations and fusion genes in oncogenesis is incomplete. Some tumors (e.g., Ewing sarcoma) may be associated with multiple different translocations, and conversely, identical translocations may occur in completely different tumors (e.g., t(12;22) in angiomyxoma and clear cell sarcoma).
- Translocations are the most utilized molecular events in soft tissue pathology for diagnostic purposes. These are used in conjunction with histomorphologic, immunohistochemical, and clinical data, not in isolation.

- FISH and RT-PCR are the most commonly employed molecular tests for detecting translocations. Each assay has its advantages and disadvantages for targeting specific molecular events.
- Prognostic and theranostic applications for molecular testing are currently limited to few isolated scenarios for sarcomas. This may change with the utilization of new powerful molecular assays like high-throughput sequencing.

## 8.1 Introduction

Soft tissue malignancies are rare. In 2013, sarcomas afflicted an estimated 11,410 Americans and resulted in 4,390 deaths, accounting for <1 % of all cancer types [1, 2]. Soft tissue tumors in the skin are even rarer, and the use of molecular testing for soft tissue tumors in the skin is rarer still. With that said, molecular diagnostics has a small but definite (and growing) role in the diagnosis and management of the sarcoma patient, and members of the patient's care team, specifically the dermatologist and dermatopathologist, need to be fully aware of these applications for appropriate utilization and interpretation.

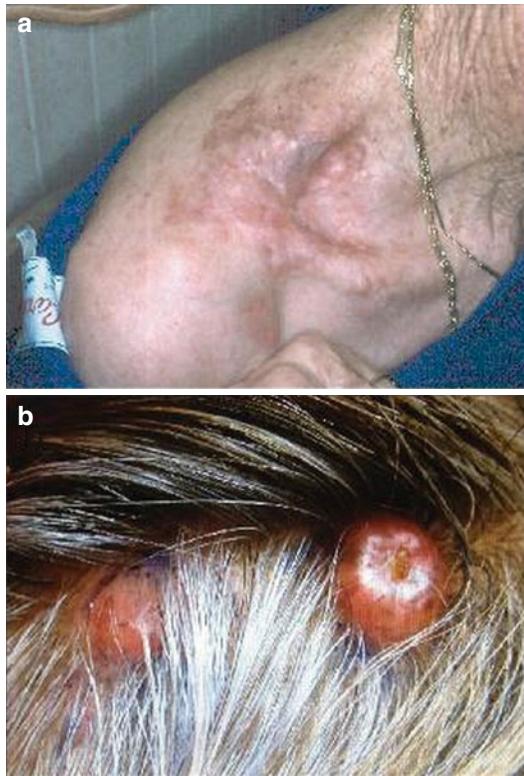
Over the past several decades, the uncovering of soft tissue tumor-specific molecular events, including chromosomal translocations with fusion genes and proto-oncogene amplifications, has advanced the understanding of the biology of these enigmatic tumors and, in some cases, has aided in their reclassification. The role of molecular events in oncogenesis remains incomplete, however. Quizzically, some tumors (e.g., Ewing sarcoma) are associated with multiple translocations, while, conversely, the identical translocation (e.g., t(12;22)) may be observed in completely different tumors (e.g., angiomyxoid fibrous histiocytoma and clear cell sarcoma). Although the roles of these molecular events are clearly complex and have not been completely elucidated, their mere presence can be exploited for diagnostic purposes with the

development of tumor-specific molecular assays. When used appropriately, as an adjunct to conventional diagnostic methods, the molecular assay can be the dermatopathologist's most powerful tool for achieving diagnostic accuracy in select settings.

As in other disciplines, the discovery of oncogenic drivers, such as fusion genes and proto-oncogene mutations, should lead to prognostic and theranostic applications. Few of these currently exist for soft tissue tumors, but the list is growing, and soon this class of tumors will join others, embarking toward the world of personalized medicine. This chapter covers select soft tissue tumors with characteristic molecular events, with an emphasis on *diagnostic* applications. Tumors that are more commonly encountered in the skin are highlighted, but it should be noted that virtually any soft tissue malignancy can expand into the skin or metastasize to the skin. Other molecular applications for soft tissue tumors, and specifics on methods and testing strategies, including practical points, are also discussed.

## 8.2 Diagnosis

As mentioned above, soft tissue tumors are rare. Because of this low incidence, most dermatopathologists are not accustomed to diagnosing these tumors. The clinical features of the soft tissue tumor offer little insight into its diagnosis (Fig. 8.1), except perhaps with assessing benign versus malignant in extreme examples. Under the microscope, tumors are diagnostically challenging because there can be stifling variability in the histomorphologic features. The tumor does not always recapitulate its normal tissue of origin, and immunohistochemistry can be vague and non-specific. There is significant and often confusing "cross-pollination" of immunomarkers between soft tissue entities (Table 8.1). In one interobserver variability study, there was a 27 % discordance rate in the diagnosis of sarcomas between primary institutions and referral centers [3]. Of course, it may not be practical to refer all soft tissue tumors for consultation. Yet alternatively, diagnostic



**Fig. 8.1** Clinical features of soft tissue tumors. The clinical features of soft tissue tumors typically provide little insight to diagnosis. Large, disfiguring, or ulcerated masses may point to malignancy, while small, freely mobile tumors are more consistent with a benign process. Other features such as firmness, color, vascularity, and anatomic site may suggest a diagnosis, but biopsy is often required. Provided are two select examples of sarcomas. This case of dermatofibrosarcoma protuberans presented as a single, ill-defined indurated plaque on the right shoulder/supraclavicular region on an elderly female (a). A separate case of angiosarcoma presented as multiple coalescing plaques and tumors on the scalp of a 77-year-old female (b). Diagnoses were confirmed histologically (Images courtesy of Dr. Alan Menter, Dallas, TX, and Dr. Jennifer Dharamsi, Dallas, TX)

accuracy is of paramount importance. Some tumors, such as nodular fasciitis and low-grade fibromyxoid sarcoma, can be histologic mimics but have divergent biologic behaviors. In more extreme examples, an inaccurate diagnosis can be the difference between local conservative excision and amputation. Ancillary studies, like molecular tests, when used appropriately, improve diagnostic accuracy, which can make a huge impact on the quality of life of some of these patients.

**Table 8.1** Select immunohistochemical markers used in soft tissue tumor diagnosis

Diagnosis	Select positive immunomarkers
Broad classifications of tumors	
Carcinoma	AE1/3, OSCAR, other keratins
Melanoma	S100, MART-1, gp100
Lymphoma/leukemia	CD45, CD30, CD3, CD20, CD56, CD68, CD34
Germ cell—sex cord stromal tumors	SALL4, CD30, D2-40, AFP, HCG, MART-1 (A103), inhibin
Neuroendocrine tumors	Chromogranin, synaptophysin
Sarcoma	Vimentin <sup>a</sup>
Vascular	CD31, CD34, FLI-1, ERG, D2-40
Muscle	Smooth muscle actin, desmin, smooth muscle myosin
Nerve	S100, SOX10
Fibrohistiocytic	Procollagen I (var), CD68 (var), CD34 (var), factor 13a (var)
Fat	S100, MDM2
Cartilage/bone	S100, osteocalcin, MDM2
Select covered tumors	
Angiomatoid fibrous histiocytoma	CD68, SMA, desmin, EMA, CD99
Angiosarcoma (post-radiation)	CD31, CD34, FLI-1, ERG, MYC
Clear cell sarcoma	S100, SOX10, MART-1, gp100, Mel-CAM, tyrosinase, MiTF
Dermatofibrosarcoma protuberans	CD34
Epithelioid hemangioendothelioma	CD31, CD34, FLI-1, ERG
Ewing sarcoma	CD99, FLI-1, NKX2.2
Low-grade fibromyxoid sarcoma	MUC4, EMA (var), SMA (var)
Myoepithelioma	S100, keratins
Nodular fasciitis	CD34 (var), smooth muscle actin (var), calponin (var)

Abbreviation: Var variable expression

<sup>a</sup>Vimentin has been shown to stain tumors of virtually all lineages. It has limited practical value

The discovery of recurrent molecular events in certain soft tissue tumors has firmly established molecular testing into the diagnostic armamen-

tarium. These tests have not replaced conventional methods, but in some cases, they have become an integral part of the diagnostic algorithm. These same molecular events have also reclassified and redefined some tumors. Low-grade fibromyxoid sarcoma and hyalinizing tumor with pseudorosettes, previously considered two separate entities, are now lumped together as histologic variants of the same entity due to the presence of a shared translocation. Myxoinflammatory fibroblastic sarcoma and hemosiderotic fibrolipomatous tumor share a similar narrative. Conversely, clear cell sarcoma is now definitively teased away from melanoma (and has now dropped its previous designation of melanoma of soft parts) following the discovery of its defining translocation. And other examples exist.

## 8.2.1 Genetic Aberrations in Soft Tissue Pathology

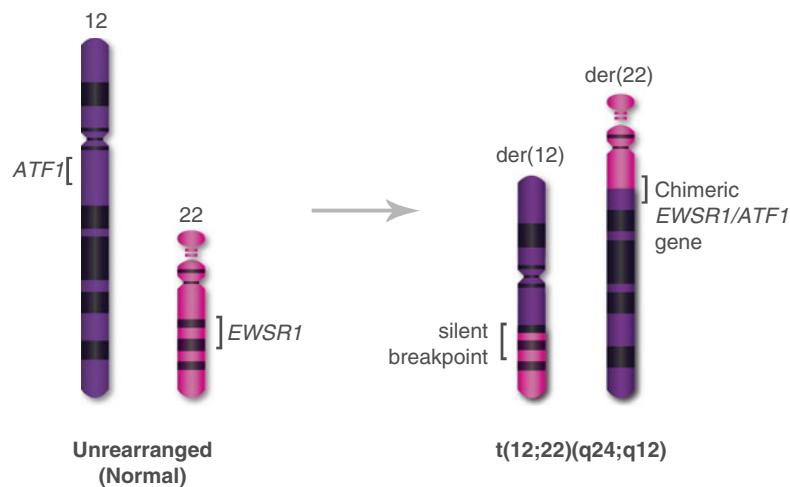
### 8.2.1.1 Translocations and Other Simple Chromosomal Abnormalities

For reasons that are poorly understood, soft tissue tumors have a disproportionate number of recurrent translocations and corresponding fusion genes compared to others in the world of solid tumors. Why these are comparatively so rare in other solid tumors, like melanoma and cutaneous

epithelial tumors, is peculiar, to say the least. Some translocations, like t(12;22) resulting in the *EWSR1-ATF1* fusion gene in clear cell sarcoma, have been known for decades and are very well characterized, while others, such as the t(17;22) translocation resulting in the *MYH9-USP6* fusion in nodular fascitis, have only recently been discovered [4, 5].

In a translocation, breakpoints occur on two separate chromosomes, swapping genetic material (Fig. 8.2). In many tumor cases, the breakpoints occur near or within genes. The chromosomes rearrange such that two genes or portions of genes become unified, forming a so-called “fusion,” or “chimeric,” gene. If the chromosomal material on the other sides of the breakpoints unifies and maintains a centromere for replication, this is called a reciprocal, or balanced, translocation. The fusion region on this partner chromosome is usually silent. If there is a net loss of genetic material, due to dissociation with a centromere or other cause, the translocation is unbalanced. There can be variations or derivatives of this rearrangement process, including the creation of small inversions and ring chromosomes, to name a few. The supernumerary ring formation of chromosome 12 found in some cases of dermatofibrosarcoma protuberans is an example [6].

Because of the consistent presence of translocations in certain tumors, it is presumed that these contribute to tumorigenesis—that is, the



**Fig. 8.2** Schematic of the *EWSR1-ATF1* translocation in clear cell sarcoma. The translocation of chromosomes 12 and 22, t(12;22), leads to the juxtaposition of *ATF1* and *EWSR1*, creating the *EWSR1-ATF1* fusion gene. This is a balanced translocation as the partner chromosome maintains its centromere and there is no net loss of genetic material. The fusion on the partner chromosome is silent.

product of the translocation is an oncogenic driver. Admittedly, these mechanisms are not fully understood. The classic paradigm is juxtaposition of a proto-oncogene with an active promoter. The promoter region of a frequently transcribed gene drives transcription of a transcription factor, or less commonly a signaling molecule, leading to cellular proliferation and survival. The generation of an oncogenic-driving fusion gene occurs in approximately 20 % of sarcomas [7]. Examples of fusion genes leading to dysregulated transcription factors include *EWSR1-FLI1* in Ewing sarcoma and *WWTR1-CAMTA1* in epithelioid hemangioendothelioma. *COLIA1-PDGFB* in dermatofibrosarcoma protuberans is an example of a fusion gene leading to increased activity of a signaling molecule or growth factor (PDGFB) under the control of an actively transcribed gene (the *COLIA1* collagen gene).

In addition to translocations, there are other simple chromosomal alterations found in soft tissue tumors. The amplification of proto-oncogenes can lead to tumor formation and, in some cases, may be a prognostic marker. In radiation-induced angiosarcoma, *MYC* is amplified, potentially aiding in diagnosis and possibly predicting a more aggressive course [8]. In osteosarcoma, atypical lipomatous tumors, and dedifferentiated liposarcomas, there are ring chromosomes (12q14-15) with amplification of *CDK4*, *MDM2*, *CPM*, and other genes with a possible tumorigenic role [9, 10].

### 8.2.1.2 Gene Mutations

Alternative to forming oncogenic drivers via macrogenetic rearrangements and the creation of fusion genes, tumors may also develop following microgenetic events, such as small point mutations, insertions, and deletions. Activating mutations of oncogenes and deactivating mutations of tumor suppressor genes are well known in the oncology literature [11]. Particularly of interest are the activating mutations since there is the potential for designer small molecules to inhibit their action and treat the tumor. There are currently only few examples of these in soft tissue pathology, however, and even fewer exam-

ples in those tumors encountered in the skin. The best characterized soft tissue example of this is the presence of *KIT* and *PDGFRA* mutations in gastrointestinal stromal tumors (GISTs). These mutations occur in up to 85 % of GISTs. Targeted therapy against the activated tyrosine kinase, such as imatinib mesylate, has proven efficacious in these patients [12, 13].

Other examples of characteristic activating mutations in soft tissue tumors include *CTNNB1* mutations in fibromatosis (leading to increased  $\beta$ -catenin expression) and *GNAS* mutations in myxomas and fibrous dysplasia. Examples of inactivating mutations, deletions, and/or epigenetic silencing include alterations of *INI1* in epithelioid sarcomas and malignant rhabdoid tumors and of *APC* in fibromatosis (reviewed in Demicco et al. [14]). There are also isolated examples of *RAS* (leiomyosarcomas, pleomorphic undifferentiated sarcomas), *BRAF* (GISTs), and *PIK3CA* (myxoid/round cell liposarcoma) activating mutations in sarcomas [15, 16]. While this latter group of mutations is of potential interest for therapeutic purposes, these mutations do not occur with sufficient consistency in any particular soft tissue tumor to suggest a diagnostic role.

### 8.2.1.3 Complex Karyotypes

Chromosomal aberrancies are not limited to translocations and amplifications in soft tissue tumors. As with many malignancies, as the tumor progresses, genomic instability increases and results in complex karyotypes. This is observed with virtually all high-grade tumors including pleomorphic undifferentiated sarcoma, leiomyosarcoma, myxofibrosarcoma, and pleomorphic rhabdomyosarcoma, among others. Extreme examples of complex karyotypes may be observed following chromothripsis, a catastrophic molecular event leading to massive exchange of genetic material between chromosomes. Complex karyotypes may confirm the malignant nature of a tumor but offer little else. A complex karyotype does not distinguish between malignant neoplasms and is not routinely performed (see Molecular Testing section below for more details on cytogenetics).

## 8.2.2 Examples of Soft Tissue Tumors with Characteristic Molecular Defects

### 8.2.2.1 Select Tumors Most Common in Superficial Skin

This group of tumors is highlighted as they may occur in the superficial skin and therefore may be encountered and first diagnosed by the dermatologist and dermatopathologist. These are, for the

most part, rare tumors. Diagnosis often relies upon standard histomorphologic and immunophenotypic criteria, although there can be significant overlap between tumors. Table 8.1 lists commonly employed immunohistochemical markers used for the diagnosis of soft tissue tumors. Characteristic molecular findings may be used to confirm the suspected diagnosis of a rare tumor or help in cases with unusual histologic/immunophenotypic features and/or small biopsies (Table 8.2).

**Table 8.2** Select tumors with recurrent molecular events most commonly encountered in superficial skin

Tumor	Chromosomal abnormality	Gene defect(s)	Testing		
			Method	Sens (%) <sup>a</sup>	Avail <sup>b</sup>
Angiomatoid fibrous histiocytoma	t(2;22)(q33;q12)	<i>EWSR1-CREB1</i>	FISH	70	↑ ( <i>EWSR1</i> )
	t(12;22)(q13;q12)	<i>EWSR1-ATF1</i>		Low	↑ ( <i>FUS</i> )
	t(12;16)(q13;p11)	<i>FUS-ATF1</i>		Rare	
Angiosarcoma (post-radiation)	8q24	<i>MYC amp</i>	FISH	55–100	↑ ( <i>MYC</i> )
Clear cell sarcoma	t(12;22)(q13;q12)	<i>EWSR1-ATF1</i>	FISH	70–80	↑ ( <i>EWSR1</i> )
	t(2;22)(q33;q12)	<i>EWSR1-CREB1</i>		5	
Dermatofibrosarcoma protuberans (DFSP) (and giant cell fibroblastoma)	t(17;22)(q21;q13)	<i>COL1A1-PDGFB</i>	FISH	80–95	↓ ( <i>PDGFB</i> )
Epithelioid hemangioendothelioma	t(1;3)(p36;q25)	<i>WWTR1-CAMTA1</i>	FISH	>95	N/A
Ewing sarcoma/primitive neuroectodermal tumor (PNET)	t(11;22)(q24;q12)	<i>EWSR1-FLI1</i>	FISH RT-PCR	85	↑ ( <i>EWSR1</i> )
	t(21;22)(q22;q12)	<i>EWSR1-ERG</i>		10	↓ (RT-PCR, <i>EWSR1-FLI1</i> first fusion only)
	t(7;22)(p21;q12)	<i>EWSR1-ETV1</i>		Low	
	t(2;22)(q35;q12)	<i>EWSR1-FEV</i>		Low	
	t(17;22)(q21;q12)	<i>EWSR1-ETV4</i>		Low	
	t(16;21)(p11;q22)	<i>FUS-ERG</i>		Low	
	t(2;16)(q35;p11)	<i>FUS-FEV</i>		Low	
Ewing-like sarcoma	t(20;22)(q13;q12)	<i>EWSR1-NFATc2</i>		Low	
	t(6;22)(p21;q12)	<i>EWSR1-POU5F1</i>		Low	
	t(4;22)(q31;q12)	<i>EWSR1-SMARCA5</i>		Low	
	inv(22) in t(1;22)	<i>EWSR1-PATZ1</i>		Low	
	t(2;22)(q31;q12)	<i>EWSR1-SP3</i>		Low	
	t(4;19)(q35;q13)	<i>CIC-DUX4</i>		Low	
Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11)	<i>FUS-CREB3L2</i>	FISH	70–90	↑ ( <i>FUS</i> )
	t(11;16)(p11;p11)	<i>FUS-CREB3L1</i>		5	
Myoepithelioma	t(6;22)(p21;q12)	<i>EWSR1-POU5F1</i>	FISH	15	↑ ( <i>EWSR1</i> ) <sup>c</sup>
	t(1;22)(q23;q12)	<i>EWSR1-PBX1</i>		15	
	t(19;22)(q13;q12)	<i>EWSR1-ZNF444</i>		Rare	
Nodular fasciitis	t(17;22)(p13;q12)	<i>MYH9-USP6</i>	FISH	90	↓ ( <i>USP6</i> )

*Abbreviations:* N/A little or no data available

<sup>a</sup>Sensitivity: references in main text

<sup>b</sup>Availability: ↓ low (few commercial laboratories); ↑ high (widely available); probe used in parentheses, fusion partner may not be available; data compiled from select websites including [www.genetests.org](http://www.genetests.org), [www.propath.com](http://www.propath.com), [www.aruplab.com](http://www.aruplab.com), [www.labcorp.com](http://www.labcorp.com), [www.mayomedicallaboratories.com](http://www.mayomedicallaboratories.com)

<sup>c</sup>Probe may not be specifically indicated on test menu for this particular tumor

### Angiomatoid Fibrous Histiocytoma

Angiomatoid fibrous histiocytoma (AFH) was originally described by Enzinger in 1979 [17]. This should not be confused with the similarly sounding aneurysmal fibrous histiocytoma, a histologic variant of a benign dermatofibroma. AFH was originally lumped with the category of malignant fibrous histiocytoma (MFH) and previously called angiomatoid malignant fibrous histiocytoma, but is now separated as a distinct entity due to its better prognosis and characteristic molecular changes. AFH has intermediate malignant potential, with approximately 1–5 % developing regional metastases and rare reported deaths [18]. Local recurrence is fairly common. This tumor affects a wide age range, but is primarily a tumor of children and young adults. AFH preferentially develops in the deep dermis and subcutis of the extremities. An interesting feature of this tumor is that, occasionally, patients have systemic findings, such as fever and weight loss and anemia, which may be due to cytokine release by the tumor.

Histologically, AFH is a nodular proliferation of plump spindled or histiocytoid cells [19]. There is usually a thick fibrous capsule, and characteristically, there is a brisk lymphocytic infiltrate with germinal center formation, mimicking a lymph node (Fig. 8.3a). Blood-filled cystic pseudovascular spaces are commonly observed. Cytologically, the tumor cells range from bland to pleomorphic. Immunohistochemical analysis reveals unusual variable expression of CD68, SMA, desmin, EMA, and CD99. Other muscle-, endothelial-, and histiocytic-specific markers are typically negative. The main differential diagnosis includes variable metastases to a lymph node and exuberant repair. The diagnosis is usually made on histologic grounds, with exclusion of other tumors by immunohistochemistry. Molecular studies may be useful when classic AFH features are not present or in other certain circumstances, such as differentiating AFH from pleomorphic undifferentiated sarcoma, rhabdomyosarcoma, or follicular dendritic cell tumors, none of which share the characteristic molecular rearrangements. Of note, AFH may rarely histologically mimic Ewing sarcoma, and because

these tumors share *EWSR1* rearrangements, molecular studies may add to the confusion.

The most common rearrangement in AFH is a t(2;22)(q33;q12) translocation resulting in the *EWSR1-CREB1* fusion gene [20]. A minority of cases have t(12;22) (*EWSR1-ATF1* fusion) or t(12;16) (*FUS-ATF1* fusion) translocations [21]. The break-apart FISH assay for *EWSR1* has a sensitivity of 76 % in AFH [22]. This would not identify cases with *FUS* rearrangements, but these appear to be rare events. Up to one-quarter of cases have no demonstrable translocation involving either *EWSR1* or *FUS*. The *EWSR1-CREB1* fusion gene is an exon 7 to exon 7 fusion (breakpoint is within exon 7 of both genes) leading to dysregulated expression of a transcription factor. The precise downstream target genes affected are not known. Interestingly, unlike with clear cell sarcoma harboring the identical translocation (see below), the fusion transcripts in AFH do not appear to activate the MiTF pathway, emphasizing the complexity of these molecular events [23].

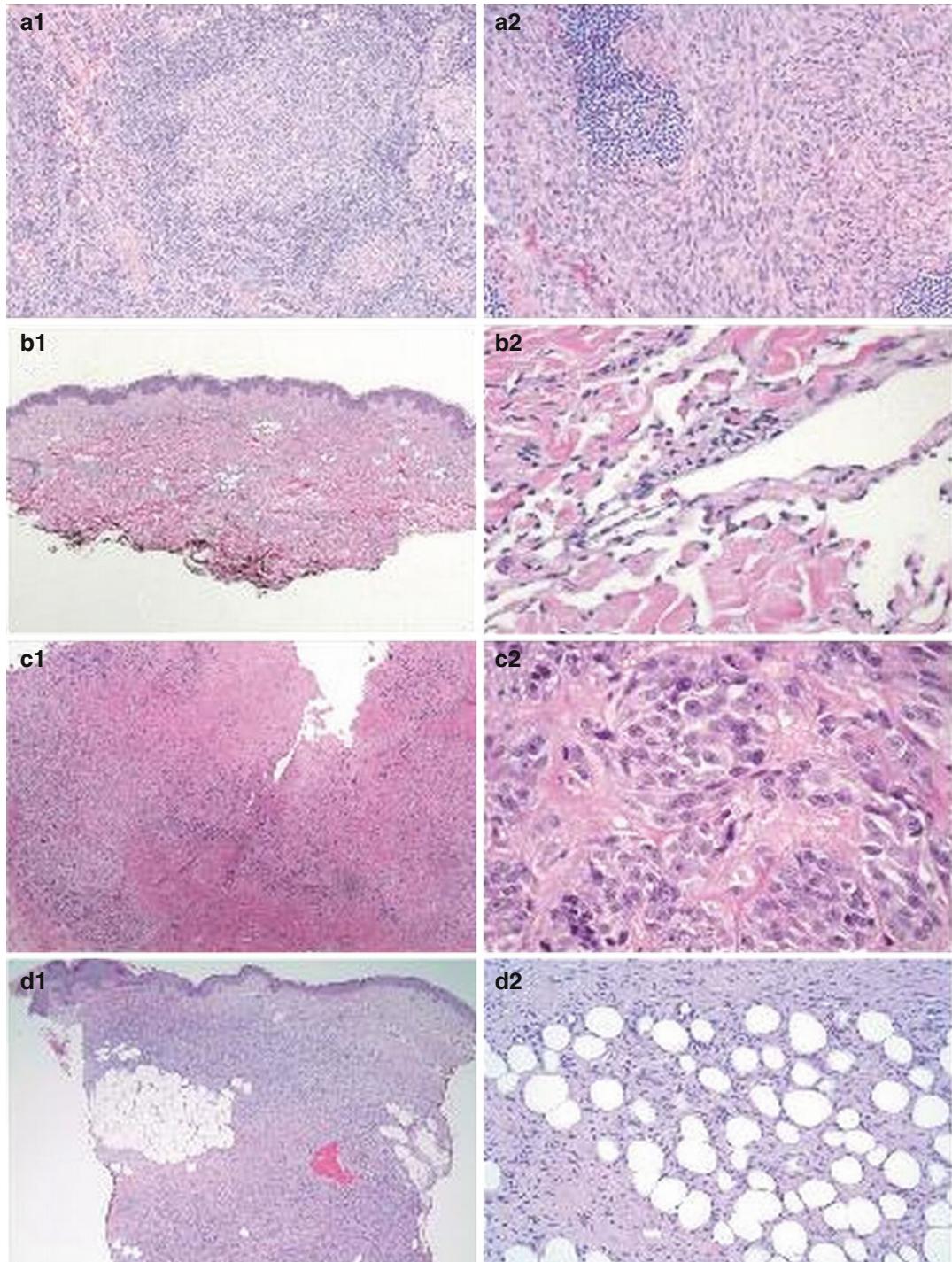
### Angiosarcoma (Post-radiation)

Post-radiation angiosarcoma is an aggressive malignant tumor with a high rate of local recurrence and up to 40 % developing metastases [24]. It is most commonly observed within 5 years following whole-breast radiation and lymphadenectomy for breast cancer, with associated lymphedema, but may occur in the skin at any anatomic site where radiation is used to treat benign or malignant conditions. The risk of this complication remains very low, estimated at around 0.1 % of treated patients [25]. Diagnosis is challenging as there is considerable clinical and histomorphologic overlap between radiation-induced angiosarcoma, radiation-associated atypical vascular lesions (AVLs), and radiation dermatitis. The distinction between these entities is paramount as the accurate diagnosis predicts benign versus malignant behavior.

Radiation-induced angiosarcoma has histologic features similar to other angiosarcomas [26]. Cytologically atypical plump endothelial cells line irregular vascular channels (Fig. 8.3b). There is a histologic spectrum between

angiosarcoma and AVL, with AVL tumors usually smaller with less cytologic atypia and only a single-cell layer lining the vascular channels.

This histologic spectrum is highlighted by the observation that both entities may coexist within the same lesion, and their distinction can be very



challenging with small or superficial biopsies [27]. Immunohistochemistry has a limited role as the vascular nature of the lesion is often not disputed.

High-level amplifications of *MYC* (defined as >8 signals per diploid cell) have recently been described in radiation-induced angiosarcoma, but not in AVLs or in angiosarcomas with no history of radiation (sporadic angiosarcomas) [8]. High-level *MYC* amplifications occur in 80–100 % of radiation-induced angiosarcoma cases. Preliminary data suggest immunohistochemistry for the Myc proto-oncogene protein may correlate well with *MYC* amplification by FISH and may, in the future, be used as an alternative. *MYC* amplification occurs in other malignancies, but these usually are not in the histologic differential diagnosis [28, 29].

### Clear Cell Sarcoma

Clear cell sarcoma (CCS) was originally described by Enzinger in 1965 [30]. It is an aggressive tumor with features of melanocytic differentiation [31]. It has previously been referred to as melanoma of soft parts but the preferred term is CCS as distinct clinicopathologic and molecular differences between CCS and melanoma are now recognized. CCS affects young adults and usually arises in the deep soft tissue of the extremities, most often the ankle region (40 %). Involvement of the deep tendons and aponeuroses are common, and some texts continue to use the designation clear cell sarcoma of tendon and aponeurosis to reflect this, but there are reported cases arising in the superficial skin [32]. The clinical course of CCS is often protracted, with an overall poor prognosis. Local

recurrences are common and multiple, with later complications of metastases and death. Patients with metastases have a 50 % 5-year survival, which drops to 10 % at 20 years. Tumors of the superficial skin appear to have a better clinical outcome.

Histologically, the tumor is usually situated in the deep soft tissues [33]. In the more unusual cutaneous variant, the tumor involves the dermis and/or subcutis, but epidermal or junctional involvement should not be present, helping to distinguish CCS from melanoma. The tumor consists of enlarged epithelioid and spindled cells, arranged in nests and fascicles, separated by hyalinized collagen (Fig. 8.3c). While, as the name implies, clear cell morphology may be observed, it is not uniformly present. Tumor cells may also be large and eosinophilic. Pleomorphism is not typical. Multinucleated giant cells are often encountered. Pigment is present in about half of cases, but melanocytic differentiation often requires immunohistochemical analysis. S100 is uniformly positive. Other melanocytic markers, including melan-A, gp100 (HMB-45), Mel-CAM, tyrosinase, and MiTF, are usually positive. Ultrastructurally, melanosomes in various stages of development are observed, but electron microscopy is rarely required for diagnosis.

The most common molecular event in CCS is the t(12;22)(q13;q12) reciprocal translocation which results in the *EWSR1-ATF1* fusion gene, occurring in approximately 70–80 % of cases (see Fig. 8.2) [4, 34, 35]. Fusion of the 3' end of *EWSR1* at exon 8 with the 5' end of *ATF1* at exon 4 is the most common breakpoint variant (type 1, 35 % of CCS breakpoints), and exon 7 of *EWSR1* fused to exon 5 of *ATF1* is the second

**Fig. 8.3** Histology of select soft tissue tumors. Angiomatoid fibrous histiocytoma (10-year-old male, arm, **a1** and **a2**). A spindle cell tumor is interrupted by nodules of inflammatory cells and germinal center formation (**a1**, H&E, 100× original magnification). The cells are plump and spindled without overt pleomorphism (**a2**, 200×). Radiation-induced angiosarcoma (56-year-old female, breast, **b1** and **b2**). The tumor inconspicuously infiltrates the superficial dermis (**b1**, 40×). It contains poorly formed anastomosing vessels lined by atypical

endothelial cells (**b2**, 400×). Clear cell sarcoma (56-year-old female, foot, **c1** and **c2**). A spindle cell tumor fills the dermis and subcutis, with areas of necrosis (**c1**, 40×). The tumor contains atypical plump spindle cells admixed with epithelioid clear cells (**c2**, 400×). DFSP (43-year-old male, back, **d1** and **d2**). Spindle cells extend from the epidermal-dermal junction through the dermis into the superficial fat (**d1**, 40×). These bland spindle cells infiltrate the fat around individual adipocytes in a honeycomb pattern (**d2**, 200×)

most common (type 2, 20 %). There are other reported variant breakpoints within these genes, and a subset of tumors may generate fusion transcripts with more than one different breakpoint [36, 37]. A minority (<5 %) of cases of CCS has a t(2;22) translocation resulting in the *EWSR1-CREB1* fusion gene. This latter fusion appears more commonly within CCS tumors of the gastrointestinal tract [38]. To date, there is no evidence that different translocations or splice variants have any clinical significance [37]. Interestingly, unlike AFH with the identical translocation, CCS fusion transcripts target the *MITF* promoter and result in increased melanocyte-specific MiTF mRNA and protein [39]. The downstream effects of this are unknown, but since MiTF has been reported to transcriptionally activate Met, a receptor tyrosine kinase, MiTF overexpression may lead to both melanocytic differentiation and transformation [40].

The main entity in the differential diagnosis of CCS is melanoma. The superficial variant of CCS may be histomorphologically and immunophenotypically indistinguishable from primary and metastatic melanoma. In these instances, molecular studies can be quite valuable since the CCS translocations are not seen in melanoma [35]. Moreover, *BRAF* and *NRAS* mutations commonly observed in melanoma are seen only rarely in CCS [41, 42]. Of course, this latter point has been disappointing given the recent success with melanoma therapies targeting the MAP kinase pathway (see Chap. 5 for in-depth discussion), but has motivated investigators to seek out other therapeutic targets for CCS.

### Dermatofibrosarcoma Protuberans and Giant Cell Fibroblastoma

Dermatofibrosarcoma protuberans (DFSP) was first described by Darier and Ferrand in 1924 as a progressive and recurrent dermatofibroma [43]. It is a low- to intermediate-grade fibrohistiocytic malignancy. Although rare, with an incidence less than 1 per 100,000, DFSP is the most common dermal sarcoma. Giant cell fibroblastoma (GCF) was first described by Shmookler and Enzinger in 1982 [44]. It is now considered a histologic and clinical variant of DFSP, primarily

afflicting children. These tumors are typically indurated plaques, usually involving the trunk and proximal extremities. They are low-grade malignancies, with a tendency to locally recur. These tumors have ill-defined borders, and recurrence is common without wide excisions. Metastases of DFSP are rare, but have been reported following multiple local recurrences and/or with fibrosarcomatous transformation.

The histology of DFSP and GCF is fairly characteristic [45, 46]. DFSP contains tumor cells that are monomorphic and bland, arranged in a storiform pattern (Fig. 8.3d). These cells usually diffusely fill the dermis, encase adnexal structures, and extend into the subcutis. Tumor cells infiltrate the fat, creating a characteristic honeycomb pattern around individual adipocytes. Plaque-like DFSP is an uncommon superficial variant. There are also pigmented (Bednar tumor), myxoid, and myoid histologic variants. Approximately 10–15 % of cases will have a high-grade fibrosarcomatous transformation. By immunohistochemistry, the tumor cells are strongly and diffusely positive for CD34. GCF resembles DFSP and has a similar immunoprofile, but differs in the presence of characteristic multinucleated giant cells and pseudovascular spaces.

Both DFSP and GCF have rearrangements of the collagen type I  $\alpha$ -1 gene on chromosome 17 and the platelet-derived growth factor  $\beta$  gene on chromosome 22 creating the *COL1A1-PGDFB* fusion gene. This fusion may occur with the unbalanced t(17;22)(q21;q13) translocation [47]. Ring chromosomes may form. A balanced t(17;22) translocation has been described in GCF but not DFSP. DFSP and GCF are usually diagnosed with characteristic histology and immunohistochemistry. Molecular studies are usually not required for diagnosis but may be useful in unusual cases or with small/superficial biopsies. These rearrangements are fairly easily detected by FISH. There are many different breakpoints within the *COL1A1* gene, making RT-PCR a less attractive testing option. And because the different breakpoints do not appear to correlate with specific histologic changes or with prognosis [48], FISH is probably the preferred modality. FISH, either by *PDGFB*

break-apart or by dual-probe techniques, will detect 80–95 % of cases, but this test is not currently widely available [49, 50].

Because tumor cells have surface PDGF- $\beta$  receptors, the product from the fusion gene auto-stimulates the tumor, acting as an oncogenic driver [51]. This provides a rationale for targeted therapy, focusing on tyrosine kinase inhibitors that act to shut down the downstream signaling of the PDGF- $\beta$  receptor. The treatment of choice for DFSP is surgical, but targeted tyrosine kinase inhibitor therapy may have utility in persistent, inoperable, or rare metastatic tumors (see Sect. 8.4.1 section below).

### Epithelioid Hemangioendothelioma

Epithelioid hemangioendothelioma (EHE) is a vascular tumor of intermediate malignancy affecting adults. It occurs anywhere, with a propensity for the extremities. EHE may occasionally present in the superficial skin [52, 53]. These tumors are indolent, but a significant percentage (up to 30 %) metastasizes.

EHE can be a challenging histologic diagnosis. Histologically, EHE is usually deep seated and may involve a large vessel [54]. The tumor cells are epithelioid and eosinophilic, some with intracytoplasmic vascular lumens (Fig. 8.4a). The cells are arranged in chains and cords. The background stroma is myxohyaline. Immunophenotypically, the cells stain with vascular immunomarkers such as CD31, CD34, FLI-1, and ERG. This can be helpful since the vascular nature of the tumor is not always evident. Keratin staining is variable.

There is a characteristic t(1;3)(p36;q25) reciprocal translocation resulting in the *WWTR1-CAMTA1* fusion gene, present in almost all cases tested and reported to date [55–57]. The *WWTR1* breakpoint appears fairly consistently within intron 4, and at least two breakpoints (exon 8 and exon 9) have been described in the *CAMTA1* gene [57]. Calmodulin-binding transcription activator 1 (*CAMTA1*) is a transcription factor that becomes activated under the *WWTR1* promoter. The downstream targets of the fusion transcription factor protein are not well characterized but presumably contribute to tumorigenesis. This

translocation is not found in histologic mimics, increasing the value of molecular testing in difficult cases.

### Ewing Sarcoma

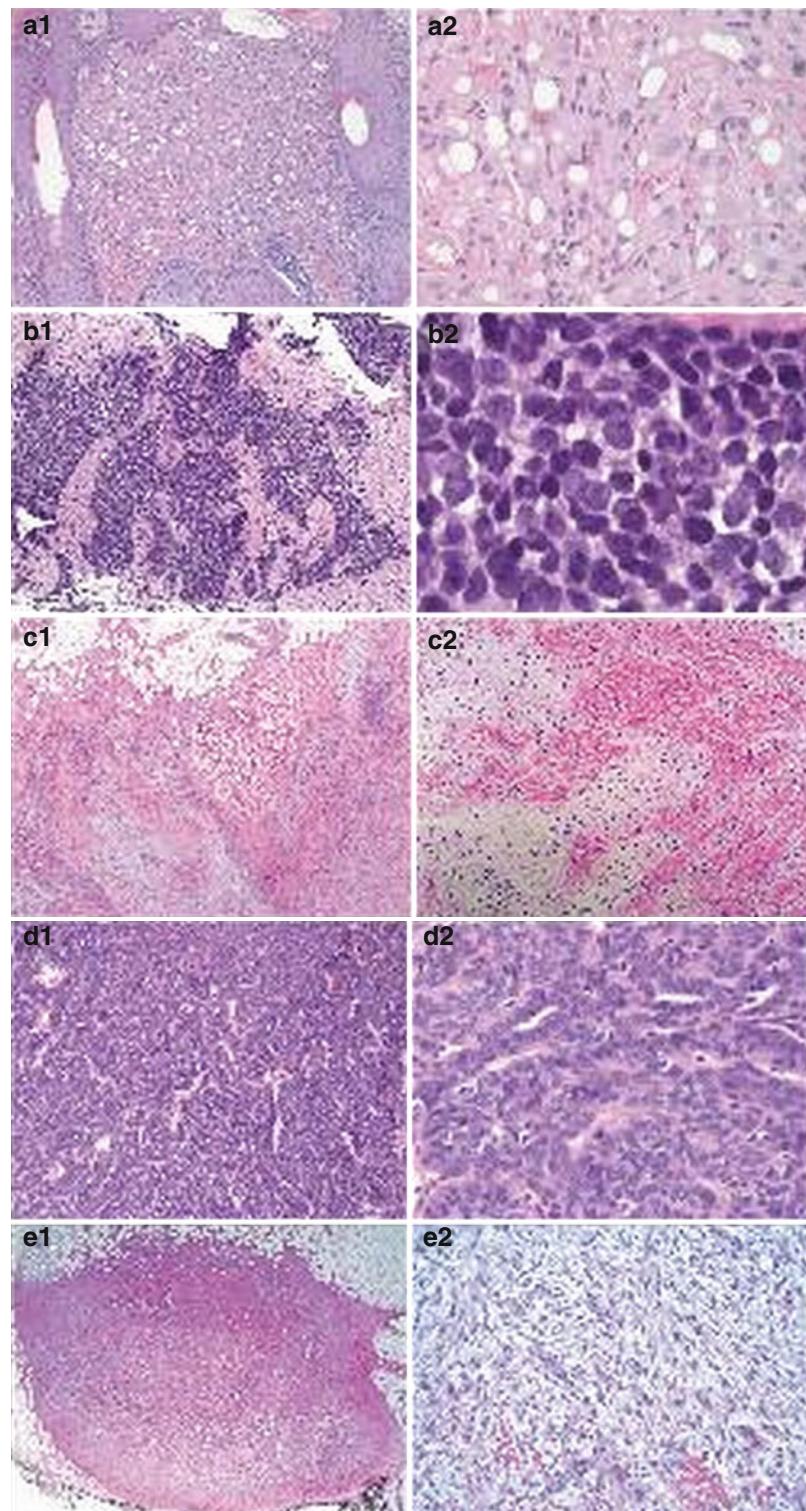
Ewing sarcoma (EWS) was first described in the radius of a 14-year-old girl by Ewing in 1921, an entity he termed “malignant endothelioma of the bone” [58]. Primitive neuroectodermal tumor (PNET) and Askin tumor were initially considered as separate entities but now are considered morphologic variants and are lumped into the extraskeletal Ewing sarcoma/primitive neuroectodermal tumor family. EWS is rare and primarily affects adolescents and young adults. Prognosis is highly dependent on the clinical stage at diagnosis—75 % survival with localized disease and 30 % for metastatic disease [59]. EWS usually originates in the deep soft tissue and bone, but tumors at other sites (extraskeletal) account for up to 20 % of cases. Rarely, the tumor is primary to the dermis or subcutis. These skin tumors are usually less than a few centimeters in size, and this more likely corresponds to localized disease and a favorable prognosis [60, 61].

EWS is a small round blue cell tumor [62]. The tumor consists of sheets of monomorphic cells with a high nuclear to cytoplasmic ratio (Fig. 8.4b). The nuclei are blast-like with finely dispersed chromatin and distinct nucleoli. Pseudorosette formation may be observed. Immunohistochemical analysis can be helpful in the distinction from other small round blue cell tumors, such as lymphoblastic lymphoma, small cell carcinoma, Merkel cell carcinoma, and rhabdomyosarcoma, to name a few. EWS is positive for CD99 and FLI-1 immunohistochemistry. It is usually negative for CD45, TdT, CD10, cytokeratins, S100, and desmin. Ambiguous cases may benefit from molecular analysis.

The t(11;22)(q24;q12) reciprocal translocation resulting in the *EWSR1-FLI1* fusion gene is the most common molecular event in EWS and is observed in >90 % of cases [63]. There are two main breakpoint variants with this translocation. Type 1 fuses exon 7 of *EWSR1* to exon 6 of *FLI1* (60 %) and type 2 fuses the same exon 7 of *EWSR1* with exon 5 of *FLI1* (25 %).

**Fig. 8.4** Histology of select soft tissue tumors.

Epithelioid hemangioendothelioma (67-year-old male, scalp, **a1** and **a2**). Plump eosinophilic cells and scattered clear spaces fill the papillary dermis (**a1**, H&E, 100 $\times$  original magnification). The cells are fairly large and eosinophilic, some with a small lumen containing red blood cells (**a2**, 400 $\times$ ). Ewing sarcoma (23-year-old female, back, **b1** and **b2**). Small round blue cells infiltrate the interstitium (**b1**, 100 $\times$ ). These cells have high nuclear to cytoplasmic ratios and fine, powdery chromatin (**b2**, 400 $\times$ ). Low-grade fibromyxoid sarcoma (47-year-old female, palm, **c1** and **c2**). An infiltrative spindle cell tumor has areas with variable degrees of cellularity (**c1**, 40 $\times$ ). The spindle cells are cytologically bland and the stroma has a prominent myxoid component (**c2**, 100 $\times$ ). Myoepithelioma (76-year-old female, neck, **d1** and **d2**). This example is very cellular, forming cords and sheets (**d1**, 100 $\times$ ). The tumor cells are monomorphic with few scattered mitoses (**d2**, 400 $\times$ ). Nodular fasciitis (32-year-old female, arm, **e1** and **e2**). This tumor has superficially infiltrative borders but is fairly well demarcated from the surrounding fat (**e1**, 20 $\times$ ). The cells are loosely arranged with splayed cytoplasmic processes and a background of inflammatory cells and red blood cells (**e2**, 200 $\times$ )



Approximately 5 % of cases have the t(12;21) (q22;q12) translocation resulting in the *EWSR1-ERG* fusion gene [64]. *EWSR1* may partner with other similar genes in the ETS (E-twenty-six) family (*ETV1*, *FEV*, *ETV4*, etc.), but this occurs with low frequency. Rare cases exhibit fusions using *FUS* instead of *EWSR1* [65]. As an aside, *EWSR1* may partner with non-ETS genes (e.g., *PATZ1*, *SMARCA5*, *NFATc2*). When this occurs, these tumors are currently designated Ewing-like sarcoma, which may represent a separate distinct family of tumors, but the classification of EWS and Ewing-like sarcoma is currently in flux. There is no clear evidence that variations in translocation partners and breakpoints impact prognosis [66]. The exact biologic functions of the fusion proteins in EWS and Ewing-like sarcoma are not well understood. They appear to function as transcriptional regulators, but it is not clear what genes or transcripts are specifically targeted [67, 68]. In some cases of EWS, in addition to *EWSR1* rearrangements, more complex karyotypes may exist, including cases with trisomy 8, gains of 12 and/or 1q, loss of 1p, and t(1;16) secondary translocations. The significance of these secondary molecular events remains unknown.

### Low-Grade Fibromyxoid Sarcoma

Low-grade fibromyxoid sarcoma (LGFMS) is the preferred designation for this tumor, originally described in two young women by Evans in 1987 [69]. Hyalinizing spindle cell tumor with giant rosettes is now considered a histologic variant [70]. Sclerosing epithelioid fibrosarcoma may also be a variant as at least some of these tumors harbor the same molecular changes as LGFMS. LGFMS usually affects adolescents and young adults. The tumor usually presents in the trunk and proximal extremities, specifically the thigh. Deep-seated tumors arising in skeletal muscle or subcutis are more common, but up to 20 % of tumors arise in the subcutis and dermis, especially in children [71]. These tumors are slow-growing, low-grade malignancies. Recurrences are uncommon within the first 5 years, but after 15 years or more, recurrences, metastases, and even death are not unusual [72]. The superficial cutaneous tumors have a better prognosis.

Histologically, LGFMS is deceptively bland [73]. It has an admixture of cellular myxoid nodules and hypocellular fibrocollagenous zones (Fig. 8.4c). The cells are arranged in whorling nodules and short fascicles, with some cases having prominent so-called collagen rosettes (thus the name “hyalinizing spindle cell tumor with giant rosettes”). These rosettes have epithelioid fibroblasts arranged around a collagen nidus. The cells are bland with a low proliferative index. Immunohistochemistry is neither diagnostic nor characteristic, often with MUC4 expression and variable expression of EMA and SMA.  $\beta$ -catenin is negative. The differential diagnosis includes a variety of benign and malignant soft tissue neoplasms including the following: fibromatosis, nodular fasciitis, DFSP, myxoma, neurofibroma, perineurioma, malignant peripheral nerve sheath tumor, and myxofibrosarcoma, among other fibrous and myxoid tumors. In these scenarios, if histologic and immunophenotypic features are nondiagnostic, molecular studies may be extremely helpful.

LGFMS is characterized by rearrangements of *FUS* on chromosome 16. The t(7;16)(q33;p11) translocation is very common in LGFMS, observed in approximately 70 % of cases [74, 75]. This translocation results in the *FUS-CREB3L2* fusion gene, and this fusion can be detected by RT-PCR in up to 90 % of cases [76]. The breakpoints are fairly consistent, occurring within either exon 6 or 7 of *FUS* and within exon 5 of *CREB3L2*. Less commonly, a t(11;16)(p11;p11) translocation resulting in the *FUS-CREB3L1* fusion gene is observed [74]. These fusion genes result in increased and unregulated expression of *CREB3L2* and *CREB3L1*, respectively, which are transcription factors [77]. Rearrangements of *FUS* are not specific to LGFMS, as they are also observed in EWS, myxoid liposarcoma and other tumors. For diagnosis, these rearrangements must be evaluated in the context of the histologic and immunophenotypic data.

### Myoepithelioma

This tumor was first described by Laskowski in 1955 under the name chordoma periphericum, or parachordoma [78, 79]. “Myoepithelioma” and “myoepithelioma of soft tissue” are now the pre-

ferred terms. Myoepitheliomas are considered related to the mixed tumor, with the latter designation used for tumors with true ductular formation. The term “myoepithelial carcinoma” can be used for malignant forms. Myoepitheliomas are rare, most commonly involving the deep soft tissues of the extremities and head/neck region of young adults. Dermal and subcutaneous tumors occur, but are even less common [80, 81]. Most of these tumors are benign (with occasional local recurrence), but a subset has foci of malignant cytology that increases the risk for metastasis [81].

Grossly, myoepitheliomas are glistening, well-circumscribed masses, typically with microscopic foci extending into the adjacent soft tissue [82]. The tumor has variable degrees of cellularity, with tumor cells arranged in small chains and cords and as single cells, often with a chondromyxoid stroma (Fig. 8.4d). The cells are usually cytologically bland but can range from epithelioid to spindled in morphology and may be vacuolated. A minority of cases have foci with cellular pleomorphism and increased mitotic activity. Immunohistochemistry yields a characteristic profile with both S100 and cytokeratin expression.

Molecular studies may be useful for diagnosis in a subset of myoepitheliomas, for example, when the above characteristic immunohistochemical profile is not observed. Similar to CCS, AFH, and others, myoepitheliomas have translocations involving *EWSR1* and less commonly *FUS*, creating fusion genes. These genetic events have been observed in both the soft tissue and cutaneous forms of myoepitheliomas [83]. The translocation partners of myoepitheliomas, however, appear different than with other *EWSR1*-rearranged tumors. *POU5F1* is the most common, resulting in the *EWSR1-POU5F1* fusion (t(6;22) translocation) [84]. The related benign and malignant mixed tumors (of both the skin and salivary glands) have a different molecular signature, often with *PLAG1*, not *EWSR1*, rearrangements [85, 86]. Extraskeletal myxoid chondrosarcoma is in the histologic differential diagnosis and carries *EWSR1* translocations [87]. Probing for the translocation partner would be useful in this scenario. Other rare entities in the

histologic differential diagnosis include extra-axial chordoma and ossifying fibromyxoid tumor. These entities do not have *EWSR1* rearrangements, with the latter carrying *PHF1* rearrangements, suggesting another useful scenario for molecular testing [88].

### Nodular Fasciitis

Nodular fasciitis was originally described by Kornwaler in 1955 as a subcutaneous pseudosarcomatous fibromatosis [89]. These tumors rapidly arise, over the course of weeks, then spontaneously resolve. Because of this characteristic clinical course, and a commonly reported history of previous trauma, nodular fasciitis was originally thought to be a reactive/reparative process. Newly discovered molecular changes have confirmed its true neoplastic (clonal) nature. Nodular fasciitis can occur at any age and any site, but is more typical in young adults, involving the trunk, upper extremities, and head and neck. Cranial fasciitis is a rare clinical variant, occurring in infants. Recurrences are rare, and if they occur, the diagnosis of nodular fasciitis should be questioned.

Nodular fasciitis is a proliferation of cells with myofibroblastic and fibroblastic differentiation [90]. The tumor is usually deep seated, in the vicinity of fascia, but dermal and intravascular variants may occur. The tumor has superficially infiltrative borders, but is overall a well-defined mass (Fig. 8.4e). The cells are spindled with a characteristic “tissue-culture” appearance, with thin splayed cytoplasmic processes. There may be storiform areas and fascicles. The cells may have enlarged nuclei with prominent reactive nucleoli and increased mitotic activity, but they are usually fairly regular and uniform. There is usually a background of inflammatory cells and extravasated red blood cells. There is usually a suggestion of myofibroblastic differentiation by SMA expression, but immunohistochemistry is usually not helpful, except in the extent of excluding other tumors with more characteristic immunoprofiles.

Chromosomal aberrancies have been previously described in isolated cases of nodular fasciitis [91, 92], but more recently, the t(17;22)

(p13;q12) translocation leading to the *MYH9-USP6* fusion gene has been reported to occur in most cases [5]. Over 90 % of lesions have rearrangements of *USP6*. The *MYH9-USP6* fusion gene upregulates the expression of *USP6*, which is a deubiquitinating protease. As ubiquitination is important in the degradation of cellular proteins, it would follow that overexpression of a deubiquitinating protein may lead to accumulation of activating proteins and cellular proliferation. However, the precise role the fusion gene plays in tumorigenesis or “transient tumorigenesis” is under investigation. Molecular analysis is not commonly employed for nodular fasciitis, but since this lesion is commonly misdiagnosed as malignant, perhaps it should be used more frequently. *USP6* rearrangements are also observed in aneurysmal bone cysts.

### 8.2.2.2 Other Select Soft Tissue Examples

The above tumors are featured as they are more commonly encountered in the skin, are recurrent diagnostic challenges for the dermatopathologist,

or both. There are many other benign and malignant soft tissue tumors that have characteristic chromosomal aberrations. Included in this group are well-characterized tumors that may metastasize or expand into the skin, such as synovial sarcoma and liposarcoma, extremely rare entities, and other miscellaneous tumors. For the most part, these are soft tissue tumors and the reader is directed to reviews and texts on soft tissue pathology for a more detailed description [93]. The tumors, their specific molecular events, and information on testing are included in tabular form (Table 8.3) [94–106].

#### 8.2.2.3 Rearrangements of *EWSR1*

As is evident from the above discussion and tables, *EWSR1* rearrangements are not limited to EWS, as the name would imply, but are commonly rearranged in soft tissue tumors, an observation that is not completely understood. Table 8.4 is a compilation of these tumors.

*EWSR1* is the EWS breakpoint region 1 gene found on chromosome 22 (22q12). It encodes for the RNA-binding protein EWS (EWSR1

**Table 8.3** Other select soft tissue tumors with molecular defects

Tumor	Chromosomal abnormality	Gene defect(s)	Testing		
			Method	Sens (%) <sup>a</sup>	Avail <sup>b</sup>
Alveolar rhabdomyosarcoma	t(2;13)(q36;q14)	<i>PAX3-FOXO1</i>	FISH	75–80	↑ ( <i>FOXO1</i> )
	t(1;13)(p36;q14)	<i>PAX7-FOXO1</i>	RT-PCR	10–15	↓ (RT-PCR, 1st fusions only)
	t(X;2)(q13;q36)	<i>PAX3-FOXO4</i>		Low	
Alveolar soft part sarcoma	t(X;17)(p11;q25) der(17)t(X;17)(p11;q25)	<i>ASPS-CTFNE-TFE3</i>	FISH	N/A	↓ ( <i>TFE3</i> )
Chondroid lipoma	t(11;16)(q13;p13)	<i>C11orf95-MKL2</i>	FISH	N/A	N/A
Congenital infantile fibrosarcoma	t(12;15)(p13;q25)	<i>ETV6-NTRK3</i>	FISH	75	↓ ( <i>ETV6</i> ) <sup>c</sup>
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	<i>EWSR1-WT1</i>	FISH RT-PCR	>95	↑ ( <i>EWSR1</i> ) ↓ (RT-PCR)
Endometrial stromal sarcoma	t(7;17)(p15;q11)	<i>JAZF1-SUZ12</i>	FISH	50	↓ ( <i>JAZF1</i> )
	t(6;7)(p21;p15)	<i>JAZF1-PHF1</i>		10	
	t(6;10)(p21;p11)	<i>EPC1-PHF1</i>		Low	
	t(10;17)(q23;p13)	<i>YWHAE-FAM22</i>		Low	
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	<i>EWSR1-NR4A3</i>	FISH	80	↑ ( <i>EWSR1</i> )
	t(9;17)(q22;q12)	<i>TAF15-NR4A3</i>		Low	↓ ( <i>NR4A3</i> )
	t(9;15)(q22;q21)	<i>TCF12-NR4A3</i>		Low	
	t(3;9)(q12;q22)	<i>TFG-NR4A3</i>		Low	
Fibroma of tendon sheath	t(2;11)(q31-32;q12)	<i>Unknown</i>	FISH	N/A	N/A

(continued)

**Table 8.3** (continued)

Tumor	Chromosomal abnormality	Gene defect(s)	Testing		
			Method	Sens (%) <sup>a</sup>	Avail <sup>b</sup>
Inflammatory myofibroblastic tumor	t(1;2)(q21;p23)	<i>TPM3-ALK</i>	FISH	N/A	↑ (ALK) <sup>c</sup>
	t(2;19)(p23;p13)	<i>TPM4-ALK</i>			
	t(2;17)(p23;q23)	<i>CLTC-ALK</i>			
	t(2;2)(p23;q12)	<i>RANBP2-ALK</i>			
	t(2;11)(p23;p15)	<i>CARS-ALK</i>			
	inv(2)(p23;q35)	<i>ATIC-ALK</i>			
Liposarcoma (well differentiated and dedifferentiated)	12q14-15 (ring)	<sup>d</sup> Amp of <i>CDK4</i> , <i>MDM2</i> , <i>CPM</i> , <i>HMG2</i> , <i>GLI1</i> , <i>TSPAN31</i>	FISH	>95	↑ ( <i>MDM2</i> , <i>CPM</i> )
Liposarcoma (myxoid and round cell)	t(12;16)(q13;p11)	<i>FUS-DDIT3</i>	FISH	>90 Low	↑ ( <i>EWSR1</i> , <i>FUS</i> , <i>DDIT3</i> )
	t(12;22)(q13;q12)	<i>EWSR1-DDIT3</i>			
Malignant gastrointestinal neuroectodermal tumor	t(12;22)(q13;q12)	<i>EWSR1-ATF1</i>	FISH	45	↑ ( <i>EWSR1</i> ) <sup>c</sup>
	t(2;22)(q33;q12)	<i>EWSR1-CREB1</i>		25	
Mesenchymal chondrosarcoma	t(8;8)(q13;q21)	<i>HEY1-NCOA2</i>	FISH	80	N/A
Myxoinflammatory fibroblastic sarcoma (and hemosiderotic fibrolipomatous tumor)	t(1;10)(p22;q24)	<i>TGFBR3-MGEA5</i>	FISH	80	↓ ( <i>MGEA5</i> )
	3p11-12 (ring)	Amp of <i>VGLL3</i> , <i>CHMP2B</i>			
Osteosarcoma	12q14-15 (ring)	Amp of <i>CDK4</i> , <i>MDM2</i> , <i>CPM</i> , others	FISH	60	↑ ( <i>MDM2</i> , <i>CPM</i> )
Pulmonary myxoid sarcoma	t(2;22)(q34;q12)	<i>EWSR1-CREB1</i>	FISH	70	↑ ( <i>EWSR1</i> ) <sup>c</sup>
Sclerosing epithelioid fibrosarcoma	t(7;16)(q33;p11)	<i>FUS-CREB3L2</i>	FISH	N/A	↑ ( <i>FUS</i> ) <sup>c</sup>
Solitary fibrous tumor	12q13 inversion	<i>NAB2-STAT6</i>	RT-PCR	90	N/A
Subungual exostosis	t(X;6)(q22;q13-q14)	<i>COL12A1-COL4A5</i>	FISH	N/A	N/A
Synovial sarcoma	t(X;18)(p11;q11)	<i>SS18-SSX1</i> <i>SS18-SSX2</i> <i>SS18-SSX4</i>	FISH RT-PCR Rare	60 25–35	↑ ( <i>SS18</i> ) ↓ (RT-PCR, 1st two fusions only)
Undifferentiated small round blue cell tumor	t(4;19)(q35;q13)	<i>CIC-DUX4</i>	FISH	70	↓ ( <i>CIC</i> )

Abbreviation: N/A little or no data available

<sup>a</sup>Sensitivity: references in main text

<sup>b</sup>Availability: ↓ low (few commercial laboratories); ↑ high (widely available); probe used in parentheses, fusion partner may not be available; data compiled from select websites including [www.genetests.org](http://www.genetests.org), [www.propath.com](http://www.propath.com), [www.aruplab.com](http://www.aruplab.com), [www.labcorp.com](http://www.labcorp.com), [www.mayomedicalaboratories.com](http://www.mayomedicalaboratories.com)

<sup>c</sup>Probe may not be specifically indicated on test menu for this particular tumor

<sup>d</sup>Amplifications mainly observed in deep, not superficial (cutaneous), tumors [94]

protein). *EWSR1* is a member of the FET (Fus-Ewsr1-Taf15) gene family of RNA-binding proteins, which includes *FUS* and *TAF15*. *FUS* is also a common player in soft tissue translocations. *EWSR1*, and *FUS* for that matter, is a promiscuous gene, having many translocation

partners. These partners are usually members of the ETS (E-twenty-six) family, which includes *FLI1*, *ERG*, *ETV1*, *FEV*, *ETV4*, and many more, but as is evident from the tables, the partners are not limited to this group in EWS or in other tumors.

**Table 8.4** Tumors with *EWSR1* rearrangements

Tumor	Chromosomal abnormality	Fusion gene
Angiomatoid fibrous histiocytoma	t(2;22)(q33;q12) t(12;22)(q13;q12)	<i>EWSR1-CREB1</i> <i>EWSR1-ATF1</i>
Clear cell sarcoma	t(12;22)(q13;q12) t(2;22)(q33;q12)	<i>EWSR1-ATF1</i> <i>EWSR1-CREB1</i>
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	<i>EWSR1-WT1</i>
Ewing sarcoma/primitive neuroectodermal tumor (PNET)	t(11;22)(q24;q12) t(21;22)(q22;q12) t(7;22)(p21;q12) t(2;22)(q35;q12) t(17;22)(q21;q12)	<i>EWSR1-FLI1</i> <i>EWSR1-ERG</i> <i>EWSR1-ETV1</i> <i>EWSR1-FEV</i> <i>EWSR1-ETV4</i>
Ewing-like sarcoma	t(20;22)(q13;q12) t(6;22)(p21;q12) t(4;22)(q31;q12) inv(22) in t(1;22) t(2;22)(q31;q12)	<i>EWSR1-NFATc2</i> <i>EWSR1-POU5F1</i> <i>EWSR1-SMARCA5</i> <i>EWSR1-PATZ1</i> <i>EWSR1-SP3</i>
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	<i>EWSR1-NR4A3</i>
<sup>a</sup> Hyalinizing clear cell carcinoma (HCCC) of the salivary gland	t(12;22)(q13;q12)	<i>EWSR1-ATF1</i>
<sup>a</sup> Malignant gastrointestinal neuroectodermal tumor	t(12;22)(q13;q12) t(2;22)(q33;q12)	<i>EWSR1-ATF1</i> <i>EWSR1-CREB1</i>
Myoepithelial tumor of soft tissue	t(6;22)(p21;q12) t(19;22)(q13;q12) t(1;22)(q23;q12)	<i>EWSR1-POU5F1</i> <i>EWSR1-ZNF444</i> <i>EWSR1-PBX1</i>
Myxoid liposarcoma (and round cell liposarcoma)	t(12;22)(q13;q12)	<i>EWSR1-DDIT3</i>
<sup>a</sup> Pulmonary myxoid sarcoma	t(2;22)(q34;q12)	<i>EWSR1-CREB1</i>

<sup>a</sup>Very rare or unreported in the skin

The precise role of *EWSR1* by itself or fused to other genes is not known. Because it encodes for an RNA-binding protein, it likely serves as some sort of cellular housekeeper or master regulator of protein expression. When fused to other genes, it may maintain this complex role or its promoter may drive expression of its translocated partner gene. Indeed, the FET-ETS family of fusion proteins has been shown to modulate transcription of genes involved in cell differentiation and survival/proliferation, but the precise role of these is still under investigation [107].

The oncogenic power of *EWSR1* is evident by its multitude of fusion partners and involvement in numerous different cancers. It is interesting, however, that with the exception of hyalinizing clear cell carcinoma of the salivary gland, these rearrangements appear restricted to tumors of mesenchymal origin. This would suggest that *EWSR1* is only turned on or has active targets in this family of

tumors. In the context of *EWSR1*-rearranged tumors, it would be convenient to attribute the divergence of tumor types to the translocation partner of *EWSR1*, but this is not the case. It is equally perplexing that the identical fusion gene can lead to completely different tumors, such as the *EWSR1-CREB1* fusion in angiomyomatoid fibrous histiocytoma and clear cell sarcoma. This observation highlights the dependence translocations have on other factors, such as cell of origin, cellular microenvironment, and epigenetic changes, among others.

### 8.3 Prognosis

The prognosis for patients with sarcomas, as with other tumor types, is reliant on the clinical and pathologic stage as dictated by the defined tumor-node-metastasis (TNM) criteria [108]. For soft tissue sarcomas, prognosis depends upon tumor

size, its location (superficial vs. deep), presence of metastases, and histologic grade. Small, superficial tumors with low histologic grade have the highest probability for cure, which is always a surgical cure. Notably absent from these criteria are molecular markers. While there is some evidence for certain molecular events to independently predict prognosis, sufficient data are not yet available for incorporation into current TNM criteria. Other theoretical applications include translocation/fusion detection of micrometastases and detection of residual/recurrent tumor for therapeutic monitoring [109, 110].

### 8.3.1 Translocations and Fusion Genes

Fusion genes are drivers of tumorigenesis. Some tumors, like low-grade fibromyxoid sarcoma, are associated with multiple different alternate translocations ( $t(7;16)$  and  $t(11;16)$ ) and corresponding alternate fusion genes (*FUS-CREB3L2* and *FUS-CREB3L1*). Other tumors, like EWS and CCS, can also have multiple variant breakpoints within the same gene, leading to the same macrogenetic translocation but with slightly different fusion genes. It is logical to hypothesize that these alternate translocations or variations of fusion genes may lead to differences in tumor biology and hence prognosis. The data on this have been mixed, however. In EWS, initial studies suggested a better prognosis in patients with type 1 *EWS-FLI* (*FLI* exon 6) fusions compared to patients with type 2 *EWS-FLI* (*FLI* exon 5) fusions. Later studies could not confirm this correlation, and now breakpoint type has been eliminated as a prognostic marker in current protocols for EWS [66, 111]. Similar lack of evidence has surfaced for clear cell sarcoma and DFSP [37, 48]. In synovial sarcoma, however, there is evidence that different rearrangements impact prognosis. In a retrospective multi-institutional multivariate analysis of 243 patients, it was determined that patients with *SS18-SSX2* tumors have a better outcome than patients with *SS18-SSX1* tumors (5-year survival 73 % vs. 53 %, respectively) [112]. The role of breakpoints and fusion

partners for patient prognosis in other soft tissue tumors is currently being investigated.

### 8.3.2 Gene Amplification

*MYC* amplifications have been reported to have prognostic value in a variety of human cancers [113, 114]. *MYC* amplifications are characteristic of post-radiation angiosarcoma and indirectly predict prognosis in these tumors, giving *MYC* amplification dual diagnostic/prognostic utility. Amplifications are observed in these tumors but not their benign counterpart and histologic mimic, the atypical vascular lesion (AVL). *MYC* may play a role in the progression of AVL to angiosarcoma in the post-radiation setting. Amplification of *MDM2*, typical of well-differentiated and dedifferentiated liposarcoma and osteosarcoma, does not have clear prognostic significance. However, recent studies have suggested *CDK4* amplification (closely linked to *MDM2* on chromosome 12 and also present within ring chromosomes) predicts worse disease-free and progression-free survival in liposarcoma patients [115]. For practical purposes, in soft tissue dermatopathology, amplifications of *MYC*, *MDM2*, and *CDK4* are considered diagnostic, not prognostic, markers limited to these settings.

## 8.4 Therapy

Surgery remains the main therapeutic approach for soft tissue sarcomas and offers the best chance for cure. Radiation and chemotherapy play a small role for preventing recurrences and modestly delaying tumor progression, respectively, but have little impact on overall survival. The overall 5-year survival for children ages 0–14 with soft tissue malignancies has improved from only 75 % (1978–1980) to 82 % (2002–2008) in the past 30 years, highlighting a need for therapeutic innovation [2].

Targeted therapy designed to shut down aberrantly activated pathways has become the treatment paradigm for oncology. In chronic myelogenous leukemia, the characteristic  $t(9;22)$

translocation leads to the Bcr-Abl fusion tyrosine kinase, which is effectively inhibited by the targeted therapy imatinib mesylate [116]. In melanoma, activating mutations such as B-Raf<sup>V600E</sup> can be effectively blocked by designed mutation-specific inhibitors such as vemurafenib and dabrafenib [117, 118]. While only few examples like this exist for soft tissue tumors, given the high frequency of tumor-specific and oncogenic-driving molecular events (such as fusion genes) in these tumors, effective therapies should soon follow.

#### 8.4.1 Fusion-Gene Targeted Therapy

While CML offered great promise for fusion gene-directed therapy in sarcomas, there have been few success stories. Most translocations in soft tissue tumors create fusion proteins involved in transcriptional regulation. We have learned that we know very little about transcriptional regulation, and attempts at blocking these fusion proteins have been unsuccessful. DFSP, on the other hand, creates a fusion protein resulting in an overactive signaling molecule, PDGFB. This more closely mimics the CML model. In fact, *COL1A1-PDGFB* and *BCR-ABL* both encode overactive kinases, and these respond to the same therapy, imatinib mesylate and its related compounds [119]. Imatinib mesylate has been shown to induce responses in surgically inoperable or the rare metastatic cases of DFSP and is recommended in those settings [120]. Of note, tumors without the *COL1A1-PDGFB* fusion do not respond to imatinib mesylate, as predicted, emphasizing the importance of molecular testing prior to the initiation of therapy [121].

There are other examples of tumors with fusion proteins being targeted by various compounds in preclinical and clinical studies. These include inflammatory myofibroblastic tumors with TPM3-ALK fusions targeted by tyrosine kinase inhibitors, myxoid/round cell liposarcomas with FUS-DDIT3 fusions targeted by trabectedin, and other examples. These are cur-

rently in the investigative stages with little current practical utility and thus are not discussed further, but are exciting potential new additions to sarcomas' theranostic repertoire [122].

#### 8.4.2 Mutation-Specific and Other Signaling Pathway-Directed Therapies

In contrast to the B-Raf<sup>V600E</sup> activating mutation in melanoma, tumor-specific activating mutations of key signaling molecules are unusual in soft tissue sarcomas. One example is with GISTs and their high frequency of tumor-driving *KIT* (or *PDGFRA*) mutations [13], but again, these are rare in the skin and are not discussed further. Mutations in *RAS*, *RAF*, and *PIK3CA* (and activation of the MAP kinase and PI3K pathways) all have been reported in various soft tissue tumors, but these mutations are neither frequent nor specific to any given tumor and often occur in high-grade tumors in the context of numerous other molecular defects, limiting the therapeutic effect of specific blockers.

Due to the heterogeneity and molecular complexity (including poorly understood fusion genes) of soft tissue tumors, different treatment strategies are being explored. One strategy is to target downstream molecules in signaling pathways known for their role in oncogenesis, irrespective of upstream mutations. Possibilities include targeting MEK or Akt, downstream molecules in the MAP kinase pathway and PI3K pathway, respectively (see Chap. 5 and Fig. 5.2 for details on oncogenic pathways). Many cancers, including sarcomas, have activation of these pathways, by a variety of upstream activating mechanisms, and have demonstrated a response to MEK inhibition [123–125]. Another possibility is Met inhibition. Met inhibitors, such as INC280 and tivantinib, are currently in clinical trials for the treatment of various cancers [126]. Preclinical data demonstrating increased expression of MiTF in clear cell sarcomas, and knowledge that MiTF transcriptionally activates Met, provide a rationale for Met inhibition in CCS. Other therapeutic strategies include

high-throughput screening of individual tumors for multiple known proto-oncogene activating mutations, irrespective of tumor type in order to tailor inhibitor therapy, and using combinations of cytotoxic chemotherapy, radiation, immunotherapy, and/or small-molecule inhibitor therapy, among others (recently reviewed in Demicco et al. [122]). A current list of clinical trials using permutations of these strategies can be found at [www.clinicaltrials.gov](http://www.clinicaltrials.gov).

## 8.5 Molecular Tests Performed on Soft Tissue Tumors and Practical Considerations

There are innumerable molecular methods in practice and in development, but only two—FISH and PCR—are routinely used in the diagnosis of soft tissue tumors on clinical samples. Other assays are briefly touched upon. Overviews of these techniques are provided in Chap. 3.

### 8.5.1 FISH

Briefly, FISH (fluorescence *in situ* hybridization) utilizes DNA probes to target chromosomal points of interest. It can be used to assess change in gene copy number (amplification, deletion, etc.) and gross chromosomal structural alterations and copy numbers (translocations, inversions, aneuploidy, etc.). Designing probes to regions involved in translocations can aid in the diagnosis of certain soft tissue tumors. There are two main types of FISH, break-apart FISH and dual-fusion FISH (Fig. 8.5). In break-apart FISH, two probes (red and green) are directed to the same region, flanking a chromosomal breakpoint. When the two signals are next to each other (no translocation), the red and green signals overlap, appearing as a yellow signal. When they are separated (translocated), the cell has two separate and distinct red and green signals. In break-apart FISH, only one translocation partner is probed. This technique may be used if there is only one main translocation for a given tumor (DFSP) or if the identity of the fusion partner does not matter

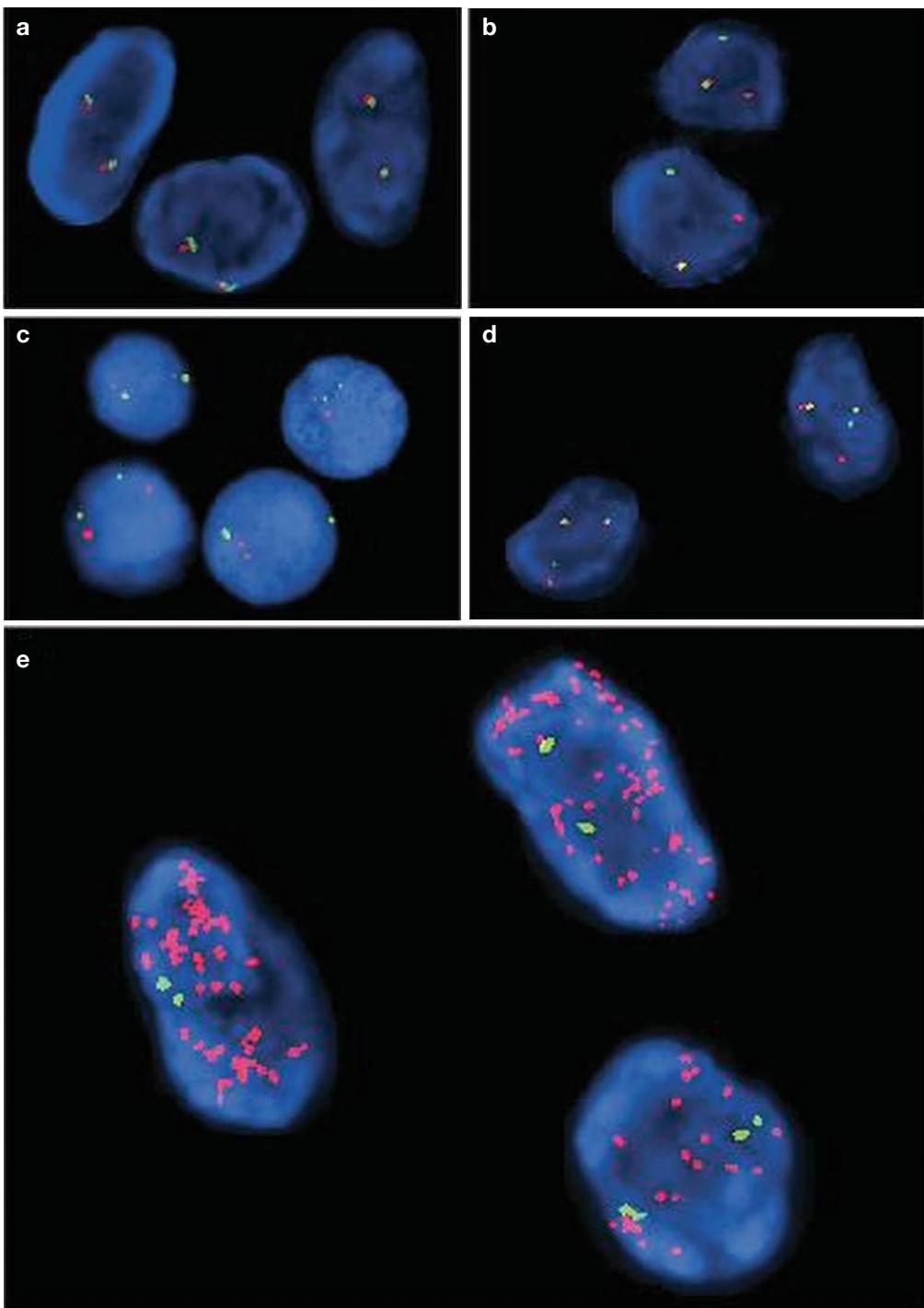
(i.e., confirming a diagnosis of EWS). This test would not be recommended if the identity of the translocation partner was important for pinning down the diagnosis or for other reasons. Dual-fusion FISH probes identify both genes involved in a translocation. In this test, a translocation is identified by the juxtaposition of red and green compared with two separate red and two separate green signals typical of a normal diploid cell. This test is useful when a gene has multiple potential fusion partners (e.g., *EWSR1*) and the identity of the partner is required to confirm a diagnosis (such as *EWSR1-NR4A3* for extraskelletal myxoid chondrosarcoma). There are potential false negatives in dual-fusion FISH, however, if alternate translocations exist.

Most laboratories use break-apart FISH over dual-fusion FISH. In addition to the flexibility described above, there are technical advantages. Because these assays are performed from formalin-fixed paraffin-embedded tissue, or PET-FISH, it is often difficult to get all chromosomes “in plane.” For that reason, any given probe may be visualized one (one allele out of plane) or three (two cells overlapping) times, instead of the normal two, and there may be “pseudo-fusions” caused by overlapping cells, challenging interpretation. Break-apart FISH eliminates some of these technical difficulties by the presence of a third color (yellow) when the probes are juxtaposed. In the exceptional case, if the fusion partner’s identity is wanted or required, break-apart FISH can reflex to dual-probe fusion FISH (often used with *EWSR1* rearrangements).

#### <sup>1</sup>CPT codes

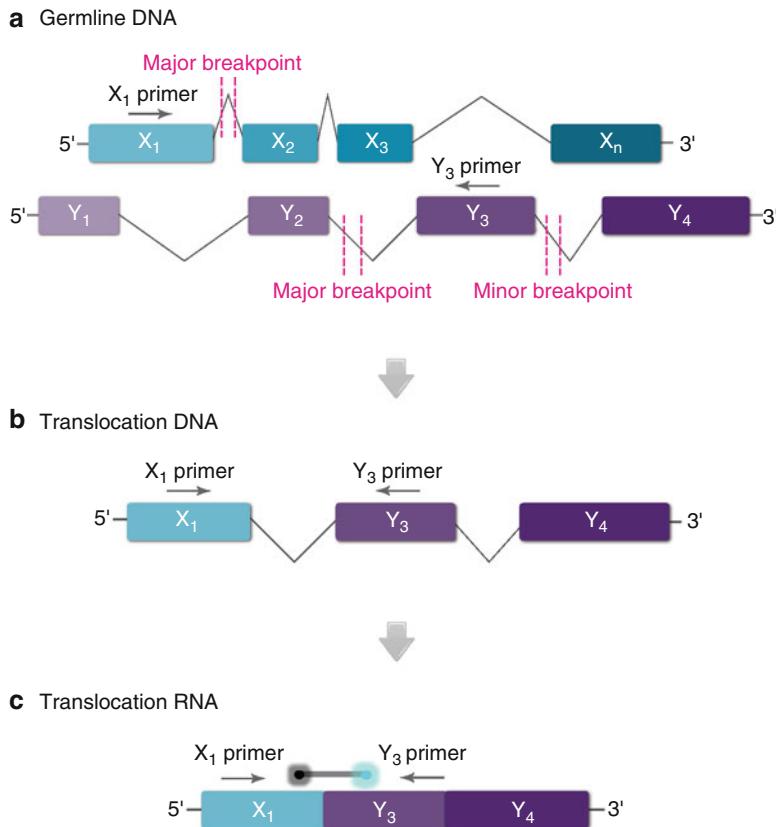
FISH probe codes include 88365 or 88367 (quantitative with computer assistance) or 88368 (quantitative, manual) (all are per probe) or the combination of 88274 (per probe) and 88291, depending on how the test is performed and interpreted.

<sup>1</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [127]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).



**Fig. 8.5** Break-apart and dual-fusion FISH. In break-apart FISH, a single locus is probed by two different probes, flanking a breakpoint region. In normal cells, the *red* and *green* probes are juxtaposed, yielding two *yellow* signals (a). If a translocation occurs in between the probed targets, the *red* and *green* signals are separated (b). In dual-fusion FISH, normal cells have two copies of each probed target, usually on separate chromosomes (c). If a translocation occurs, the *red* and *green* signals, targeting respective partners of the

fusion gene, are united and yield a *yellow* signal (d). Modern probes are designed to both sides of the translocation, and therefore, two unified *red-green* (*yellow*) signals are observed per cell with balanced translocations. Amplifications can easily be detected by probing the gene of interest and comparing the number of signals per cell with a centromere probe, to control for aneuploidy/polyplioidy and determine low versus high amplification. An example of *MDM2* high amplification is shown (e, *MDM2 red*, centromere 12 *green*)



**Fig. 8.6** RT-PCR. RT-PCR is a commonly employed technique for the detection of soft tissue tumor translocations. In this schematic, genes X and Y are shown in their germline configuration with their respective exons (boxes) and introns (lines) (a). Generalized breakpoint regions are indicated by dashed lines. Following rearrangement of the DNA at the major breakpoint regions, the first exon of gene X (X<sub>1</sub>) is juxtaposed with the third exon on gene Y (Y<sub>3</sub>) (b). Primers directed to these exons will not yield an amplicon by DNA PCR because the interval genetic mate-

rial is too large. Following splicing, transcription to mRNA, and then in vitro conversion to cDNA, RT-PCR will yield a detectable amplicon using the same primer set and probe (c). Note that if the translocation occurred at the minor breakpoint on Y, this would still be detectable by FISH (but major and minor breakpoints could not be distinguished) but not RT-PCR using the X<sub>1</sub>-Y<sub>3</sub> primer set. A separate RT-PCR primer set, X<sub>1</sub>-Y<sub>4</sub>, would be capable of distinguishing breakpoints

### 8.5.2 RT-PCR

RT-PCR (reverse transcription polymerase chain reaction) is another method to detect soft tissue tumor translocations. “Regular” PCR (using genomic DNA as a template) is not practical because the translocation breakpoints are usually within introns, making distances between primers long and variable (Fig. 8.6). In RT-PCR, mRNA transcripts from the tumor sample are converted to cDNA, providing a PCR template. The primers are designed to exons flanking the breakpoint regions of the fusion genes. A detect-

able PCR product is only generated if the fusion occurs. RT-PCR can be used to detect any fusion product so long as the breakpoints are known and consistent, and primers are designed accordingly. For example, if multiple translocations occur in a tumor, such as t(12;22) *EWSR1-ATF1* and t(2;22) *EWSR1-CREB1* in clear cell sarcoma, primers would need to be designed to *EWSR1* and both *ATF1* and *CREB1*, in a multiplex reaction, to maximize sensitivity. The translocations can be differentiated by amplicon size, if needed, with appropriate primer design. Until fairly recently, RT-PCR was only performed on fresh and fro-

**Table 8.5** FISH versus RT-PCR

	FISH	RT-PCR
Availability	High	Low
Cost	High	Variable
Insurance coverage	Covered by most plans	Variable
Utility on paraffin	Used regularly (PET-FISH), but variations in fixation materials/procedures can be problematic	Can use, but may deal with RNA degradation and requires small amplicons
Used for tumors with multiple translocations	Yes	Yes, but requires multiplex or separate reaction for each
Used for tumors with breakpoint variants	Yes, but cannot distinguish	Yes, but not if there are numerous variants
Used for tumors with unknown fusion partners	Yes	No
Sensitivity	Requires at least 50 cells of relatively pure tumor	Very high, sample can be scant and heterogeneous
Detection range for macrochromosomal aberrancies	Translocations, amplifications, inversions, deletions	Translocations
Resolution	Low (50–100 kb), cannot detect small insertions, deletions, point mutations	High (single base pair)
Inhibitors and other limitations	Decalcification procedures, non-formalin fixatives, tumor necrosis	Hemosiderin, EDTA, melanin, divalent cation chelators, others
False positives	Unusual	Can occur with contamination/carryover
Technical expertise required	Very high	High

Abbreviation: EDTA ethylenediaminetetraacetic acid

zen tissue samples, not paraffin-embedded tissue, due to problems with mRNA degradation. This technical hurdle has been largely overcome with the development of new RNA extraction techniques, allowing for more widespread RT-PCR applications.

#### <sup>2</sup>CPT codes

88401 (translocation analysis for EWS, alveolar rhabdomyosarcoma, desmoplastic small round cell tumor, myxoid liposarcoma); 88402 (translocation analysis for DFSP); 88479 (unlisted procedure code) for all others.

FISH and RT-PCR both have their advantages and disadvantages regarding the detection of tumor-specific translocations, and these must be factored when choosing to order or perform such

an assay (Table 8.5). FISH is more available commercially, with only few laboratories offering select RT-PCR assays (see Tables 8.2 and 8.3). There are several reasons for this. FISH is more flexible regarding sample source, able to be performed on paraffin, frozen samples, single-cell spreads, cytologic smears, and FNAs. As mentioned above, RT-PCR can be used on paraffin but requires more delicate handling to ensure intact mRNA, and the amplicon size should be restricted to a few hundred base pairs to minimize the effects of unavoidable degrees of mRNA degradation. Other disadvantages of RT-PCR include the following: Multiplex reactions are required for tumors with more than one breakpoint (or translocation) and may be prohibitive in tumors with numerous breakpoint variants (DFSP); false negatives will occur for omitted minor breakpoints; the fusion partner must be known; it cannot detect other chromosomal aberrances like amplifications and deletions; and the reaction can be inhibited by hemosiderin and melanin, among other biologic and synthetic components.

<sup>2</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [127]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

RT-PCR does have its advantages over FISH. For one, RT-PCR is very sensitive, able to detect low levels of tumor. This may be important with small tissue samples, early metastatic deposits, heavily inflamed tumors, or identifying minimal residual disease or recurrence following therapy. RT-PCR can detect translocations in tumors with complex karyotypes, which may be challenging to interpret by FISH. RT-PCR has a higher resolution than FISH. With appropriate assay design, RT-PCR can detect point mutations, small insertions/deletions, and breakpoint variants within genes. FISH cannot distinguish changes smaller than 50–100 kb. While the significance of different breakpoints in translocations and fusion genes remains unclear, there are always potential prognostic and/or therapeutic implications [111, 112], and some tumors carry small intra-chromosomal rearrangements, like solitary fibrous tumors, out of the resolution range of FISH [128].

From a laboratory standpoint, the decision to offer RT-PCR versus FISH requires consideration of all the above factors and more. FISH may not be an option for smaller laboratories as there can be prohibitive startup and operating costs. Usually, FISH laboratories are only practical when there is a significant hematopathology volume, as soft tissue pathology alone cannot generate enough volume for its support. *EWSR1* and *FUS* break-apart FISH are logical first offerings, for a soft tissue FISH laboratory, given their diverse applications (see Table 8.4). FISH probes are easily purchased, but not always available for specific rare or partner loci. For smaller fledgling laboratories or those with specific research interests, RT-PCR may be a better choice. In addition to the above advantages of RT-PCR, it is potentially more inexpensive to perform and may require less technical expertise for certain platforms, although costs can be extraordinary for FDA-approved tests.

### 8.5.3 Others

#### 8.5.3.1 Cytogenetics

The main advantage cytogenetic analysis has over other methods is its ability to evaluate the entire genome, not just a specific translocation.

This may be useful as a “shotgun” approach in challenging cases, when a specific diagnosis is required but the histology offers few clues. Rarely, it may also be helpful in distinguishing benign from malignant tumors by identifying a complex karyotype. It can also help uncover secondary molecular events, aiding in the understanding of pathogenesis. As a general rule, however, the above scenarios are rarely encountered and cytogenetic analysis has little role in diagnosing or predicting therapeutic response of soft tissue tumors in the skin. The identification of a complex karyotype is common for high-grade sarcomas, with increasing genomic instability, but this is not specific for any tumor type. FISH and RT-PCR are preferred modalities to look for specific chromosomal aberrancies as they require fewer restrictions on tissue handling and are easier to interpret.

In the rare event that karyotypic analysis is required for one of the above reasons or if FISH/RT-PCR is not available (e.g., if there is no commercially available FISH probe), the following should be considered. Cytogenetic analysis requires fresh tissue, not frozen or paraffin fixed tissue, because the tumor cells need to be kept alive for culture and cellular division. Therefore, tissue is often procured in the operating room or grossing room, using sterile technique. Most laboratories prefer a 1.0-cm<sup>3</sup> block of representative, non-necrotic tumor, but smaller samples may also yield results. Approximately 1 week is required to establish the culture and achieve dividing cells, and additional time is required for analysis. This turnaround time can be shortened with rapidly dividing cells or the use of mitosis-arresting agents such as colchicine. Once cells are dividing, a metaphase spread is created and stained using G-banding techniques. This is in contrast to FISH, which has the distinct advantage by being able to probe targets on interphase (and thus paraffin fixed) cells. Because of these restrictive requirements, a failed cytogenetic assay is not an uncommon event, with reported failure rates up to 60 %, depending on tissue type and transport time. Additionally, false-negative results may occur with preferential growth and division of nonmalignant cells (stromal fibroblasts) or when

tumors have small molecular abnormalities— inversions, insertions, deletions, mutations, etc.—beyond the resolution of the assay (which is roughly 10 Mb). False-positive results are considered unusual.

#### <sup>3</sup>CPT codes

88239, 88264, 88280, 88291.

#### **8.5.3.2 Immunohistochemistry**

Immunohistochemistry is not a molecular assay, but is included here for completeness. Immunohistochemistry continues to play an integral role in the diagnosis of soft tissue neoplasms. Panels of antibodies directed to proteins expressed by the tumor can be extremely helpful in identifying the tumor's lineage and often the specific diagnosis (see Table 8.1). Most dermatopathologists continue to use the systematic diagnostic approach of histology → immunohistochemistry → molecular. There is nothing wrong with this conventional approach, but in cases that exhibit a characteristic morphology (e.g., angiomyxoid fibrous histiocytoma), a molecular test may abrogate the need for extensive and expensive immunohistochemical workups. Alternatively, immunohistochemical analysis for Myc or Alk expression (in post-radiation angiosarcoma and inflammatory myofibroblastic tumor, respectively) may be a cheaper and better alternative than FISH, depending on the laboratory arrangements [129–131].

#### <sup>3</sup>CPT codes

88342 (per antibody).

#### **8.5.3.3 Comparative Genomic Hybridization and Sequencing (Including High-Throughput Sequencing)**

Array comparative genomic hybridization (aCGH) has emerged as a powerful tool for determining chromosomal copy number gains and

losses. Arrays can be designed to evaluate the entire patient or tumor genome, a single chromosome, or more focused areas, with a resolution as high as 200 bp [132]. These assays can use arrays of single nucleotide polymorphisms (SNPs), oligonucleotides, or other markers to determine gains and losses. While aCGH is clearly powerful, and may supplant conventional cytogenetics for the detection of macrogenetic aberrancies, it has not gained much traction in soft tissue pathology. The main reason for this, to date, is that losses and gains are common in high-grade sarcomas, but this provides little added information for the diagnosis and/or management of the sarcoma patient. Interestingly, translocations in soft tissue pathology are often balanced, and therefore, there are no net gains and losses, and these would be undetected by most aCGH assays.

High-throughput sequencing (aka next-gen, deep, massively parallel array sequencing) is the next technological wave in the evaluation of soft tissue tumors. Conventional sequencing methods have been hampered by cost, time, and restrictions to focused targets, as well as other limitations. Only few tumor-associated mutations exist in the soft tissue world—*KIT* for GISTs, *CTNNB1* for fibromatosis, *GNAS* for myxomas, etc.—and the scope of testing is too limited (perhaps with the exception of GISTs) for most laboratories to maintain valid assays. These types of mutational assays have been limited to small “home brew” operations and investigational settings. With newer technologies, however, massive amounts of sequencing data can be generated at relatively low cost and over a shorter time period. Entire panels of genes can be assessed simultaneously, evaluating for multiple tumor-specific mutations as well as translocations. Indeed, this technology was used to identify the small 12q13 inversion and *NAB2-STAT6* fusion in solitary fibrous tumors, for example [101, 133]. Moreover, as demonstrated in other oncologic fields, high-throughput sequencing can simultaneously search for a multitude of proto-oncogene mutations, offering a roadmap for targeted therapy. Anticipate high-throughput sequencing playing a larger role in the diagnosis and management of the sarcoma patient in the near future.

<sup>3</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [127]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

**<sup>4</sup>CPT codes**

88479 (unlisted molecular procedure code).

## 8.6 Summary

The high frequency of translocations and fusion genes in soft tissue tumors has shaped our understanding of these nebulous tumors and shaped our management of the sarcoma patient. These fusion genes are of particular interest because they are shared across mesenchymal lines of differentiation in sarcomas, but, for the most part, are only rarely observed in other solid tumors or normal or senescent tissue. These sarcoma-specific oncogenic-driving events have not only furthered our understanding of oncogenesis but now provide a rationale for the development of diagnostic assays and therapeutic strategies.

Some molecular events, such as t(7;16) in low-grade fibromyxoid sarcoma, have helped distinguish tumors from histologic mimics. Other events, like *EWSR1* partnering with multiple genes in multiple cancers and the identical t(12;22) found in both clear cell sarcoma and angiomyxoma, have found diagnostic utility and have provided some insight into post-stem cell oncogenesis. FISH and RT-PCR are effective tools at identifying these rearrangements, and their role in the diagnostic repertoire of soft tissue tumors has become firmly established. FISH has become the more widely used commercial offering but RT-PCR can be equally or more effective, depending on the differential diagnosis or the specific question being asked.

Prognostic and theranostic utilities in soft tissue oncology remain limited to few isolated scenarios. Unlike other tumor types, such as melanoma, activating mutations in signaling molecules are neither prevalent nor specific to sarcoma. Therapeutic targeting of fusion genes

has been largely unsuccessful, although tyrosine kinase inhibition in DFSPs and inflammatory myofibroblastic tumors has provided some initial hope in this area. Until there is a better understanding of the complex master-regulating roles of the FET (*EWSR1*) and ETS (*ATF1*) family of genes, which are ubiquitous translocation fusion partners in soft tissue pathology, therapeutic strategies may be less “targeted,” focusing on the generic blocking of downstream signaling molecules within commonly hijacked oncogenic signaling pathways.

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<sup>4</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [127]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

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# Genodermatoses. Part I: Muir-Torre Syndrome

9

## Contents

9.1	<b>Introduction.....</b>	232
9.2	<b>Pathophysiology of MMR-Defective MTS.....</b>	233
9.3	<b>Clinical Features .....</b>	236
9.4	<b>Histologic Features.....</b>	237
9.5	<b>Immunohistochemical Features.....</b>	238
9.6	<b>Assessing MMR Defects: Immunohistochemistry and PCR-Based Assays.....</b>	239
9.6.1	Immunohistochemistry for MMR.....	239
9.6.2	Molecular MSI Testing .....	239
9.6.3	IHC Versus MSI.....	241
9.6.4	Genetic Testing .....	243
9.7	<b>Approach to the Suspected MTS Patient.....</b>	245
9.7.1	Defining MTS .....	245
9.7.2	An Algorithmic Approach to the Diagnosis of MTS .....	246
9.8	<b>Summary.....</b>	250
	<b>References.....</b>	250

## Key Points

- The Muir-Torre syndrome (MTS) bridges the fields of genodermatoses and oncology, with individuals developing cutaneous and visceral tumors due to germline defects.
- MTS individuals are clinically defined by the presence of sebaceous neoplasms in conjunction with visceral tumors (colon cancer most common) *or* multiple keratoacanthomas in the context of a positive family history.
- MTS tumors develop at a younger age and have a less aggressive biology than their sporadic counterparts.
- A subset of clinically defined MTS individuals has germline defects in mismatch repair (MMR) genes, which are critical for preventing the propagation of mutations leading to oncogenesis. MTS, Lynch syndrome, and Turcot syndrome are all autosomal dominant MMR gene defect-associated cancer syndromes with different phenotypes.
- MSH2* is the most common gene altered in MTS, followed in frequency by *MLH1*. *MSH6* and *PMS2* are other MMR genes that are only rarely (if at all) altered in MTS.
- Immunohistochemistry (IHC) can be used to assess defects in MMR protein

expression and therefore acts as a surrogate for MMR gene defects.

- Molecular microsatellite instability (MSI) assays evaluate the function of the MMR machinery by assessing the tumor's ability or inability to replicate microsatellite (mononucleotide and dinucleotide) DNA.
- IHC and MSI testing are complementary, and when used together, can effectively evaluate the MMR machinery within tumors.
- Direct gene sequencing of the MMR genes can help confirm the diagnosis of MTS and identify carriers among family members.
- Diagnostic algorithms for MTS should incorporate clinical, histologic, IHC and/or MSI, and MMR gene sequencing data to appropriately classify and manage MTS individuals and their family members.

**Table 9.1** Muir-Torre syndrome clinical diagnostic criteria

Group 1	Group 2
Visceral malignancy <i>and</i>	Multiple keratoacanthomas <i>and</i>
Cutaneous sebaceous tumor <sup>a</sup>	Multiple visceral malignancies <i>and</i>
Sebaceous adenoma <i>or</i>	Family history of MTS
Sebaceous carcinoma	

Patients meet the criteria for MTS if they satisfy group 1 or group 2 criteria, without predisposing conditions to cutaneous or visceral malignancies including but not limited to nevus sebaceus, prior radiotherapy, and immunodeficiency. Adapted from Schwartz et al. [3]

<sup>a</sup>May also include what other authors describe as sebaceousoma, sebaceous epithelioma, and keratoacanthomas with sebaceous differentiation

polyp, and over 100 cutaneous tumors including sebaceous adenomas, sebaceous carcinomas, and basal cell carcinomas with sebaceous differentiation [2]. These original patients and subsequent studies of large affected families led to the clinical definition of MTS, which is still used today (Table 9.1) [3]. Individuals meet these clinical criteria if they have both a *visceral malignancy* and a *cutaneous sebaceous neoplasm* OR a *visceral malignancy* and *multiple keratoacanthoma-like lesions* in the context of a family history of MTS.

A large subset of MTS is now considered allelic to (or a phenotypic variant of) Lynch syndrome, also known as hereditary nonpolyposis colon cancer (HNPCC) [4]. Lynch syndrome is more common than MTS and has been more extensively studied. As with MTS, Lynch syndrome was previously defined by personal and family malignancy history. For Lynch syndrome, individuals were identified by documenting the presence of colon cancer and tracking colon cancer within the patient's family using the Amsterdam criteria [5]. Later, cancers of the endometrium, small bowel, ureter, and pelvis were added to the list of Lynch syndrome-associated cancers, and new criteria were developed (Amsterdam criteria II) [6]. Now, a clear association between Lynch syndrome and defects in the MMR machinery is recognized. The Bethesda Guidelines, and subsequent "revised" Bethesda

## 9.1 Introduction

The Muir-Torre syndrome (MTS) bridges the fields of genodermatoses and oncology and is a natural segue between the previous discussions on dermatologic tumors and the next chapter on inherited dermatologic disease. MTS is characterized by cutaneous and visceral tumors, and most of these individuals have inheritable gene defects of DNA mismatch repair (MMR). Molecular testing plays an important role in the diagnosis/subclassification of MTS as well as in the understanding of its pathophysiology.

MTS was independently described by both E. G. Muir and Douglas Torre in 1967 and 1968, respectively [1, 2]. Muir described a Maltese man with laryngeal cancer, four primary colon cancers, two primary duodenal cancers, intestinal polyps, and multiple facial sebaceous adenomas and keratoacanthoma-like lesions [1]. Torre described a 57-year-old man with a carcinoma of the ampulla of Vater, colon cancer, an intestinal

Guidelines, are now used to select tumors from high-risk individuals, based on personal and family malignancy history and histologic features, and specifically direct them for MMR-defect testing [7]. The closely intertwined relationship between Lynch syndrome and MTS is highlighted by several points: (1) Lynch syndrome and a large subset of MTS have defects in the MMR machinery [8, 9], (2) up to 28 % of Lynch syndrome families contain individuals with the MTS phenotype [4, 8, 10], and (3) up to 10 % of Lynch syndrome individuals also meet the criteria for MTS. This close relationship is underscored by the addition of sebaceous tumors and keratoacanthomas to the list of Lynch syndrome-associated tumors in the most recent revised Bethesda Guidelines, firmly linking these two syndromes [7].

The clinical criteria for MTS are useful for identifying individuals and family members who may benefit from increased tumor surveillance, but using *only* clinical criteria is problematic. Some MTS individuals may not fulfill these criteria for decades, delaying diagnosis and intervention. Conversely, using clinical criteria alone will capture a genetically heterogeneous group of people with biologically diverse tumors. As mentioned, it is now recognized that only (approximately) two-thirds of individuals with clinically defined MTS have defects in their MMR machinery [9]. The other one-third has apparently normal MMR machinery and either has MMR defects undetectable by our current methods or develops sebaceous and visceral tumors by a completely different (non-MMR-related) mechanism. Therefore, MTS can be broadly divided into two groups: (1) MMR-defective MTS and (2) MTS by clinical criteria only (MMR normal). Distinction between these groups is important because MMR-defective tumors appear to have a different biology (occur at younger age, less aggressive, etc.) than their MMR-normal counterparts, and, if germline mutations are identified, familial carriers can be flagged for increased tumor surveillance.

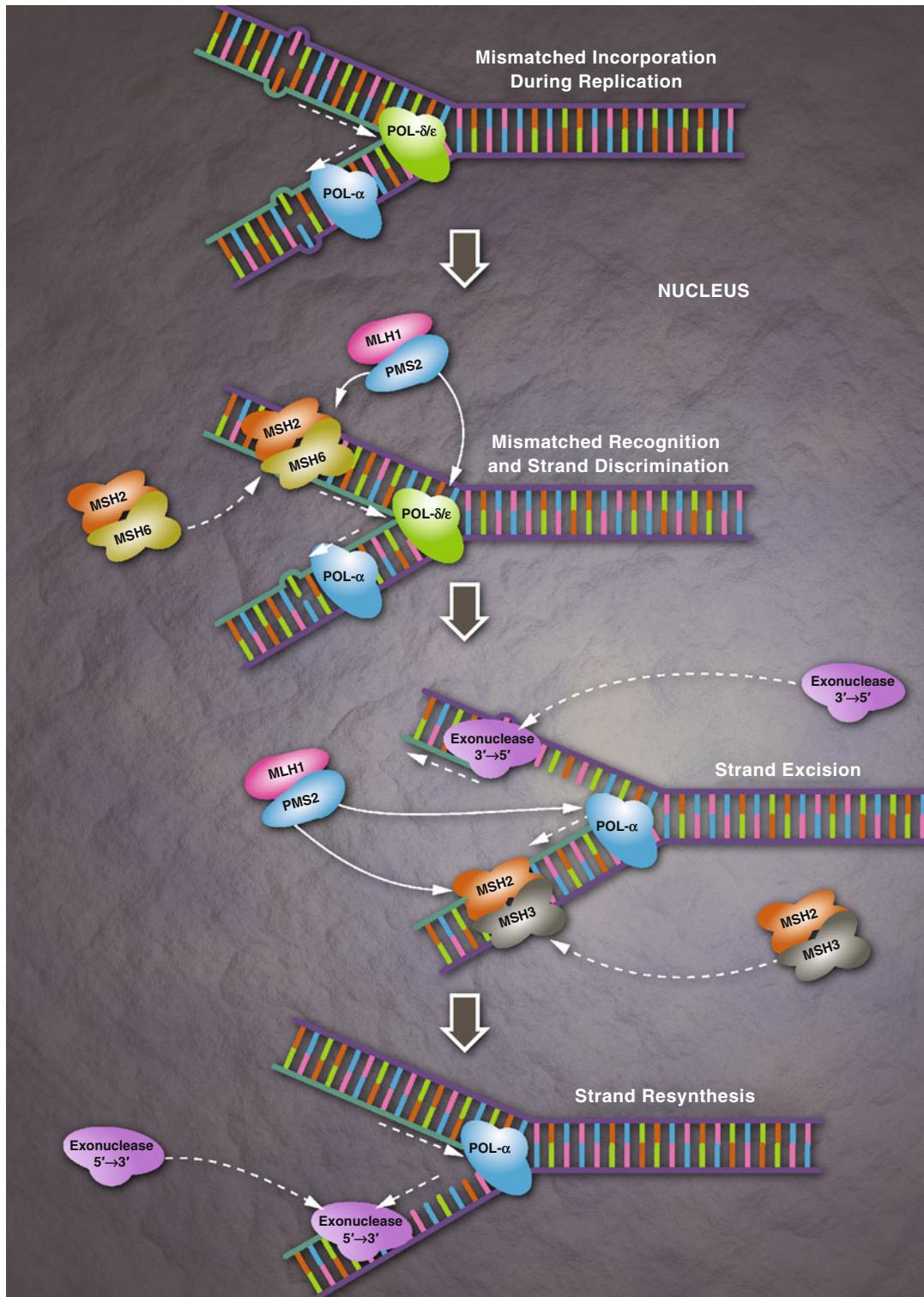
As the genetic and biologic underpinnings of these tumors continue to unfold, molecular testing will increasingly shape the definitions of

MTS and related entities. The emerging hallmark of MTS is the presence of genetic defects in DNA MMR machinery and, thus, microsatellite instability (MSI) [8]. This hallmark is shared with its phenotypic variants, Lynch syndrome and Turcot syndrome (central nervous system tumors) [11]. It is not unreasonable to now define these entities based on the presence of MMR defects and, until the link between MMR gene status and clinical expression becomes more fully understood, unify them under the header “familial MMR-defect-associated cancer syndromes.” Additionally, due to the differences in the biology of MMR-defective and MMR-normal tumors, it is not unreasonable to reclassify the non-MMR-associated MTS as a separate entity altogether, perhaps “familial cancer syndrome, not otherwise specified,” until the genetics underlying these tumors is more fully understood.

## 9.2 Pathophysiology of MMR-Defective MTS

During DNA synthesis, in preparation for cell division, incorrect or “mismatched” bases are incorporated into newly synthesized strands with relative frequency. MMR proteins along with DNA polymerases and exonucleases act in concert, through a series of complex steps, to identify and correct these errors (Fig. 9.1). Several key genes have been implicated as important in the MMR process—*human homologue of E. Coli MutL 1 (MLH1)* (located on 3p22.2), *human homologue of E. Coli MutS 2 (MSH2)* (2p21), *human homologue of E. Coli MutS 6 (MSH6)* (2p16.3), and *postmeiotic segregation increased, S. cerevisiae, 2 (PMS2)* (7p22.1) [12, 13]. Other family members—*PMS1*, *MLH3*, *MSH3*, *MSH4*, and *MSH5*—are also involved in the MMR process but have a less clear role in oncogenesis [14].

Most individuals with MTS have a germline defect in one allele of one of the repair genes involved in MMR [9, 15, 16]. This germline defect can be passed to the individual’s offspring, in an autosomal dominant fashion. Most commonly, the inherited mutated gene leads to a truncated non-functional protein. Inactivation of the other allele,



**Fig. 9.1** The DNA mismatch repair (MMR) process. MMR is a complex process that recognizes, removes, and fixes errors in DNA synthesis/replication. These functions are achieved by MMR molecules, exonucleases, and DNA polymerases, respectively, with a series of molecular

interactions (*solid arrows*). Molecules constantly move on, off, and along the DNA strand (*dotted arrows*). Defects in this machinery will cause propagation of errors and potentially oncogenesis. Key molecules in MMR are shown. POL—DNA polymerase

in accordance with the Knudson 2-hit model (leaving no remaining normal gene), leads to functional loss of the corresponding MMR protein [17]. Inactivation of the second allele may occur with alterations in the DNA sequence (somatic mutation) or with epigenetic silencing, such as hypermethylation. Without well-functioning MMR machinery, DNA errors (mutations) propagate and accumulate throughout the genome of dividing cells [18]. Most of these errors are biologically silent, but some may be detrimental to the cell. Bi-allelic (and inactivating) mismatch hits to tumor suppressor genes or, less commonly, single (activating) mismatch hits to oncogenes can drive oncogenesis in MTS [19].

In addition to oncogenesis, defects in the MMR machinery lead to a detectable epipheno-menon termed “microsatellite instability” (MSI). Microsatellites are noncoding stretches of DNA containing tandem repeats of one to six base pair segments, and the combined lengths of the tandem repeats vary between individuals. AAAA...AAAAAAA is an example of a mono-nucleotide repeat microsatellite, and ACGACGACGACG is an example of a trinucleotide repeat microsatellite. Stretches of DNA with mono- or dinucleotide repeats are particularly vulnerable to errors during DNA replication, possibly due to slippage by the polymerase. Tumors with MMR defects will exhibit changes in the lengths of these microsatellites, and when

this is detected, the tumor is said to have microsatellite instability, or MSI. Tri-, tetra-, and pentanucleotide repeats appear less vulnerable to errors. Normal nonneoplastic tissue and tumors without MMR defects maintain consistent microsatellite lengths with cell division, and these tumors are designated microsatellite stable, or MSS.

As mentioned, MMR defects and MSI are recurrent findings in tumors from MTS and Lynch syndrome patients [8, 9, 20–22] (Table 9.2). There are some important distinctions, however. In Lynch syndrome, over 95 % of colorectal tumors have MSI. Of these, defective MMR protein expression is observed with the following frequencies: MLH-1 (40 %), MSH-2 (40 %), MSH-6 (10 %), PMS-2 (5 %), and unknown (5 %) [30]. Frequencies of gene mutations mirror those of protein expression [16, 23]. With MTS, approximately two-thirds of patients (defined clinically) have sebaceous tumors with MSI [9]. Of these tumors, MSH-2 protein loss and *MSH2* gene mutations are more common than MLH-1 protein loss and *MLH1* mutations, respectively (and rare reports of *MSH6* mutations have been described [24, 25]). In some studies, the *MSH2:MLH1* ratio of germline mutations is as high as 10:1 in MTS as compared to the fairly even *MSH2:MLH1* ratio of germline mutations in Lynch syndrome [26]. Despite this skewed ratio in MTS, and suggestions that certain genes may

**Table 9.2** MMR proteins and gene mutations in Muir-Torre and Lynch syndromes

MMR protein	Protein loss in unselected sebaceous tumors (IHC) (%)	Protein loss in MSI sebaceous tumors (IHC) (%)	Gene mutations in MTS <sup>a</sup> (%)	Gene mutations in Lynch syndrome (%)
MSH-2	35–40	60–80	60–65	35–45
MLH-1	5–15	10–35	5–8	30–35
MSH-6	35–40	60–80	rare	5–10
PMS-2	5–20 <sup>b</sup>	25–35 <sup>b</sup>	NR	1–5
PMS-1	NR	NR	NR	rare
MLH-3	NR	NR	NR	rare
MSH-3	NR	NR	NR	NR
MSH-4	NR	NR	NR	NR
MSH-5	NR	NR	NR	NR

Abbreviation: NR not reported

Data compiled from multiple sources and presented as ranges [12, 13, 15, 16, 22–29]

<sup>a</sup>Percentages refer to relative frequencies of mutations in patients meeting clinical criteria for MTS or with MSI sebaceous tumors (percentages were similar in both groups) [15, 26]

<sup>b</sup>Only limited data available [13, 22, 27]

have a higher penetrance or a weaker phenotype (*MSH6*) [31, 32], to date, mutations in specific MMR genes are not linked to any specific clinical presentation [8, 10].

Another distinction between MTS and Lynch syndrome is the prevalence of MSI in their sporadic sebaceous and colon tumor counterparts. Approximately 15–20 % of all (unselected) colorectal cancers have MSI [33–35]. The majority of these MSI tumors are due to sporadic, not germline, defects in the MMR machinery. A common mechanism for sporadic MSI is the epigenetic silencing of *MLH1*, primarily by promoter hypermethylation [35]. In contrast, MSI does not appear to be a common event in sporadic (non-MTS) sebaceous tumors [9, 36].

### 9.3 Clinical Features

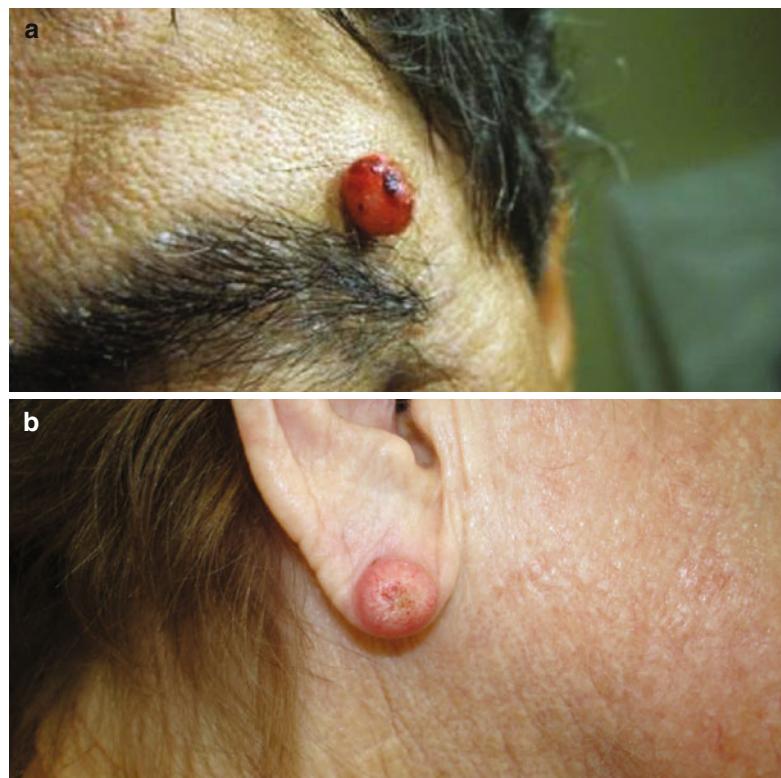
MTS is inherited in an autosomal dominant fashion with variable expression and high penetrance. Because MTS lacks a precise pathophysiologic or molecular definition, the true incidence is unknown. It is estimated that 0.05–0.5 % of individuals from Western countries carry a defect in a known MMR gene associated with Lynch syndrome. It is also estimated that 1–10 % of patients with Lynch syndrome fulfill the diagnostic criteria for MTS [10, 37]. Using these data, MTS may be expressed in roughly 1 in 20,000 individuals, but this is likely an underestimation. Cutaneous tumors (and therefore MTS diagnoses) are often under-recognized and underreported among Lynch syndrome patients, and this calculation does not include the 30 % of MTS patients that fulfill clinical criteria but lack known MMR defects.

As was observed in Muir's and Torre's original patients, MTS patients develop both visceral and cutaneous tumors [1–3]. The tumors may be synchronous or metachronous, sometimes separated in time by decades. By convention, a clinical diagnosis of MTS is reserved until individuals meet the full criteria, leaving many classified as “suspected” MTS, potentially for years. Patients with MTS often develop multiple primary visceral malignancies. The visceral tumors

occur at a younger age (usually under 50 years old) and behave in a slightly less aggressive fashion than their sporadic counterparts. Colorectal carcinoma accounts for 50 % of the MTS-related visceral malignancies and is usually right sided, especially in older patients. Genitourinary malignancies (endometrium, ureter, renal pelvis, prostate) account for about 25 %. The remaining 25 % is a mixture of breast, lymphoreticular (leukemias and lymphomas), laryngeal, and other malignancies [3, 38]. Colorectal, small bowel, endometrial, and ureter tumors have shown clear association with MSI, but less is known about the MSI status of the other less commonly reported malignancies.

There is a variety of cutaneous tumors associated with MTS (Fig. 9.2). The hallmark is the sebaceous neoplasm. A sebaceous adenoma, particularly outside of the head and neck region, is virtually pathognomonic for MTS. Sebaceous adenomas more often, however, occur on the head and neck and consist of flesh-colored or yellow papules. They may become large and cystic. Sebaceous carcinomas can occur anywhere but usually (75 %) on the head and neck [39]. Sporadic sebaceous carcinomas also occur on the head and neck but tend to occur on older individuals and aggregate around the eyelids, associated with meibomian glands. Sebaceous carcinomas also often have a yellowish hue but may become large and hemorrhagic and ulcerate. They may clinically overlap with other malignancies like basal cell carcinoma as well as inflammatory processes such as rhinophyma or a chalazion. Other sebaceous tumors—sebaceous epithelioma, sebaceoma, basal cell carcinoma with sebaceous differentiation, and keratoacanthomas with sebaceous differentiation—have all been described in MTS. A minority of patients, approximately 20 %, have keratoacanthomas without sebaceous tumors [40]. Other sebaceous lesions such as sebaceous hyperplasia, nevus sebaceus of Jadassohn, and steatocystomas have been reported in MTS, but these lesions have no direct association with MTS or direct evidence for MSI [41]. Sebaceous tumors are sufficiently unusual that their identification often prompts an initial evaluation for MTS. *Approximately*

**Fig. 9.2** Clinical features of Muir-Torre syndrome. MTS patients develop cutaneous tumors, specifically sebaceous tumors and keratoacanthomas. This elderly man developed a red and yellow polypoid, focally ulcerated and vascular-appearing sebaceous carcinoma on the left temple (a). Keratoacanthomas are also common in MTS, as shown with this dome-shaped, crateriform, keratotic tumor on the earlobe of this 49-year-old woman (b). Diagnoses were confirmed by histology (Images courtesy of Dr. Alan Menter, Dallas, TX, and Dr. Stephen Weis, Ft. Worth, TX)



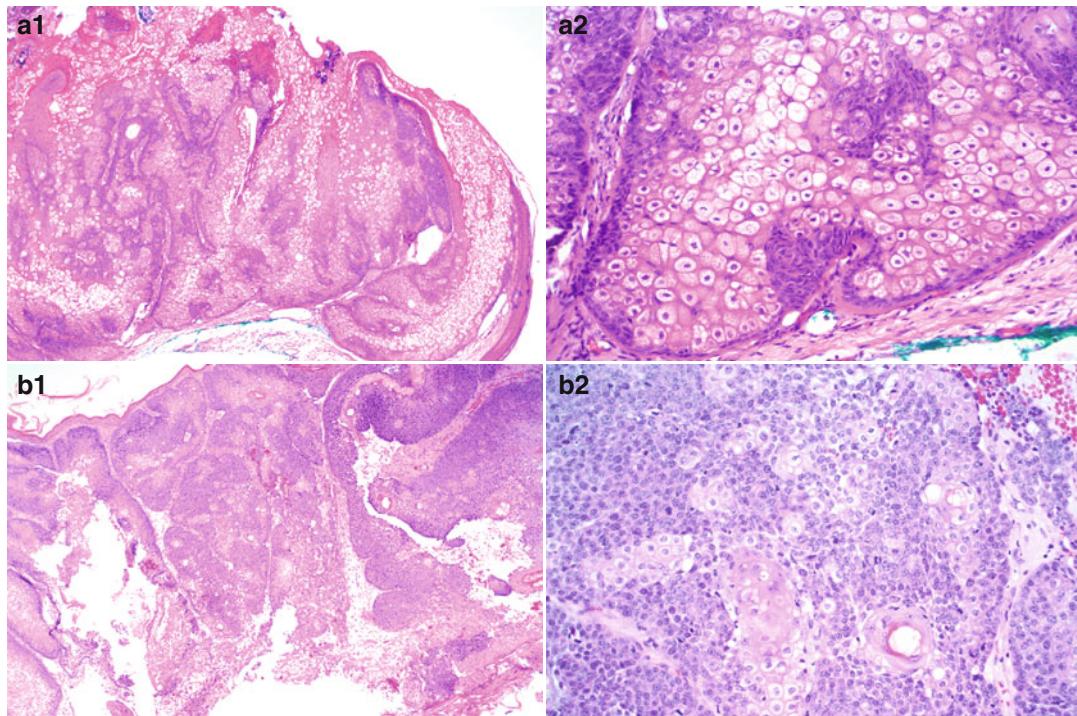
50–60% of patients ultimately diagnosed with MTS are first identified by a dermatologist or dermatopathologist following a biopsy of a sebaceous lesion, prior to the development of a visceral malignancy [10, 42].

#### 9.4 Histologic Features

MTS is associated with a histologic spectrum of sebaceous tumors. For practical purposes, these can be divided into sebaceous adenomas and sebaceous carcinomas, differentiated by standard morphologic criteria for malignancy (Fig. 9.3). Some authors have described intermediate entities—termed “sebaceomas” and “sebaceous epitheliomas,” for example—both of which have been reported in MTS [43, 44]. In general, these terms should be avoided as their criteria are ill defined, resulting in poor inter-observer variability [45], and their use causes confusion among clinicians.

Sebaceous adenomas are well-defined proliferations, arranged in lobules. These may have a

central cyst or dell and may appear keratoacanthoma-like. This tumor is usually superficial and directly attaches to the epidermis, without a mature follicle. The lobules are lined by germinative cells, usually two to three layers, or so, in thickness, but may account for a significant portion of the tumor. This periphery of the lobules contains very basaloid cells, with high nuclear to cytoplasmic ratios, and usually stands out in comparison to common sebaceous hyperplasia. Few scattered mitoses are typical. The basaloid cells transition to cells with more obvious sebocytic differentiation in the central portion of the lobules. These central cells have clear, bubbly cytoplasm, often indenting a small, crenulated nucleus, and usually account for the majority of the cells in the lesion. Benign lesions with a prevalent basaloid component may fall into the category of sebaceoma. Small ductules may be present. There is no evidence that sebaceous hyperplasia is a precursor to an adenoma, and sebaceous hyperplasia, as mentioned, is not directly associated with MTS.



**Fig. 9.3** Histologic features of sebaceous tumors. MTS patients can develop a range of sebaceous tumors. A 54-year-old man had multiple sebaceous tumors. This sebaceous adenoma has well-defined borders and is arranged in lobules (**a1**, H&E, 40 $\times$  original magnification). The lobules have a thin basaloid peripheral layer transitioning to mature sebocytes in the center of the lob-

ules. Overt cytologic atypia is not present (**a2**, H&E, 200 $\times$ ). In contrast, this well-differentiated sebaceous carcinoma from the cheek of a 47-year-old man is large and poorly organized with focal necrosis (**b1**, H&E, 40 $\times$ ). Sebaceous carcinomas have a greater proportion of basaloid cells with more mitoses and more prominent cytologic atypia (**b2**, H&E, 200 $\times$ )

Sebaceous carcinomas are histologically less organized than their benign adenoma counterpart. The architecture may appear well demarcated from the adjacent dermis or may be quite infiltrative. Pagetoid growth and invasion of adnexal units may be observed, more commonly in a periocular location. True necrosis (and not mere holocrine secretion) is a helpful clue for malignancy when present. The cytologic atypia and mitotic activity usually exceed those of adenomas. Regarding the atypia, there is often pleomorphism and the most mature-appearing cells with bubbly cytoplasm often retain their enlarged nuclei and nucleoli, unlike the small crenulated nuclei observed in adenomas and hyperplasia.

The sebaceous tumors in MTS have been reported to be cystic or even keratoacanthoma-like [46]. There is likely an overlap between these keratoacanthoma-like sebaceous tumors and true

keratoacanthomas in MTS. This cystic morphology, including keratoacanthomas with sebaceous elements, seems to be fairly specific for MTS, but in most cases, the clinical and histologic features of MTS sebaceous tumors and sporadic (non-MTS) tumors are indistinguishable.

## 9.5 Immunohistochemical Features

Immunohistochemistry can be helpful in the diagnosis of a sebaceous tumor, in some circumstances, but its main role is for identifying defects in the MMR machinery (see below). Usually, the sebaceous origin of the tumor is morphologically evident, but sometimes sebocytic differentiation is not so clear. Tumors may be pleomorphic and clear or virtually all basaloid. In ambiguous

cases, the differential diagnosis includes a range of epidermal, follicular, eccrine, and metastatic tumors. Some authors have reported utility of EMA, cytokeratin 7, androgen receptor, Ki-67 (K2 clone), adipophilin, and/or factor XIIIa (nuclear staining) (all positive in sebaceous tumors) to help in challenging cases. Sebaceous tumors are usually negative for CEA and BerEP4, the latter often diffusely positive in basal cell carcinomas. Oil Red O can be effective for identifying fat (or sebocytic differentiation) on frozen material/sections.

## 9.6 Assessing MMR Defects: Immunohistochemistry and PCR-Based Assays

Typically, a diagnosis of MTS is first suspected after identifying a sebaceous adenoma or carcinoma (or, less commonly, multiple keratoacanthomas) on biopsy. Complete evaluation for MTS should include assessment for MMR gene defects. This can be achieved by evaluating the tumor for loss of protein expression involved in DNA repair or looking for MSI. The former utilizes immunohistochemical analysis, and the latter is a PCR-based assay. These assays provide different, and potentially complementary, data.

### 9.6.1 Immunohistochemistry for MMR

Immunohistochemistry (IHC) is the most common modality for assessing the MMR machinery, as testing is easily assimilated into the pathology laboratory. IHC is performed on the tumor, usually a sebaceous neoplasm, but keratoacanthomas may also be tested. There are four main MMR proteins commonly tested—MLH-1, MSH-2, MSH-6, and PMS-2 (Fig. 9.4). In the IHC analysis for MMR defects, the *absence* of expression is considered a *positive* or *abnormal* test, suggestive of MTS. MMR proteins are normally expressed in virtually all human cells, and therefore, positive nuclear staining in adjacent keratinocytes, inflammatory cells, and stromal cells is expected and acts as a

good internal control. Approximately 60 % of unselected sebaceous tumors have abnormal MMR protein expression by IHC [12, 27]. And as presented in Table 9.2, the majority of unselected and MTS-associated sebaceous tumors have abnormal loss of MSH-2 and/or MSH-6 expression, and the most common mutated gene in MTS is *MSH2* [12, 13, 15, 16, 22, 25–29]. Concurrent loss of MSH-2 and MSH-6 expression is common, and the percentage of cases with MSH-2 loss often mirrors the percentage with MSH-6 loss in most studies. MSH-6 and MSH-2 form a stable heterodimer, and MSH-6 becomes unstable when not bound to MSH-2 [47]. This likely explains the high frequency of observed MSH-6 loss by immunohistochemistry but low frequency of *MSH6* gene mutations [12]. Similarly, it is not uncommon to observe concurrent MLH-1 and PMS-2 loss [27]. This phenomenon is underreported but also likely attributed to dimerization. In this setting, *MLH1* is the more commonly mutated gene. Other combinations of protein losses can also occur, but these are rare events.

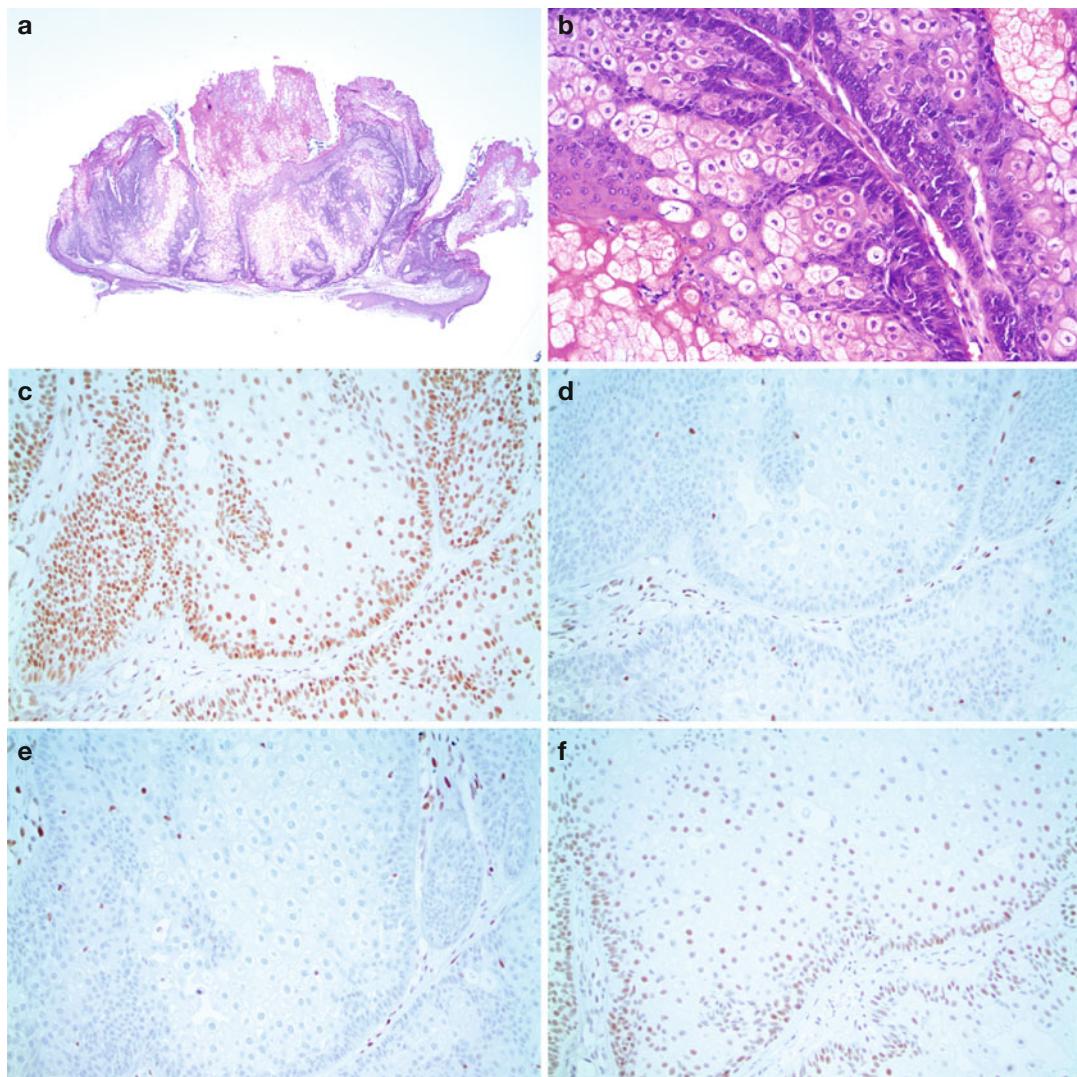
<sup>1</sup>CPT coding:

88342 (immunohistochemistry, per antibody).

### 9.6.2 Molecular MSI Testing

An alternative approach to detecting MMR defects in MTS is by a molecular PCR-based assay. This is a functional assay, assessing the tumor's ability to repair errors in DNA replication. Newer generation MSI assays focus on mononucleotide microsatellites, as these are the most vulnerable to DNA errors and are most likely to be altered in tumors with MMR machinery defects. The following are examples of mononucleotide microsatellites used in commercial assays: NR-21 (poly A), BAT-26 (poly A), BAT-25 (poly A), NR-24 (poly A),

<sup>1</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [48]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).



**Fig. 9.4** Immunohistochemical analysis for mismatch repair defects. Sebaceous adenomas are virtually pathognomonic for MTS, as in this case from the back of a 54-year-old man (same patient as Fig. 9.3a). The tumor is arranged in lobules (a, H&E, 20 $\times$  original magnification) with a basaloid peripheral layer transitioning to mature seocytes in the center (b, H&E, 200 $\times$ ).

Immunohistochemical analysis for MMR proteins is interpreted as normal (nuclear staining present) or abnormal (nuclear staining absent). This tumor has the following pattern, which is typical for MTS: MLH-1, normal (c, 200 $\times$ ); MSH-2, abnormal (d, 200 $\times$ ); MSH-6, abnormal (e, 200 $\times$ ); PMS-2, normal (f, 200 $\times$ )

and MONO-27 (poly A) (Promega Corporation, Madison, WI) [49]. Most MSI assays amplify the microsatellite regions by PCR and separate the amplicons by size using capillary electrophoresis, creating an electropherogram. In patients with MMR defects, the sizes of the amplicons from tumor and normal tissue DNA will vary since the tumor-specific DNA is prone

to errors, or slippage, during DNA replication. The end result is a shift, or other alteration, in the amplicon size profile (Fig. 9.5). By convention, if  $\geq 30\%$  of microsatellites *or* at least 2 of 5 mononucleotide microsatellites *or* at least 2 of 5 of the National Cancer Institute (NCI) mono-nucleotide and dinucleotide microsatellites demonstrate an altered profile, this is considered

MSI high (or MSI-H) and has a high probability for MTS (or Lynch syndrome) [20, 50–52]. If 1–29 % of microsatellites *or* only 1 of 5 of the mononucleotide microsatellites *or* 1 of 5 NCI loci is affected, this is MSI low (or MSI-L), and further testing is recommended. If no microsatellite loci demonstrate this shift, the tumor is considered microsatellite stable (MSS), and MTS (or Lynch syndrome) is considered unlikely. Practically speaking, using newer methods with only mononucleotide repeats as microsatellites, MSI is usually evident at all five loci (MSI-H) or at no loci (MSS), eliminating the MSI-L category.

Interestingly, electropherograms generated by MTS sebaceous tumors and Lynch syndrome colorectal tumors yield different profiles. In Lynch syndrome, colon tumors with MSI usually cause a leftward shift in all five mononucleotide microsatellite profiles (decreasing its length), with each microsatellite shifting by fairly consistent sizes [53]. Specifically, BAT-25 shifts leftward 5 bases (-5) and the others have similarly consistent leftward shifts: BAT-26 (-9), NR-21 (-7), MONO-27 (-6), and NR-24 (-7). Of course, there are exceptions, but these leftward shifts highlight the fact that deletions of mononucleotides are more common than insertions in Lynch syndrome colon tumors with MSI, and the consistency behind the microsatellite size changes suggests there may be some structural (deletion loops) or other physiologic explanation behind the DNA replication errors. In MTS MSI sebaceous tumors, however, there are many different ways that microsatellites get altered. They may increase, decrease, or increase and decrease, usually by several base pairs. The resulting electropherograms have left or right shoulders or become broadened in both directions (Fig. 9.6) [27]. Overall, the changes in microsatellite length in MTS sebaceous tumors are more variable but less dramatic than the microsatellite length changes in colorectal cancers of Lynch syndrome. This implies there may be different cellular mechanisms of MMR in the sebaceous tumors of MTS versus colon tumors of Lynch syndrome, but the pathophysiology behind these differences is not fully understood.

#### <sup>2</sup>CPT coding:

81301 (molecular MSI).

### 9.6.3 IHC Versus MSI

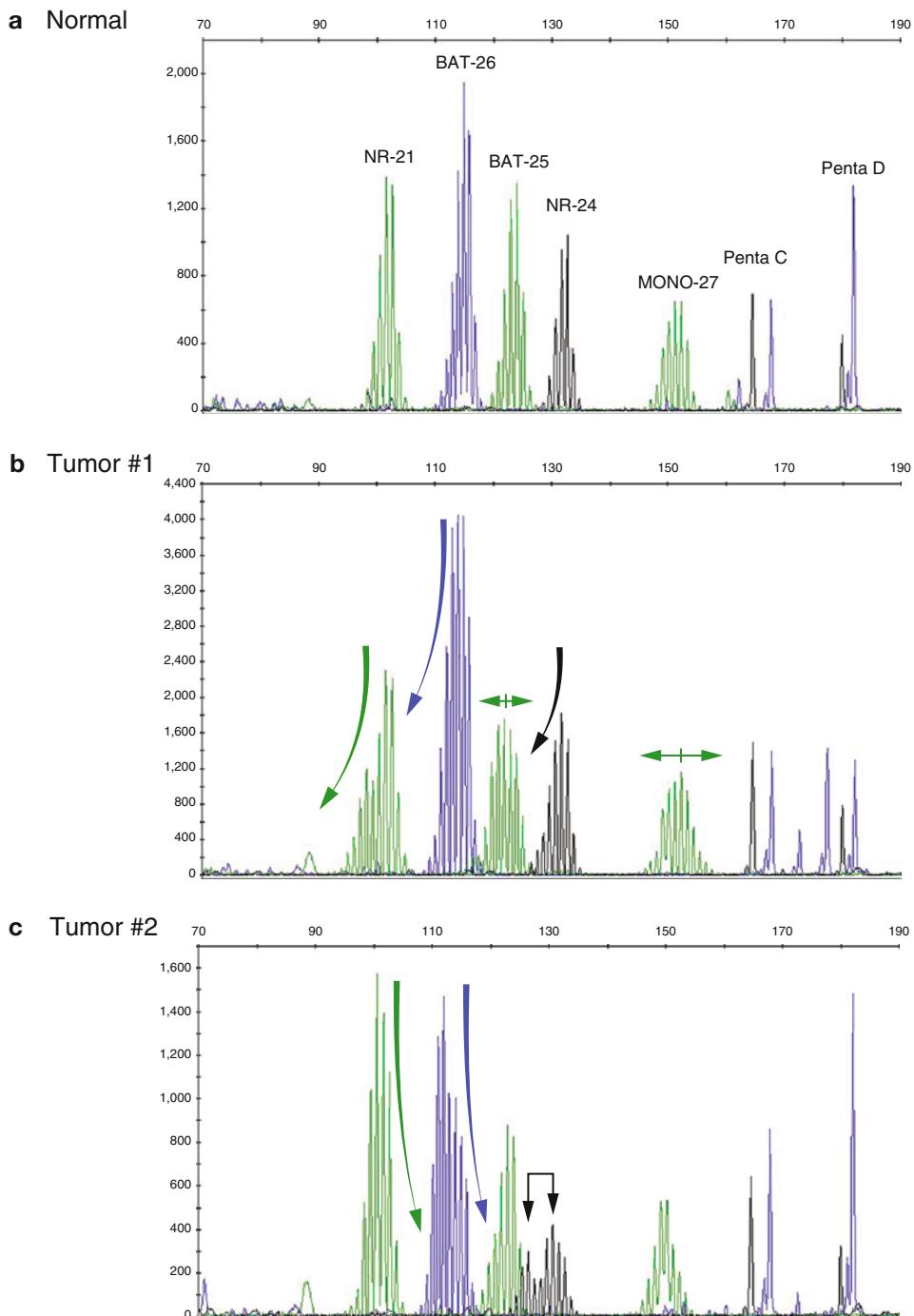
The concordance between IHC and MSI testing is strong, but not 100 % [54]. Both tests evaluate the MMR machinery but in a slightly different manner, and the positive and negative results of the two methods can have different meanings (Table 9.3). By performing both assays, the results can be complementary. Immunohistochemistry assesses the presence or absence of proteins involved in MMR, as detected by an antibody. A negative signal may be due to a mutation causing a truncated or absent protein, bi-allelic somatic mutation or promoter hypermethylation, an altered protein unrecognized by the antibody (functional or nonfunctional), or a false-positive result (negative staining) due to technical failure or flawed interpretation, among others. Of note, in the latter instances, the protein may still be functional and therefore not equate to the MSI-H phenotype [55, 56]. Conversely, expression of the MMR protein does not confirm its functionality, as it could be mutated or posttranslationally altered (but still recognized by antibody), or, again, there may have been a technical failure or flawed interpretation. Moreover, there are likely other genes/proteins directly or indirectly involved in MMR that are not yet discovered or routinely assayed for. Therefore, a subset of MSI-H patients may be missed by immunohistochemical screening alone [51].

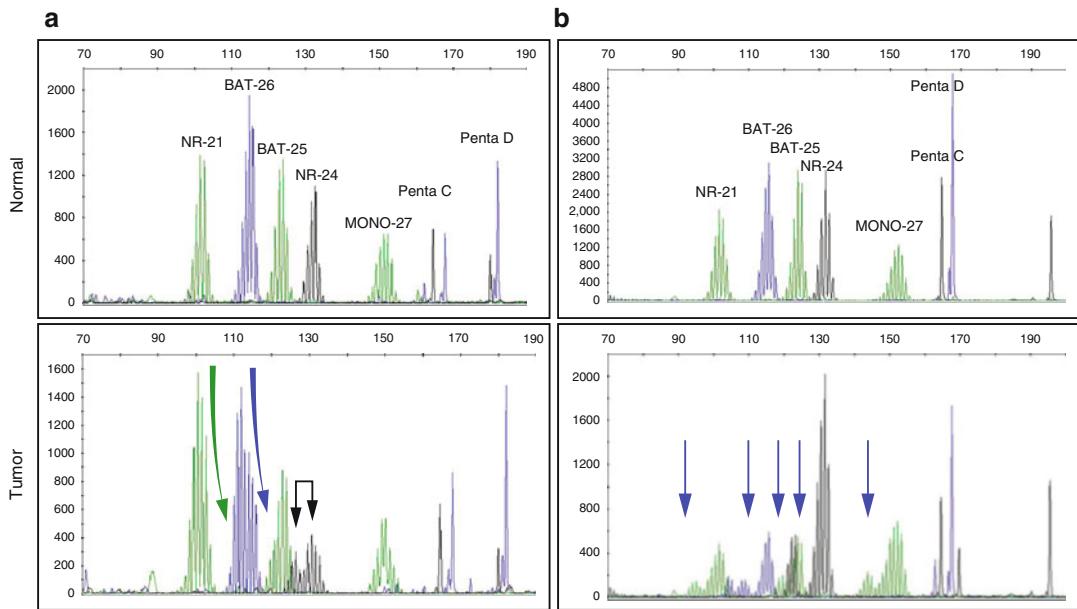
PCR-based MSI testing approaches 100 % sensitivity and specificity for identifying MSI-H tumors, regardless of the gene/protein affected. Interestingly, some MMR gene defects, such as *MSH6*, may not always result in MSI-H tumors, but may be MSI-L or even MSS [12]. These could be missed by standard MSI testing. MSI

<sup>2</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [48]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

testing does not distinguish germline from sporadic or epigenetic causes (nor does immunohistochemistry), and it does not identify the defective gene/protein. This latter point is not trivial

because identifying MMR gene defects can be arduous. These defects have been reported as point mutations, insertions, and deletions and span all exons, thus requiring direct sequencing





**Fig. 9.6** MSI electropherograms for MTS and Lynch syndrome. Molecular MSI testing yields different electropherogram patterns when comparing sebaceous tumors of MTS patients (**a**, same patient as Fig. 9.5) and colon cancers of Lynch syndrome patients (**b**, 42-year-old female). As described in Fig. 9.5, normal and tumor tissue were microdissected and DNA was extracted. MSI analysis was performed and analyzed per manufacturer instructions (Promega Corporation, Madison, WI) [49]. In normal tissue with intact MMR machinery, amplification of the five

mononucleotide microsatellites results in five peaks with a Gaussian distribution of amplicon sizes (**a, b, top panels**). In sebaceous tumors from MTS patients, microsatellite amplicon sizes have more subtle shifts, resulting in left or right shoulders, broadened peaks, or, occasionally, completely shifted peaks (**a, bottom panel, arrows**). Colon cancers from Lynch syndrome patients have more dramatic and consistent shifts in microsatellite amplicon sizes (**b, bottom panel, arrows**) compared to the electropherograms of sebaceous tumors from MTS patients

of the entire gene(s) and increasing cost for their detection [57]. Immunohistochemical loss offers the advantage of knowing which gene to target for sequencing.

#### 9.6.4 Genetic Testing

Genetic testing is an important component in the workup for MTS. Not only does genetic

**Fig. 9.5** Molecular analysis for microsatellite instability. Molecular MSI was performed on normal tissue (**a**), a sebaceous adenoma from the jaw (**b**), and a sebaceous adenoma from the thigh (**c**), all from the same 65-year-old man. Following microdissection of normal and tumor from formalin-fixed and paraffin-embedded tissue, DNA was extracted and MSI analysis was performed per manufacturer instructions (Promega Corporation, Madison, WI) [49]. Five mononucleotide microsatellites—NR-21 (green, left), BAT-26 (blue), BAT-25 (green, middle), NR-24 (yellow, changed to black), and MONO-27 (green, right)—and two pentanucleotide microsatellites, Penta C and Penta D, were amplified by PCR. The amplicons were separated by size using capillary electrophoresis, creating electropherograms, and analyzed with GeneMapper software. In normal tissue with intact MMR machinery,

amplification of the five mononucleotide microsatellites results in five peaks (**a**, normal tissue). Slight variations in amplicon size result in a Gaussian distribution for each peak. In tumor tissue with defective MMR machinery, microsatellite amplicons will have more gross variations in sizes, resulting in different electropherogram patterns (**b**, sebaceous adenoma of jaw; **c**, sebaceous adenoma of thigh). The Gaussian distributions may change to profiles with left or right shoulders, broadened peaks, or shifts with separate peaks (*arrows*). In MSI-H tumors, usually all five mononucleotide microsatellites are affected, as in this case. Pentanucleotide microsatellites usually do not change sizes with MSI tumors (although it did in the tumor from “b” and has been reported in a subset of tumors) and are useful to double-check that the tumor and normal tissue are from the same patient

**Table 9.3** Causes of normal and abnormal MMR immunohistochemistry and molecular MSI test results in the suspected MTS patient

Result	Primary causes	
	IHC	MSI
Normal	No MMR gene defect present Defect in other, non-tested MMR protein (or <i>MUTYH</i> mutation) Protein expressed but abnormal Flawed interpretation High background Other	No MMR gene defect present <i>MUTYH</i> or other non-MMR gene defect Insufficient tumor in sample Flawed interpretation
Abnormal	MMR gene defect present No protein Altered, nonfunctional protein Altered protein not recognized by Ab but functional Protein degraded due to mutated heterodimer partner gene  Somatic MMR gene defect Hypermethylation Bi-allelic somatic MMR gene mutations Defect in MMR gene/protein modifier (e.g., <i>EPCAM</i> ) Flawed interpretation Poor Ab/Ag reaction Other	Germline MMR gene defect (do not know which gene) Defect in MMR gene/protein modifier (e.g., <i>EPCAM</i> ) Somatic MMR gene defect Hypermethylation Bi-allelic somatic MMR gene mutations Patient heterozygous at microsatellite locus or loci Flawed interpretation

testing confirm the diagnosis and tumor biology of the tested patient, but it allows for a familial genetic signature to screen for carriers within families.

Most genetic testing is performed following extraction of DNA from peripheral blood leukocytes of the patient with suspected MTS. DNA from formalin-fixed biopsy material is often compromised by degradation and may be contaminated with tumor DNA and, therefore, should not be used for genetic testing. Testing usually requires full-gene sequencing as mutations can span virtually all exons of any known MTS-associated MMR gene. Many laboratories use some form of comprehensive sequencing (Sanger sequencing, next-gen or high-throughput sequencing, etc.) to evaluate the gene or genes. In cases with a known familial signature mutation, more pointed (and discounted) approaches, such as focused direct sequencing or pyrosequencing, for example, can be used.

Mutational analysis can be challenging to interpret. If the mutation results in an absent or

truncated protein, or if it has been previously characterized in Lynch syndrome or MTS families, then the mutation is likely biologically significant. These patients are considered to have MTS, and this mutation can be used to screen for carriers. In many cases, however, mutations (or variants) are identified and have unknown significance. These mutations may result in loss of protein function but may also be biologically irrelevant. Assuming these mutations are responsible for the MTS phenotype may inappropriately classify individuals and their family members. Current lists of MTS/Lynch syndrome MMR mutations can be found at [www.insight-group.org](http://www.insight-group.org) [23].

In most cases, a negative mutational analysis suggests the individual has a sporadic tumor, but this does not exclude the possibility of MTS or other familial cancer syndromes [15]. Germline mutations may be present in non-tested MMR genes or yet-to-be discovered genes that play a direct or indirect role in MMR. Individuals with *MUTYH* mutations of the related *MUTYH*-associated polyposis may clinically mimic MTS

and will have a normal MMR genetic screen. Moreover, the tested gene could have a large deletion or duplication or intronic/splice sequence mutations that may go undetected using a standard exon-only sequencing approach. Many laboratories will offer or reflex to more extensive sequencing protocols, to include splice regions, and deletion/duplication analysis (e.g., by array comparative genomic hybridization) when the initial mutational analysis is normal. A current list of laboratories that offer genetic testing can be found at [www.genetests.org](http://www.genetests.org) [58].

<sup>3</sup>CPT coding:

81292 (full *MLH1* gene analysis), 81293 (*MLH1* known familial mutation analysis), 81294 (*MLH1* deletion/duplication screen); 81295–81297 (similar *MSH2* gene analyses); 81298–81300 (similar *MSH6* gene analyses); 81317–81319 (similar *PMS2* gene analyses); 81401, 81406 (*MUTYH* common variants or full-gene sequence, respectively); 81479, which is the unlisted molecular procedure code, for all others.

## 9.7 Approach to the Suspected MTS Patient

Dermatologists' and dermatopathologists' practices vary greatly with respect to the evaluation and management of the suspected MTS patient. There are differences in patient selection for initiating a MTS workup, the extent of the workup, and the inclusion of a genetic counselor, to name a few. The primary reason for these differences is confusion on what defines or what is needed to define MTS. Additionally, the availability of specific tests and cost constraints also shape diagnostic algorithms.

<sup>3</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [48]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

### 9.7.1 Defining MTS

Given our current understanding of clinical features, genetics, and pathophysiology of MTS, patients who present to the dermatologist with sebaceous tumor(s) and/or keratoacanthoma(s) can be divided into four main groups: (1) *MTS* (MMR defect with a known mutation); (2) *MTS* (MMR defect but *no* known mutation); (3) *familial cancer syndrome, other* (increased genetic risk but MMR normal); and (4) *sporadic tumor* (no increased genetic risk).

The first two groups constitute what may be “true” MTS. These are the individuals with germline MMR defects and MSI tumors, allelic to Lynch and Turcot syndromes. Group 1 is further characterized by having a detectable loss-of-function mutation in a known MMR gene, and group 2 has the same phenotype, with MSI tumors, but apparently normal MMR genes (presumably due to a mutation in a modifier gene or a yet-to-be discovered MMR gene).

Group 3 includes patients with a strong family history of cancer, by using Amsterdam or equivalent criteria, but no evidence of MMR defects. These patients may meet the clinical criteria for MTS, but their tumors are MSS and therefore they are best separated from “true” MMR-defective MTS. There is no standard definition for this group, but these patients should be segregated from MTS and reclassified as having some alternate familial non-MMR-defect-related familial cancer syndrome.

The sporadic tumor group, group 4, is the largest. Most of these patients will have a single cutaneous tumor with no MMR defect. This group also includes patients with single tumors exhibiting MSI (sporadic MSI, such as hypermethylation) and patients with noninheritable multiple tumors, cutaneous and/or visceral. Note that this latter group of patients does not have a familial cancer syndrome but could be inappropriately classified as MTS using clinical criteria alone (see Table 9.1).

## 9.7.2 An Algorithmic Approach to the Diagnosis of MTS

Algorithms are useful to standardize the approach to the suspected MTS patient. Appropriately classifying patients based on tumor biology and heritable risk will allow for better management of these patients and their family members. While a standard approach would be ideal, algorithms for dermatologists, dermatopathologists, and laboratorians vary depending on patient demographics, test availability, cost, and expertise, among others. Variations are acceptable as long as there is full understanding of the meaning of the results and patients are appropriately classified.

Figure 9.7 is a suggested approach to the suspected MTS patient who presents with a MTS-related cutaneous tumor (sebaceous tumor or keratoacanthoma). Patients belonging to a family with a known MTS-related gene mutation should receive genetic counseling and be tested for that mutation. If the mutation is present, then that patient has MTS and should be managed accordingly. If the mutation is absent, the patient's pretest probability for MTS is reevaluated in the context of a *negative* family history.

All tests and testing algorithms should take into account the pretest probability for disease, since tests with very low pretest probability will have a false-positive rate that approaches or exceeds the true positive rate, questioning the meaning of a positive result. In the case of MTS,

the pretest probability is very low with a single keratoacanthoma on an elderly individual without personal or family history of malignancy, since keratoacanthomas are prevalent in the general population. In this scenario, this tumor should be considered sporadic and further testing is not warranted. The pretest probability for MTS cumulatively increases with each of the following: (1) keratoacanthoma on a younger patient, on non-sun-exposed areas, or with sebaceous differentiation; (2) multiple keratoacanthomas; (3) any sebaceous tumor; (4) any sebaceous tumor with cystic or keratoacanthoma-like histology; (5) any sebaceous carcinoma outside the periocular region or any sebaceous adenoma; (6) any sebaceous tumor outside the head and neck region; (7) any sebaceous tumor at age <50; (8) multiple sebaceous tumors; (9) personal history of malignancy, especially colon or endometrial cancer; and (10) family history of malignancy, especially colon or endometrial cancer [3, 39, 46, 59].

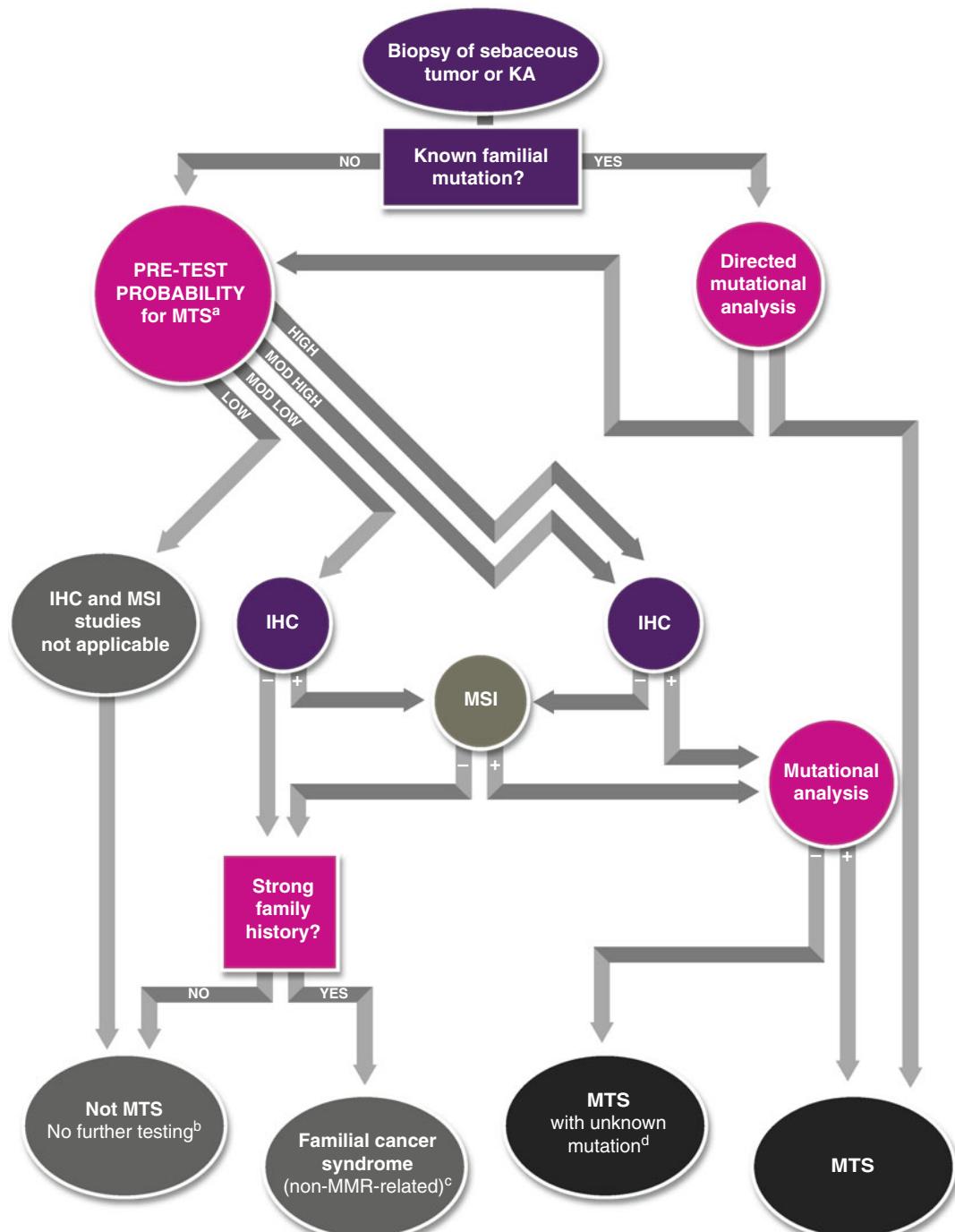
The overall pretest probability for MTS should determine whether or not to proceed with MMR testing. It is now fairly established that the presence of *a single sebaceous neoplasm* provides sufficient pretest probability to go forward [3, 7, 8, 28, 60]. A similar approach was recently adopted for Lynch syndrome, with many centers now proceeding with MMR testing on all colon cancers [6]. This approach was determined to be cost-effective, when taking into account cancer prevention for individuals

**Fig. 9.7** Flowchart algorithm to the presumed MTS patient. Testing progression and interpretation depend on the pretest probability for MTS. Most patients will funnel into one of four categories: (1) MTS with detectable mutation, (2) MTS with no known mutation, (3) a non-MMR-defect-associated familial cancer syndrome, and (4) sporadic tumors. Appropriate classification into these categories will help with management decisions of patients and their family members, including but not limited to cancer screening, treatment, and genetic testing.  
\*Pretest probability increases with any of the following: any keratoacanthoma on a younger patient, on non-sun-exposed areas, or with sebaceous differentiation; multiple keratoacanthomas; any sebaceous tumor; any sebaceous tumor with cystic or keratoacanthoma-like histology; any sebaceous carcinoma outside the periocular region or any sebaceous adenoma; any sebaceous tumor outside the

head and neck region; any sebaceous tumor at age <50; multiple sebaceous tumors; personal history of malignancy, especially colon or endometrial cancer; and family history of malignancy, especially colon or endometrial cancer sebaceous tumor. <sup>b</sup>Baseline colonoscopy and increased tumor surveillance of the patient should still be considered, especially if the original pretest probability for MTS was moderately high to high. <sup>c</sup>Testing for *MUTYH* mutations should be considered in patients with high pretest probability. <sup>d</sup>A small percentage within this group may have sporadic MSI (hypermethylation of *MLHI* promoter or bi-allelic somatic mutations) or may have *MSH2* promoter hypermethylation due to a germline deletion in *EPCAM* (these are primarily described with Lynch syndrome and would be considered rare for MTS). Testing for these can be considered in suspected cases

and their family members [61]. For MTS, the argument is even stronger with the relative rarity of sebaceous tumors, the low frequency of sporadic sebaceous tumors with MSI, and the high

correlation between sebaceous tumors and MTS (thus the high rate of informative MTS workup) when compared to colon cancer and Lynch syndrome. Moreover, because sebaceous tumors may



precede visceral malignancies by decades, there is an added benefit—identifying MTS individuals prior to the development of visceral tumors [3, 10, 42, 60].

As mentioned above, both immunohistochemistry (IHC) and molecular MSI testing can be used as surrogates for MMR gene defects. Currently, it is more practical to begin the MTS evaluation with IHC, since this is more widely available to most dermatopathologists [29, 62]. It should be reemphasized that IHC is not superior to MSI testing, but complementary (refer back to section on 9.6.3). Initial screening by MSI testing is a valid approach and may be preferred when available since costs of testing have dropped and testing identifies tumors with actual MSI, eliminating interpretive challenges of IHC.

There are several permutations to IHC panels for MTS. Some laboratories cut costs by testing for only one protein of each heterodimer, either a panel of MLH-1/MSH-2 or a panel of MSH-6/PMS-2 [63]. These approaches are valid as a screen, but, if possible, the preferred panel includes four antibodies—MLH-1, MSH-2, MSH-6, and PMS-2. Using this panel, loss of protein expression will point to the mutated gene, allowing for directed mutational analysis (Table 9.4). Another advantage to using the 4-antibody panel is that it may identify potentially false-positive and false-negative results. If there is equivocal staining by one antibody, a clue to the correct interpretation may reside with the staining for the heterodimer partner. In ambiguous cases or in cases with unusual patterns of protein loss, repeat testing is recommended, and if the results are still not clear, MSI testing should be performed. Immunohistochemistry for MLH-3, PMS-1, or other MMR protein has no current role in routine diagnostic testing.

MSI testing can be extremely valuable as an arbiter of equivocal IHC results and to confirm IHC results that are out of alignment with the pretest probability. For example, for patients with a very high pretest probability of MTS (sebaceous tumor, strong family history, etc.), normal IHC results should be followed by MSI testing. Likewise, in patients with a moderately low pretest probability for MTS (e.g., an elderly man

with sebaceous carcinoma on the eyelid with no personal or family history of malignancy), abnormal IHC results should be confirmed by MSI. A recent study by Roberts et al. determined that an abnormal MMR immunohistochemical result had a sensitivity of 85 % and specificity of only 48 % when evaluating for MTS (using gene mutations as a gold standard) [55]. The positive predictive value for immunohistochemistry became significantly higher if there was a personal or family history of colorectal cancer, prompting the authors to discourage genetic testing for isolated sebaceous tumors without a suggestive clinical history. While it is debatable that gene mutational analysis should be used as a gold standard for MTS, their findings support the notion that the pretest probability should be high prior to MMR-defect analysis and perhaps MSI should be used more often when the IHC result conflicts with the pretest probability.

Once defects in MMR have been confirmed, the patient should be referred for genetic testing. Ideally, presumed MTS patients should be consented prior to initiating any MMR analysis. This is not routinely performed, but because sporadic MSI is rare in cutaneous tumors, MMR defects by IHC or MSI testing are strong surrogates for germline mutations, and individuals should, at the very least, be made aware of the implications. For those who do not adhere to this logic (arguing that IHC/MSI testing only *indirectly* assesses gene defects), consent with genetic counseling is still required at the stage of gene mutational analysis. Genes should be tested for mutations using IHC analysis as a guide (see Table 9.4). If IHC data is not available, mutational analyses should proceed based on reported frequencies of mutations in MTS: *MSH2>>MLH1>>MSH6, PMS2*.

Using this testing progression, from pretest probability assessment to genetic testing, most presumed MTS patients can be appropriately classified into one of the following groups: (1) MTS (MMR defect with a known mutation); (2) MTS (MMR defect but no known mutation); (3) familial cancer syndrome, other (increased genetic risk but MMR normal); and (4) sporadic tumor (no increased genetic risk and MMR normal). There

**Table 9.4** Gene testing strategy for most common MMR IHC panel results

MMR IHC result <sup>a</sup>				Targeted MMR gene
MLH-1	MSH-2	MSH-6	PMS-2	
N	N	N	N	None <sup>b</sup>
A	N	N	A	<i>MLH1&gt;&gt;PMS2</i>
A	N	N	N	<i>MLH1</i>
N	A	N	A	<i>MSH2&gt;&gt;MSH6<sup>c</sup></i>
N	A	N	N	<i>MSH2<sup>c</sup></i>
N	N	A	N	<i>MSH6&gt;MSH2</i>
N	N	N	A	<i>PMS2&gt;MLH1</i>
N	N	?	?	None <sup>b</sup>
A	N	?	?	<i>MLH1&gt;&gt;PMS2</i>
N	A	?	?	<i>MSH2&gt;&gt;MSH6<sup>c</sup></i>
?	?	N	N	None <sup>b</sup>
?	?	A	N	<i>MSH2&gt;&gt;MSH6<sup>c</sup></i>
?	?	N	A	<i>MLH1&gt;&gt;PMS2</i>
?	?	?	?	<i>MSH2&gt;&gt;MLH1&gt;&gt;MSH6,PMS2</i>

*Abbreviations:* N normal (present) expression, A abnormal (absent) expression, ? unknown or not performed

<sup>a</sup>Other combinations may occur but are rare, and MSI testing should be considered in those cases

<sup>b</sup>*MUTYH* testing can be considered if pretest probability for familial cancer syndrome is high

<sup>c</sup>*EPCAM* deletion testing can be considered when no *MSH2* mutation is identified (more commonly described for Lynch syndrome)

are several scenarios that do not fit neatly into these categories but should also be considered:

- *MUTYH* gene defects (mutY homologue, 1p34.1). A percentage of individuals with sebaceous tumors may have a strong family history of malignancies, but no evidence for MMR defects by IHC, MSI, or MMR gene mutational analysis (segregating into diagnostic group 3 above). A subset of these individuals may have *MUTYH*-associated polyposis (MAP), and testing for *MUTYH* gene mutations should be considered [64].
- *EPCAM* deletions (epithelial cell adhesion molecule, 2p21). Up to 20 % of Lynch syndrome patients with abnormal *MSH2* expression by IHC have no identifiable *MSH2* mutation. Up to 20 % of this group has a deletion in *EPCAM*, leading to hypermethylation of the *MSH2* promoter, leading to MSI [65]. While this phenomenon is not well described in the setting of MTS, *EPCAM* deletion testing can be considered when *MSH2* gene mutations are suspected but genetic testing is normal (diagnostic group 2).
- Sporadic MSI. Hypermethylation of the *MLH1* promoter leading to MSI is well described for

colon cancer. These individuals do not have a germline mutation and therefore do not have Lynch syndrome. Hypermethylation of *MLH1* is considered a rare event in sebaceous tumors, but if this occurs, individuals will have abnormal IHC/MSI with no identifiable mutation of *MLH1* (segregating into diagnostic group 2). *MLH1* promoter methylation studies can be performed. These individuals should be moved to diagnostic group 4.

Patients in the MSI/MMR-defective groups (groups 1 and 2) should be monitored with increased tumor surveillance and treated as outlined for Lynch syndrome [6]. If a signature mutation has been established, mutational analysis should be offered to family members to determine carrier status. Genetic screening can also be considered (with appropriate counseling) when there is no known signature mutation or when tumor is not available for IHC/MSI testing. For group 3 patients and family members, increased tumor surveillance remains recommended. Patients with sporadic tumors can be reassured, but baseline colonoscopy and increased tumor surveillance should still be considered on a case by case basis.

## 9.8 Summary

The Muir-Torre syndrome, as originally defined, casts a wide net, capturing a group of genetically heterogeneous individuals linked only by the presence of cutaneous and visceral tumors. As the genetics underlying these tumors are revealed, molecular testing is at the forefront of MTS reclassification. Defects in the mismatch repair (MMR) machinery with tumor microsatellite instability are hallmarks of MTS and are emerging as requisites for diagnosis. MMR defects are now identified using a multipronged diagnostic approach, with most testing algorithms following variations of the following template: *clinical features → histology → immunohistochemical and/or molecular assessment of MMR → MMR gene sequencing*. By using this approach, with molecular testing playing a central role, most presumed MTS individuals and their family members can be appropriately classified and managed.

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# Genodermatoses. Part II: Other Hereditary Dermatologic Disease

10

## Contents

10.1	<b>Introduction</b> .....	254
10.2	<b>Genodermatoses Associated with Cutaneous and/or Visceral Tumors (Inheritable Tumor Disorders)</b> .....	257
10.3	<b>Inheritable Vascular Disorders</b> .....	263
10.4	<b>Inheritable Bullous Disorders</b> .....	263
10.5	<b>Inheritable Keratinization Disorders</b> .....	270
10.6	<b>Ectodermal Dysplasias and Other Inheritable Disorders of the Sweat Glands, Hair, Nails, and/or Teeth</b> .....	278
10.7	<b>Inheritable Connective Tissue Disorders</b> .....	278
10.8	<b>Inheritable Disorders of Pigmentation</b> .....	278
10.9	<b>Inheritable Metabolic Disorders</b> .....	278
10.10	<b>Miscellaneous Disorders</b> .....	301
10.11	<b>Practical Issues of Testing</b> .....	301
10.11.1	Testing Strategy .....	301
10.11.2	Interpretation .....	309
10.11.3	Cost and CPT Coding .....	309
10.12	<b>Summary</b> .....	311
	<b>References</b> .....	312

### Key Points

- Genodermatoses are inherited disorders with prominent dermatologic manifestations.
- These are a heterogeneous group of disorders with underlying DNA alterations ranging from single base substitutions to complex polygenic mechanisms.
- Molecular testing can confirm the diagnosis of a genodermatosis as well as identify genetic carriers and, in some cases, provide a rationale for therapy. Genetic counseling is an important component to molecular testing.
- Full-gene sequencing is the most common genodermatosis testing strategy, as most disorders are associated with not one specific mutation, but many different mutations spanning many exons.
- Other techniques, such as array comparative genomic hybridization, are often used in conjunction with (or as a reflex to) full-gene sequencing in order to identify large gene deletions and duplications.
- High-throughput (next-gen) sequencing is a powerful new technology capable of generating immense quantities of data in short periods of time. More and more commercial laboratories are transitioning to this technology for genetic screening, but this technology still has its limitations.

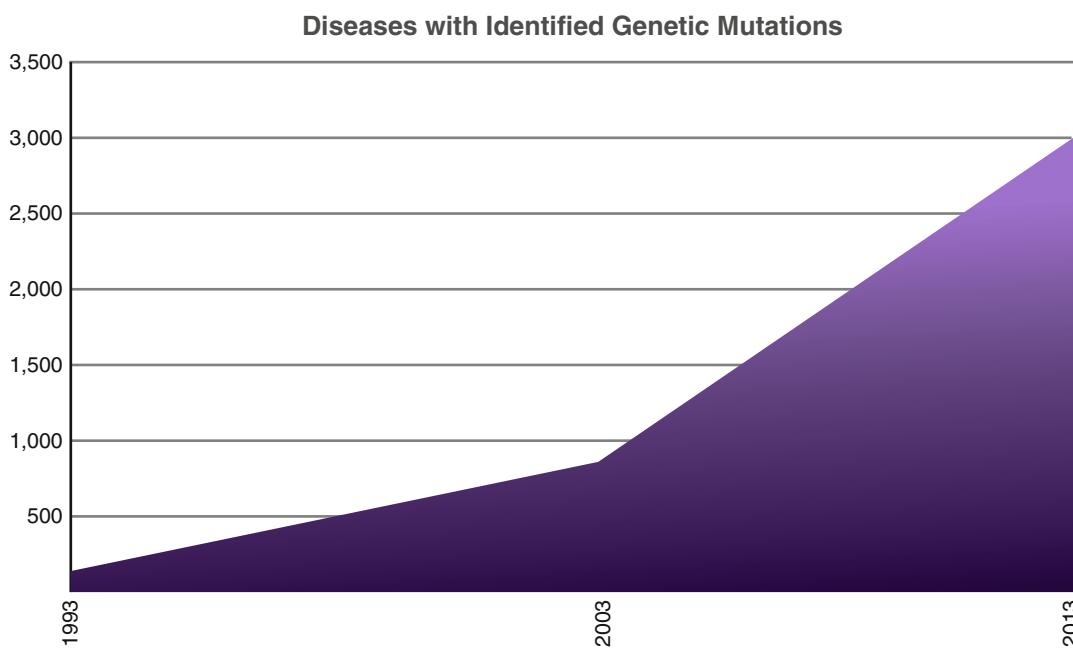
- Interpretation of genetic tests remains a challenge as more and more genetic variants are discovered with unknown biologic significance. The considerable sequence variation of the human genome highlights the importance of interpreting genetic results in the context of the individual patient and, when appropriate, involving genetic counseling.

ichthyosis [3]. Since the completion of the Human Genome Project in 2003 and the development of rapid, high-throughput sequencing technologies, the number of disorders linked to specific mutations has exploded (Fig. 10.1) [4, 5].

Genodermatoses are a heterogeneous group of disorders with a wide range of underlying DNA alterations. These alterations range from single gene mutations to contiguous gene deletions or larger chromosomal abnormalities to complex and polygenic mechanisms. By definition, genodermatoses are potentially inheritable since the mutations are germline, not somatic. Genodermatoses are typically inherited in classic Mendelian fashion—autosomal dominant (AD), autosomal recessive (AR), X-linked dominant (XLD), and X-linked recessive (XLR). As with other genetic diseases, more complex inheritance patterns exist, including X-inactivation, imprinting, and mitochondrial inheritance, to name a few. With many of the genodermatoses, individuals have sporadic, or de novo, mutations (not inherited by the parents, but in the individual's germline due to a mutation during gamete

## 10.1 Introduction

Genodermatoses are inherited disorders with prominent dermatologic manifestations. The Mendelian inheritance of human disease has been recognized for over a century [1, 2]. The discovery of specific gene mutations leading to genodermatoses is a more recent event, beginning approximately 25 years ago with the identification of steroid sulfatase mutations in patients with X-linked recessive



**Fig. 10.1** Trends in the identification of human disease-specific mutations. The number of disorders with a recognized genetic mutation has increased exponentially according to registered entities on the public website [www.genetests.org](http://www.genetests.org) [5]

production). And in many of these conditions, mutations are not passed on to offspring, because the conditions themselves preclude procreation.

In addition to differences in inheritance patterns, most genodermatoses have differences in disease expression. For example, individuals with a given disorder may range anywhere from mild to severe disease. *Penetrance* refers to the variable percentage of family members with the same germline mutation who express the disorder's phenotype. Complete penetrance means 100 % of individuals carrying the mutation have the disorder. Penetrance as well as clinical severity of disease can vary depending on differences in modifier genes, activation of alternate genetic pathways, and environmental factors. *Mosaicism* refers to an individual having more than one genetic population of cells in their body. Depending on the relative contribution of the mutated population, there can be quite variable disease expression. In dermatologic disease, mosaicism often manifests as affected skin or soft tissue in a segmental or Blaschkoian distribution.

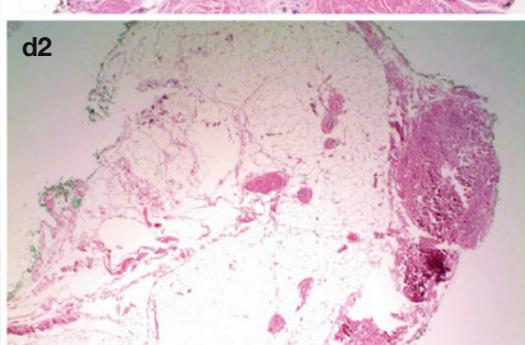
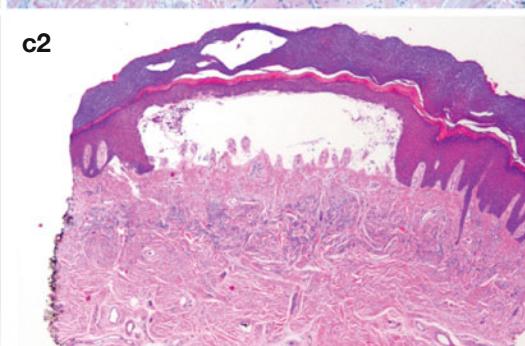
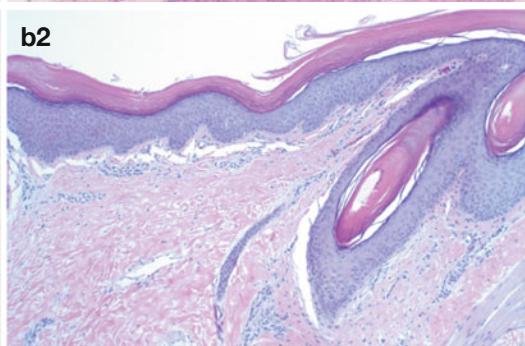
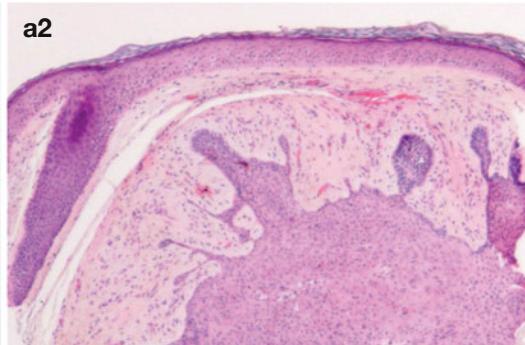
A variety of tools can be used to diagnose the genodermatoses, and with each disorder, different emphasis is placed on each tool (Fig. 10.2). Examples include the following:

- Clinical features and inheritance patterns (e.g., many)
- Laboratory studies (e.g., X-linked recessive ichthyosis, porphyria cutanea tarda)
- Tissue biopsy (e.g., Birt-Hogg-Dubé syndrome, Darier-White disease)
- Immunofluorescence (e.g., inheritable mechanobullous disease)
- Electron microscopy (e.g., inheritable mechanobullous disease, ichthyosis hystrix, Curth-Macklin type)
- Molecular analysis (e.g., many)

Regardless of the method, identification of patients with genodermatoses is important. Accurate diagnosis can lead to a better prediction of disease course and better management of the individuals and their family members through monitoring for other associated disease, determining appropriate cancer screening, and planning treatment strategies. Molecular studies have added potential benefits, which include the

following: (1) confirming the diagnosis, (2) providing prenatal information, (3) identifying a family signature mutation for screening carriers, and (4) predicting disease course by determining the specific mutational variant. Of course, there are many genodermatoses without known mutations. These are presumed to have a genetic basis due to inheritance patterns in families, and in some cases, linkage analysis to small regions of chromosomes. Every year, more and more gene mutations are linked to specific disorders, adding to our understanding of the pathophysiology and shaping the classification of the genodermatoses.

Genodermatoses are historically divided into several main categories, based on the primary dermatologic expression of the disease, for example, the bullous diseases, the ectodermal dysplasias, etc. These are subdivided based on inheritance pattern. As with other organ systems, the discovery of disease-driving mutations has dramatically revamped historical designations. An individual with a mutation in gene *A* may develop the same disorder as an individual with a mutation in gene *B*, a phenomenon termed "locus heterogeneity" (one clinical phenotype caused by mutations in different genes, e.g., *KRT5* and *KRT14* mutations in epidermolysis bullosa simplex, Dowling-Meara). Locus heterogeneity has caused some entities (many historically tagged with eponymic names) to be divided into two or more distinct entities based on the mutated gene. Relatedly, an individual with a mutation in gene *A* may develop disorder *X*, while an individual with a mutation in the same gene *A* may develop disorder *Y*, a phenomenon termed "allelic heterogeneity" (mutation in one gene causing two different clinical phenotypes, e.g., *GJB2* mutation in both keratitis-ichthyosis-deafness syndrome and Vohwinkel syndrome). In some cases, two disorders have been merged or reclassified into one based on this finding. Moving forward, classifications will undoubtedly continue to evolve to include gene mutation status, further reshuffling current classification schemes. For now, categorization by clinical phenotype remains useful, as, in many cases, the clinician observes a phenotype but needs a "differential diagnosis" of genes to



assay. This classic clinically oriented organization of the genodermatoses is preserved in this chapter, as it is probably the most convenient and practical method. Many genodermatoses, however, do not fit into a specific category and others bridge multiple categories. This must be kept in mind when searching for a particular entity.

As the list of genodermatoses continues to grow, for the purposes of this text, information on these entries is most efficiently presented in tabular form. For clarity, this chapter focuses on the genodermatoses with known gene mutations. Somatic mutations may also lead to cutaneous disease but are not included here. As diseases and gene names are in constant flux, the reader is directed to several websites for the most up-to-date designations. There is now a public online version of McKusick's Mendelian Inheritance in Man (OMIM) database that provides each genetic disorder with a unique six-digit identification number based on its inheritance pattern and gene mutation, found at [www.omim.org](http://www.omim.org) [6]. This database can be helpful when searching for the most current official name of a disease, which may oscillate from a syndromic name or eponym to a more descriptive label and, at times, back to the eponym. The most current gene names and chromosomal locations can also be found on the public website [www.hgmd.cf.ac.uk](http://www.hgmd.cf.ac.uk) [7]. Practical considerations regarding ordering and performing genetic tests are also briefly discussed.

## 10.2 Genodermatoses Associated with Cutaneous and/or Visceral Tumors (Inheritable Tumor Disorders)

The previous chapter provided an in-depth look at the Muir-Torre syndrome (MTS). MTS is a prototype for an inheritable cancer syndrome and utilizes molecular testing in multiple ways (microsatellite instability and gene mutational analysis) (see Chap. 9). Many other genodermatoses are associated with malignancies (Table 10.1). Identification of these patients is important because they may require a higher degree of cutaneous and visceral tumor surveillance. The genodermatoses with cutaneous tumors are somewhat unique because dermatopathologists often provide the first clue to the presence of a genetic disorder.

Because these genodermatoses are associated with tumor formation, many (especially the ones associated with malignancies) are characterized by defects in tumor suppressor genes (e.g., *CDKN2A*, *MSH2*). With tumor suppressor gene mutations, one affected allele is passed to the offspring with the other allele deactivated at a later date, leading to the disorder's phenotype according to Knudson's 2-hit model. Because the second hit occurs at a later date, the inherited mutated allele has the ability to be passed on to subsequent generations. Oncogenic-driving mutations are less common in the inheritable disorders (as opposed to somatic mutations) because their presence often prevents live births.

**Fig. 10.2** Select genodermatoses. Genodermatoses are a heterogeneous group of disorders. Depending on the disorder, diagnosis relies upon differently weighted combinations of clinical, laboratory, histologic, immunofluorescent, electron microscopic, and molecular data. Shown are examples of Birt-Hogg-Dubé syndrome (a), lamellar ichthyosis (b), hereditary porphyria (c), and mosaic overgrowth syndrome (d). The Birt-Hogg-Dubé syndrome patient is a 54-year-old female who presented with numerous, small, flesh-colored papules, most prominent on the lateral aspects of the nose and onto the nasomalar region (a1). Histologically, there was a central distorted follicular structure with delicately emanating follicular strands, diagnostic of fibrofolliculoma (a2, H&E, 40 $\times$  original magnification). This 6-year-old girl with lamellar ichthyosis presented with hyperkeratosis and increased skin markings from head to toe (b1). Histologically, there was orthokeratotic hyperkeratosis with a retained granular

layer, consistent with lamellar ichthyosis but requiring genetic studies for confirmation (b2, H&E, 100 $\times$ ). A 25-year-old man with hereditary porphyria cutanea tarda presented with skin fragility of the dorsal hands, consisting of milia and blistering (c1). Histologically, there was a paucicellular subepidermal blister with papillary festooning, suggestive of porphyria cutanea tarda (c2, H&E, 40 $\times$ ), and the diagnosis was confirmed by immunofluorescence, porphyrin, and genetic studies. This 15-year-old female presented with scoliosis and a soft tissue back mass, present since birth (d1). Histologically, the mass contained unremarkable mature fibroadipose tissue (d2, H&E, 20 $\times$ ). A diagnosis of the rare entity mosaic overgrowth syndrome (related to Proteus syndrome) was confirmed by molecular studies that identified the characteristic *PIK3CA* mutation in the mass but not in uninvolved skin (Several images courtesy of Dr. Travis Vandergriff, Dallas, TX, and Dr. Stephen Weis, Ft. Worth, TX)

**Table 10.1** Genodermatoses associated with cutaneous and/or visceral tumors

Disorder	Other names	Tumors	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Ataxia-telangiectasia AT Louis-Bar		Leukemia/lymphoma	AR	11q22.3	<i>ATM</i>	Ataxia-telangiectasia mutated	DNA repair defect	208900 607585
Basal cell nevus syndrome	BCNS Gorlin Gorlin-Goltz	Basal cell carcinoma, odontogenic keratocysts of jaw, fibromas, medulloblastoma	AD	9q22.32	<i>PTCH1</i>	Patched 1	Signaling pathway defect	109400 601309
			1p34.1	<i>PTCH2</i>	Patched 2			109400 603673
			10q24.32	<i>SUFU</i>	Suppressor of fused			109400 607035
			7q32.1	<i>SMO</i>	Smoothened			109400 601500
Bazex syndrome	Bazex-Dupré-Christol	Basal cell carcinomas	XLD	Xq24-q27	?	?		301845
Beckwith-Wiedemann syndrome (exomphalo-macroGLOSSIA-gigantism syndrome)	BWS	Wilms tumor, hepatoblastoma	AD sporadic Complex, imprinting	5q35.2-q35.3 11p15	<i>NSD1</i> <i>H19</i>	Nuclear receptor-binding Su-var, domain 1 Non-translated	Signaling pathway defect Epigenetic defect	130650 606681 130650 103280
					<i>KCNQ1OT1</i>	Antisense transcript	Epigenetic defect	130650 604115
Birt-Hogg-Dubé syndrome	BHD Hornstein-Knickenberg	Facial fibrofolliculomas, trichodiscomas, acrochordons; renal tumors	AD	17p11.2	<i>FLCN</i>	Folliculin	Signaling pathway defect Energy-nutrient sensing defect	130650 600856 607273
Bloom syndrome	BLS	Multiple cancers of skin, heme, connective tissues, germ cells, nerves, kidneys	AR	15q26.1	<i>BLM</i> ( <i>RECQL3</i> )	RecQ protein-like 3	DNA stability/repair defect	210900 604610
Brooke-Spiegler syndrome	BRSS	Cylindromas, spiradenomas, trichoepitheliomas	AD	16q12.1	<i>CYLD</i>	CYLD	Deubiquinating enzyme defect	605041 605018
		Multiple familial trichoepitheliomas						601606 605018
		Familial cylindromatosis						132700
Costello syndrome (faccutaneoskeletal syndrome)	FCS	Facial and perianal papillomas, rhabdomyosarcomas	AD	11p15.5	<i>HRAS (p211)</i>	GTPase HRas	Signaling pathway defect	218040 190020

Cowden syndrome	CWS1	Trichilemmomas Breast, thyroid, endometrial cancers	AD	10q23.31	<i>PTEN</i>	Phosphatase and tensin homologue	Signaling pathway defect	158350 601728
	CWS2			1p36.13	<i>SDHB</i>	Succinate dehydrogenase complex B	Mitochondrial enzyme defect	612359 185470
	CWS3			11q23.1	<i>SDHD</i>	Succinate dehydrogenase complex D		615106 602690
	CWS4		Hypermeth	10q23.31	<i>KLLN</i>	Killin	Promoter shared with <i>PTEN</i>	615107 612105
	CWS5		AD	3q26.32	<i>PIK3CA</i>	Phosphatidylinositol 3-kinase α	Signaling pathway defect	615108 171834
	CWS6			14q32.33	<i>AKT1</i>	RAC-α serine/threonine- protein kinase		615109 164730
Cowden-like	Cowden CWS1	Trichilemmomas Breast, thyroid, endometrial cancers	AD	10q23.31	<i>BMPR1A</i>	Bone morphogenetic protein receptor type IA		601299
	Bannayan-Riley- Ruvalcaba	Hamartomatous intestinal polyposis; breast, thyroid, endometrial cancers			<i>PTEN</i>	Phosphatase and tensin homologue	Signaling pathway defect	158350 601728
PTEN hamartoma tumor syndrome	Proteus-like syndrome	Generalized or localized hamartomatous overgrowth; ovarian cystadenomas, CNS, parotid, testicular tumors						153480 601728
	Lhermitte-Duclos disease	Same as Cowden with cerebellar gangliocytomas						176920 601728
	Cutaneous malignant melanoma susceptibility (familial atypical mole malignant melanoma syndrome)	Melanomas	AD	1p36	?	?		158350 601728
	CMM1	Melanomas nevi, pancreatic cancer (FAMMM)	AD	9p21.3	<i>CDKN2A</i>	Cyclin-dependent kinase inhibitor 2A	Signaling pathway defect	155600 155601
	CMM2	Melanomas nevi, pancreatic cancer (FAMMM)	AD	12q14.1	<i>CDK4</i>	Cyclin-dependent kinase 4	Signaling pathway defect	600160 609048
	CMM3	Melanomas nevi, pancreatic cancer (FAMMM)	AD	1p22	?	?		123829 608035
CMM4	CMM4	Melanomas	AR	16q24	<i>MC1R</i>	Melanocortin-1 receptor	Pigmentation variation	613099 155555
	CMM5	Melanomas	AR	14q32.33	<i>XRCC3</i>	X-ray repair, complementing defective 3	DNA repair defect	613972 600675
	CMM6	Melanomas		20q11.22	?	?		612263
	CMM7	Melanomas	AD	3p14-p13	<i>MTIF</i>	Microphthalmic transcription factor	Melanocyte development defect	614456 156845
	CMM8	Melanomas	AR/AD	5p15.33	<i>TERT</i>	Telomerase reverse transcriptase	Telomere maintenance defect	615134 187270
	CMM9	Melanomas						

(continued)

**Table 10.1** (continued)

Disorder	Other names	Tumors	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Dyskeratosis congenita	DKCX Zinser-Cole-Engman Hoyeraaal-Hreidarsson DKCA1 Scoggins DKCA2	Variable malignancies	XLR	Xq28	<i>DKC1</i>	Dyskerin	Telomere maintenance defect	305000 300126
DKCA3 Revesz			AD	3q26.2	<i>TERC</i>	Telomerase RNA component		127550 602322
DKCB1				5p15.33	<i>TERT</i>	Telomerase reverse transcriptase		613989 187270
DKCB2				14q12	<i>TINF2</i>	TRF1-interacting nuclear factor 2		613990 604319
DKCB3			AR	15q14	<i>NOP10 (NOLA3)</i>	Nucleolar protein family A, member 3		224230 606471
DKCB4				5q35.3	<i>NHP2</i>	Nucleolar protein family A, member 2		613987 606470
DKCB5				17p13.1	<i>WRAP53 (TCAB1)</i>	WD-encoding RNA antisense to p53		613988 612661
Epidermolyticusplasia verruciformis	EDV	Squamous cell carcinoma	AR	5p15.33	<i>TERT</i>	Telomerase reverse transcriptase		613989 187270
Familial adenomatous polyposis	FAP1 APC Gardner	Colon polyps and carcinomas; Pilomatrical/epidermoid cysts, osteomas, desmoids (Gardner)	AD	20q13.33	<i>RTELI</i>	Regulator of telomere elongation helicase 1		615190 608833
Hereditary leiomyomatosis and renal cell cancer	HLRCC Reed	Cutaneous leiomyomas, uterine leiomyomas, leiomyosarcomas, papillary renal cell carcinoma	AD	17q25	<i>TMC6 (EVER1)</i>	Transmembrane channel-like 6	Immune regulation defect	226400 605828
Lipomatosis, familial multiple		Lipomas		5q22.2	<i>TMC8 (EVER2)</i>	Transmembrane channel-like 6		226400 605829
				1q42.1	<i>APC</i>	Adenomatous polyposis coli	Signaling pathway defect	175100 611731
					<i>FH</i>	Fumarate hydratase	Enzyme defect	150800 136850
					?	?		151900

Muir-Torre syndrome (familial cancer syndrome)	MTS Muir-Torre Lynch Turcot	Colon, endometrial carcinomas (all) Sebaceous tumors, keratoacanthomas (MTS) CNS tumors (Turcot)	AD	2p21 3p22.2 2p16.3 7p22.1	<i>MSH2</i> <i>MLHI</i> <i>MSH6</i> <i>PMS2</i>	MutS homologue of <i>E. coli</i> 2 MutL homologue of <i>E. coli</i> 1 MutS homologue of <i>E. coli</i> 6 Postmeiotic segregation increased 2	DNA mismatch repair defects	158320 609309 158320 120436 158320 600678 158320 600239
Multiple encephalomatosis Maffucci type	Maffucci	Enchondromas, chondrosarcomas, subcutaneous hemangiomas	AD	?	<i>?</i>	?	?	614569
Multiple endocrine neoplasia	MEN1 Wermer	Tumors of pituitary, parathyroid, AD pancreas; acromegaly, facial angiofibromas, collagenomas	AD	11q13.1	<i>MEN1</i>	Menin	Transcription factor defect	131100 613733
MEN2A Sipple	MEN2A Sipple	Medullary carcinoma, pheochromocytoma, parathyroid tumors	AD	10q11.21	<i>RET</i>	RET proto-oncogene (extracellular domain)	Signalling pathway defect	171400 164761
MEN2B Wagemann-Frobose	MEN2B Wagemann-Frobose	Mucosal neviomas, pheochromocytomas, medullary carcinoma	AD	10q11.21	<i>RET</i>	RET proto-oncogene (substrate binding)	Signalling pathway defect	162300 164761
MEN4	MEN4	Similar to MEN1	AD	12p13.1	<i>CDKN1B</i>	Cyclin-dependent kinase inhibitor 1B	Signalling pathway defect	610755 600778
Multiple self-healing squamous epithelioma syndrome	Ferguson-Smith	Multiple but spontaneously involuting squamous proliferations	AD	9q22	<i>TGFBR1</i>	TGF-β receptor	Signalling pathway defect	132800 190181
MUTYH-associated polyposis	MAP Familial adenomatous polyposis 2	Colorectal carcinoma, sebaceous tumors, squamous and basal cell carcinomas, melanomas	AR	1p34.1	<i>MUTYH</i>	MutY, homologue of <i>E. coli</i>	DNA mismatch repair defect	608456 604933
Neurofibromatosis, type I	Von Recklinghausen	Plexiform neurofibromas, malignant peripheral nerve sheath tumors	AD	17q11.2	<i>NFI</i>	Neurofibromin 1	Signalling pathway defect	1622006 131113
Neurofibromatosis, type II	Gardner Wishart NF3	Meningiomas, schwannomas, neurofibromas	AD	22q12.2	<i>NF2</i>	Neurofibromin 2 (merlin)	Transmembrane signaling defect	101000 607379
Schwannomatosis		Cutaneous and spinal schwannomas	AD	22q12.2	<i>NF2</i>	Neurofibromin 2 (merlin)	Transmembrane signaling defect	162091 607379
				22q11.23	<i>SMARCB1</i> ( <i>INI1</i> )	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, B1	Signalling pathway defect	162091 601607

(continued)

**Table 10.1** (continued)

Disorder	Other names	Tumors	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Peutz-Jeghers syndrome	PJS Hamartomatous intestinal polyposis	Gastrointestinal polyps, ovarian/testicular tumors	AD	19p13.3	<i>STK11</i>	Serine/threonine-protein kinase 11	Signaling pathway defect	175200 602216
Rothmund-Thomson syndrome (poikiloderma atrophicans and cataract)	RTS	Osteosarcoma, squamous and basal cell carcinomas	AR	8q24.3	<i>RECQL4</i>	RecQL protein-like 4	DNA helicase defect	268400 603780
Scleromylosis	Huriez	Skin and bowel cancers	AD	4q23	?	?	?	181600
Tuberous sclerosis	TS Bourneville	Angiofibromas, ungual fibromas, cortical tubers, angiomyolipomas, renal cell carcinomas, CNS tumors	AD	9q34.13	<i>TSC1</i>	Hamartin	Signaling pathway defect	191100 605284
Tylosis with esophageal cancer	TOC Howel-Evans	Esophageal carcinoma	AD	16p13.3	<i>TSC2</i>	Tuberin		191100 191092
Von Hippel-Lindau syndrome	VHL	Hemangioblastomas, renal cysts and carcinoma, pheochromocytoma	AD	17q25.1	<i>RHBDF2</i> ( <i>TOC</i> )	Rhomboid 5, homologue of drosophila 2	Signaling pathway defect	148500 614404
Werner syndrome	WRN	Sarcomas, melanomas, thyroid cancers	AD	3p25.3	<i>VHL</i>	Von Hippel-Lindau protein	Signaling pathway defect	193300 608537
Xeroderma pigmentosum	XPA DeSanctis-Cacchione	Squamous and basal cell carcinomas, melanomas	AR	8p12	<i>WRN</i> ( <i>RECQL2</i> )	RECQL protein-like 2	DNA helicase defect	277700 604611
XPD	XPC		AR	9q22.33	<i>XPA</i>	XPA	DNA nucleotide-excision repair defect	278700 611153
XPE			AR	2q14.3	<i>ERCC3</i> ( <i>XPB</i> )	Excision repair cross-complementing 3		610651 133510
XPF			AR	3p25.1	<i>XPC</i>	XPC		278720
XPG	Cockayne Variant		AR	19q13.32	<i>ERCC2</i> ( <i>XPD</i> )	Excision repair cross-complementing 2		613208 278730
DeSanctis-Cacchione syndrome	Same as XP		AR	11p11.2	<i>DDB2</i> ( <i>p48</i> )	DNA damage-binding protein 2		126340 278740
				16p13.12	<i>ERCC4</i> ( <i>XPF</i> )	Excision repair cross-complementing 4		600811 278760
				13q33.1	<i>ERCC5</i> ( <i>XPG</i> )	Excision repair cross-complementing 5		133520 278780
				6p21.1	<i>POLH</i>	DNA polymerase-η	DNA post-replication repair defect	133530 278750
				10q11.23	<i>ERCC6</i>	Excision repair cross-complementing 6	DNA nucleotide-excision repair defect	603968 278800
								? Unknown 609143

Gene mutational analysis for most of these disorders consists of full-gene sequencing. Exceptions include FISH and methylation analysis for imprinting in Beckwith-Wiedemann syndrome and mutational hotspot analysis, which may be performed prior to reflexing to full-gene sequencing and/or deletion/duplication analysis for disorders with few or clustered gene mutations (e.g., *RHBD2*, *RET*) (see Testing Strategy section below). Several laboratories are beginning to offer next-gen sequencing panels, which may be useful for disorders that have potential mutations across many different genes. Examples include xeroderma pigmentosum and chromosomal instability syndromes panels.

Basal cell nevus syndrome (Gorlin syndrome) is worth special mention because it is one of a few disorders with available targeted therapy. Individuals with basal cell nevus syndrome develop multiple basal cell carcinomas at an early age. Odontogenic keratocysts, palmar-plantar pits, ovarian fibromas, undescended testes, bifid ribs, and craniofacial defects are also characteristic. Medulloblastomas occur in a small subset of these individuals. The majority of cases of basal cell nevus syndrome have a mutation in the *PTCH1* gene, which encodes for the sonic hedgehog receptor, a large cell membrane protein. This Patched protein binds and suppresses the activity of Smoothened, a seven-transmembrane G-protein receptor. Because Smoothened, when unbound to Patched, is a pro-growth/anti-apoptosis molecule (via activity on the transcription factor Gli), mutated *PTCH1* results in tissue growth and oncogenesis. Vismodegib is a targeted Smoothened inhibitor and has been shown to be effective in the treatment of unresectable basal cell carcinomas, both sporadic tumors and tumors in patients with the basal cell nevus syndrome [8, 9]. Molecular testing for a *PTCH1* mutation is not routinely performed prior to treatment but should be considered prior to therapy, and mutational analysis of other genes in the Patched-Smoothened pathway (*PTCH2*, *SMO*, etc.) could be considered in vismodegib-refractory cases.

### 10.3 Inheritable Vascular Disorders

There is a group of genetic mutations that result in vascular-specific defects (Table 10.2). These mutations may result in defects of capillaries, veins, arteries, arteriovenous shunts, lymphatics, or combinations of various vessels. The mutations are heterogeneous, but many affect signaling pathways that are directly or indirectly involved in angiogenesis. Clinical manifestations include angiomas, malformations, telangiectasias, and/or lymphedema. These may occur anywhere in the body and can potentially cause complications with spontaneous bleeding (especially in the brain and viscera) and with platelet consumption.

There are a few disorders that can be screened by focused mutational analysis due to hotspot mutations (e.g., *KRIT1* exon 10 mutations in cerebral cavernous malformation 1), but virtually all others require full-gene sequencing. The relatively small size of many of these genes simplifies analysis. Due to the clinical similarities in many of these disorders, which may encompass many mutations spanning multiple genes, next-gen sequencing screening panels are beginning to surface. Examples include hereditary hemorrhagic telangiectasia and cerebral cavernous malformation panels.

### 10.4 Inheritable Bullous Disorders

The inheritable bullous diseases present with variable degrees of skin fragility. Patients blister following minor skin trauma. They are typically divided into groups based on the microanatomic level of the split and its relation to the basement membrane, and more recently, by their molecular defects [10, 11] (Table 10.3). Genetic defects associated with these diseases lead to abnormal or absent structural proteins, including keratins and basement membrane elements. The level of the split is dictated by microanatomic location of

**Table 10.2** Inheritable vascular disorders

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Apert syndrome	Apert-Crouzon	AD/most sporadic	10q26.13	<i>FGFR2</i>	Fibroblast growth factor receptor 2	Fibroblast development defect	176943 101200
Ataxia-telangiectasia	AT Louis-Bar	AR	11q22.3	<i>ATM</i>	Ataxia-telangiectasia mutated	DNA repair defect	208900 607385
Capillary infantile hemangioma		AD	2p13.3	<i>ANTXR1 (TEM8)</i>	Anthrax toxin receptor 1	Complex defect	602889 606410
Capillary malformation-arteriovenous malformation		AD	5q14.3	<i>RASA1</i>	RAS p21 protein activator 1	Signaling pathway defect	602089 191306
Cerebral cavernous malformations	CCM1	AD	7q21.2	<i>KRIT1 (CCM1)</i>	Krev interaction trapped-1	Signaling pathway defect	116860 604214
CCM2		AD	7p13	<i>CCM2</i>	CCM2 (malcavernin)	Signaling pathway defect	603284
CCM3		AD	3q26.1	<i>PDCD10 (CCM3)</i>	Programmed cell death 10	Signaling pathway defect	607929
Congenital disorder of glycosylation	CDG1E	AR	20q13.13	<i>DPM1</i>	Dolichyl-phosphate mannosyltransferase 1	Glycosylation defect	603285 609118
CDG1F		AR	17p13.1	<i>MPDU1</i>	Mannose-P-dolichol utilization defect 1	Glycosylation defect	608799 603303
Gliomovenous malformations		AD	1p22.1	<i>GLMN</i>	Glonulin	Signaling pathway defect	609180 604041
Heredity hemorrhagic telangiectasia of Rendu, Osler, and Weber	HHT1 Osler-Rendu-Weber	AD	9q34.11	<i>ENG</i>	Endoglin (CD105)	Angiogenesis defect	138000 601749
Heredity hemorrhagic telangiectasia	HHT2		12q13.13	<i>ACVR1L (ALK1)</i>	Activin A receptor type II-like 1	Angiogenesis defect	187300 131195
HHT3			5q31.1-q32	?	?		601101
HHT4			7p14	?	?		610655
Hereditary lymphedema	HL Ia	AD/AR	5q35.3	<i>FLT4</i>	FMS-like tyrosine kinase 4	Angiogenesis defect	153100 136352
Milroy							600376 601284
HL IB		AD	6q16.2-q22.1	?	?		611944
HL IC		AD	1q42.13	<i>GJC2</i>	Gap junction protein, γ2 (connexin 26)	Gap junction defect	613480 608803
HL II Meige		AD	16q24.1	<i>FOXC2</i>	Forkhead box C2	Transcription development defect	153200 602402

Hypotrichosis-lymphedema-telangiectasia syndrome	HLTS	AD/AR	20q13.33	<i>SOX18</i>	Sex-determining region Y-box 18	Complex defect	607823 601618
Juvenile polyposis/hereditary hemorrhagic telangiectasia syndrome	JPHT	AD	18q21.2	<i>SMAD4</i>	Mothers against decapentaplegic 4	Angiogenesis defect	175050 600993
Lymphedema-distichiasis syndrome		AD	16q24.1	<i>FOXC2</i>	Forkhead box C2	Transcription development defect	153400 602402
Mulibrey nanism	Perheentupa	AR	17q22	<i>TRIM37</i>	Tripartite motif-containing protein	Zinc finger development defect	253250 605073
Noonan syndrome	NS1 Pterygium colli	AD	12q24.13	<i>PTPN11</i>	Protein tyrosine phosphatase, non-receptor type 11	Signaling pathway defect	163950 176876
	NS2	AR	?	?	?	GTPase KRas	605275 609942
	NS3	AD	12p12.1	<i>KRAS</i>			190070 610733
	NS4		2p22.1	<i>SOS1</i>	Son of sevenless homologue 1		182330 610733
	NS5		3p25.2	<i>RAF1</i>	v-raf-1 murine leukemia viral oncogene homologue 1		611553 164760
	NS6		1p13.2	<i>NRAS</i>	GTPase NRas		613224 164790
	NS7		7q34	<i>BRAF</i>	Serine/threonine-protein kinase B-raf		613706 164757
Parkes Weber syndrome		?	5q14.3	<i>RASA1</i>	RAS p21 protein activator 1	Signaling pathway defect	608355 139150
Primary erythromelalgia		AD	2q24.3	<i>SCN9A</i>	Sodium channel voltage-gated, type IX, $\alpha$ subunit	Sodium channel defect	133020 603415
Rubinstein-Taybi syndrome	RSTS1	AD	16p13.3	<i>CREBBP</i>	CREB binding protein	Transcription defect	180849 600140
	RSTS2	AD	22q13.2	<i>EP300</i>	E1A binding protein	Cell proliferation defect	613684 602700
SC phocomelia syndrome	Roberts	AR	8p21.1	<i>ESCO2</i>	Establishment of cohesion 1, homologue 2	Mitosis defect	269000 609353
Smith-Lemli-Opitz syndrome	SLOS	AR	11q13.4	<i>DHCR7</i>	7-dehydrocholesterol reductase	Complex developmental defect	270400 602858

(continued)

**Table 10.2** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Stuve-Wiedemann syndrome	STWS Stuve-Wiedemann-Schwartz-Jampel 2	AR	5p13.1	<i>LIFR</i>	Lekemia inhibitory factor receptor	Signaling development defect	601559 151443
Syndromic microphthalmia 7 (microphthalmia with linear skin defects)	MIDAS	XLD	Xp22.2	<i>HCCS</i>	Holocytchrome c synthase	Oxidation-cytochrome defect	309801 300056
Venous malformations, multiple cutaneous and mucosal ? Unknown		AD	9p21.2	<i>TEK (TIE2)</i>	TEK tyrosine kinase	Angiogenesis defect	600195 600221

**Table 10.3** Inheritable bullous disorders

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Epidermolysis bullosa (suprabasal epidermal split)	EBLA EB lethal acantholytic	AR	6p24.3	<i>DSP</i>	Desmoplakin	Structural defect	609638 125647
McGrath	Ectodermal dysplasia-skin fragility	AR	1q32.1	<i>PKP1</i>	Plakophilin 1	Structural defect	604536 601975
Dowling-Meara	Dowling-Meara simplex (basal epidermal split)	AD	12q13.13	<i>KRT5</i>	Keratin 5	Keratin defect	131760 148040
			17q21.2	<i>KRT14</i>	Keratin 14		131760 148066
Weber-Cockayne localized		AD	12q13.13	<i>KRT5</i>	Keratin 5	Keratin defect	131800 148040
			17q21.2	<i>KRT14</i>	Keratin 14		131800 148066
Koebner generalized		AD	12q13.13	<i>ITGB4</i>	β4-integrin	Structural defect	131800 147557
			17q25.1				
EBS with mottled pigmentation		AD	12q13.13	<i>KRT5</i>	Keratin 5	Keratin defect	131900 146040
			17q21.2	<i>KRT14</i>	Keratin 14		131900 148066
EBS-AR		AR	17q21.2	<i>KRT14</i>	Keratin 14	Keratin defect	601001 148066
EBS with muscular dystrophy		AR	8q24.3	<i>PLEC1</i>	Plectin 1	Structural defect	226670 601282
EBS with pyloric atresia		AR	8q24.3	<i>PLEC1</i>	Plectin 1		612138 601282
Ogna		AD	8q24.3	<i>PLEC1</i>	Plectin 1		131950 601282
EBS with migratory circinate erythema		AD	12q13.13	<i>KRT5</i>	Keratin 5	Keratin defect	609352 148040

(continued)

**Table 10.3** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OIMM
Junctional epidermolysis bullosa (intra-lamina lucida split)	JEB, Herlitz	AR	18q11.2	<i>LAMA3</i>	Laminin-5 ( $\alpha 3$ )	Structural defect	226700 600805
			1q32.2	<i>LAMB3</i>	Laminin-5 ( $\beta 3$ )		226700 150310
			1q25.3	<i>LAMC2</i>	Laminin-5 ( $\gamma 2$ )		226700 150292
JEB—non-Herlitz inversa, progressive, severe nonlethal, disentis, generalized benign, localized		AR	10q24.3-q25.1	<i>COL7A1</i> ( <i>BPGAG2</i> )	Collagen XVII ( $\alpha 1$ )	Structural defect	226650 113811
			18q11.2	<i>LAMA3</i>	Laminin-5 ( $\alpha 3$ )		226650 600805
			1q32.2	<i>LAMB3</i>	Laminin-5 ( $\beta 3$ )		226650 150310
			1q25.3	<i>LAMC2</i>	Laminin-5 ( $\gamma 2$ )		226650 150292
JEB with pyloric atresia Carmi		AR	2q31.1	<i>ITGA6</i>	$\alpha 6$ -integrin	Structural defect	226730 147556
			17q25.1	<i>ITGB4</i>	$\beta 4$ -integrin		226730 147557
Epidermolysis bullosa dystrophica, autosomal dominant (sublamina densa split)	Pasini, Cockayne-Touraine DDEB, pretibial split)	AD	3p21.31	<i>COL7A1</i>	Collagen VII ( $\alpha 1$ chain)	Structural defect	131750 120120 131850 120120
DDEB, pruriginosa							604129 120120
DDEB, toenails only		AD					607523 120120
DDEB, transient bullous dermolysis of the newborn							131705 120120
Bart		AD					132000 120120

Recessive dystrophic epidermolysis bullosa	Hallopeau-Siemens Non-Hallopeau-Siemens	AR AR	3p21.31	<i>COL7A1</i>	Collagen VII ( $\alpha 1$ chain)	226600 120120
RDEB, inversa	RDEB, centripetalis	AR AR				226600 120120
Kindler syndrome		AR	20p12.3	<i>FERMT1</i> ( <i>KIND1</i> )	Kindlin-1	Structural defect
Laryngo-onycho-cutaneous syndrome	LOCS Shabbir	AR	18q11.2	<i>LAMA3</i>	Laminin-5 ( $\alpha 3$ )	173650 607900
Macular-type hereditary bullous dystrophy	Mendes da Costa	XR	Xq27.3-pter	?	?	245660 600805
Nephropathy with pretibial epidermolysis bullosa and deafness		AR	11p15.5	<i>CD151</i>	Platelet-endothelial cell tetraspanin antigen 3	302000
Skin fragility-woolly hair syndrome	SFWHS	AR	6p24.3	<i>DSP</i>	Desmoplakin	Structural defect
? Unknown						607655 125647

affected protein. The diagnosis is often made with a combination of clinical and biopsy findings. Immunofluorescence studies, and more rarely electron microscopy, can be very useful for localizing the microanatomic site of the absent or defective protein. Genetic studies offer confirmation.

Some of the inheritable bullous disorders have specific mutations that occur with high frequency. An example is the p.R661X mutation in junctional epidermolysis bullosa. In these cases, mutant-specific PCR can be performed. In most cases, however, full-gene sequencing is required. The structural proteins can be quite large, with some containing over 70 exons (e.g., *LAMA3*, *COL7A1*), and thus full-gene sequencing leads to increased costs of testing. Stepwise approaches can be used, but this impacts turnaround time. Molecular panels have been developed to screen individuals based on level of split and/or inheritance pattern, which offers convenience for the clinician but also adds to overall cost. To counter these effects, many of these panels are being transitioned from direct sequencing to next-gen sequencing platforms. Comprehensive next-gen sequencing panels, which include mutational analyses for 28 genes spanning simplex, junctional, and dystrophic forms, are now emerging.

## 10.5 Inheritable Keratinization Disorders

Ichthyoses and erythrokeratodermas are disorders of keratinization resulting in variable degrees of hyperkeratosis, ranging from mild and focal hyperkeratosis to collodion membranes and potential death in utero. Erythrokeratoderma refers to variable degrees of cornification along with erythema. Ichthyoses and erythrokeratodermas are typically grouped based on inheritance pattern, presence or absence of clinical features at birth, and whether the cutaneous manifestations are isolated or part of multisystem disease [12]. The majority of these disorders are caused by mutations that disrupt

epidermal maturation and/or its barrier function (Table 10.4). These affected genes encode for gap junction proteins, keratins, and enzymes involved in the biosynthesis of the lipid barrier, among others.

Diagnosis of the ichthyoses is often suspected clinically. Some conditions, however, may utilize other diagnostic tools. Steroid sulfatase levels can be measured for diagnosing X-linked recessive ichthyosis. Trichorrhexis invaginata and trichorrhexis nodosa, characteristic of Netherton syndrome, can be diagnosed on hair pulls. Biopsy may be useful in some cases. “Epidermolytic hyperkeratosis” (EHK), typical of bullous congenital ichthyosiform erythroderma, is a term used to describe the histologic pattern of suprabasal keratinocytic cytolysis with vacuolization and clumping of keratin filaments. Ichthyosis vulgaris has a diminished granular layer. Ichthyosis hystrix, Curth-Macklin type, has a characteristic electron microscopic finding of perinuclear vacuolization and collections of keratin filaments forming a shell in keratinocytes of the upper epidermis. Many of the other ichthyoses and erythrokeratodermas require molecular testing for confirmation.

Palmoplantar keratoderma (PPK) refers to hyperkeratosis, largely restricted to the palms and soles. Hereditary PPK varies in severity and may be associated with multisystem disease. The hyperkeratosis can be diffuse (entire palm or sole), focal, or punctuate (<1 cm lesions). The genetic defects are similar to those observed in the ichthyoses, affecting function of structural and barrier proteins, and there is also some clinical overlap (Table 10.5) [13]. There are other genodermatoses with defective keratinization that do not quite fit into the categories of ichthyosis or PPK (Table 10.6). Some are quite common, such as Darier-White disease and Hailey-Hailey disease, caused by mutations in two different  $\text{Ca}^{2+}$  ATPase genes.

Mutational analyses of most of the disorders of keratinization require full-gene sequencing. Some of the disorders have specific mutations found in a majority of cases, and mutation-specific PCR can be performed. An example is p.R501X in ichthyosis vulgaris.

**Table 10.4** Inheritable ichthyoses and erythrokeratodermas

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Arthrogryposis-renal dysfunction-cholesterol syndrome	ARCS1	AR	15q26.1	<i>VPS33B</i>	Vacuolar protein sorting 33 yeast homologue B	Trafficking defect	208085 608552
	ARCS2		14q24.3	<i>VIPAR</i>	VP33B-interacting protein, apical-basolateral polarity regulator		613404 613401
Autosomal recessive congenital ichthyosis with hypotrichosis		AR	11q24.3	<i>STI4</i>	Matriptase	Metabolic-barrier defect	610765 606797
Autosomal recessive exfoliative ichthyosis	Siemens-like	AR	3q21.1	<i>CSTA</i>	Cystatin A	Metabolic-barrier defect	607936 184600
Cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma	CEDNIK	AR	22q11.21	<i>SNAP29</i>	Synaptosomal-associated protein, 29 kDa	Intracellular trafficking defect	609528 604202
Chanarin-Dorfman syndrome Ichthyotic neural lipid storage disease		AR	3p21.33	<i>ABHD5</i> ( <i>CG158</i> )	Abyhydrolase domain-containing 5	Lipid metabolism-barrier defect	275630 604780
Chondrodyplasia punctata	CDPX1	XLR	Xp22.33	<i>ARSE</i>	Arylsulfatase E	Metabolic-barrier defect	302950 300180
CDPX2-Conradi-Hünermann-Happle	CDPX2	XLD	Xp11.23	<i>EBP</i>	Enopamil binding protein	Cholesterol biosynthesis defect	302960 300205
Chondrodyplasia punctata, rhizomelic form	Peroxisome disorder 9	AR	6q23.3	<i>PEX7</i>	Peroxin 7	Peroxisome defect	215100 601757
Congenital disorder of glycosylation, type IIm (dolichol kinase deficiency)		AR	9q34.11	<i>DOLK</i>	Dolichol kinase	Metabolic defect	610768 610746
Congenital hemidysplasia with ichthyosiform erythroderma and limb defects	CHILD	XLD	Xq28	<i>NSDHL</i>	NAD(P)H steroid dehydrogenase-like protein	Cholesterol biosynthesis defect	308050 300275

(continued)

**Table 10.4** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Congenital ichthyosis, autosomal recessive	ARCI1 Lamellar ichthyosis 1	AR	14q11.2	<i>TGM1</i>	Transglutaminase 1	Barrier defect	242300 190195
	ARCI2 NCIE-1		17p13.1	<i>ALOX12B</i>	12(R)-lipoxygenase	Lipid enzyme and barrier defect	242100 603741
	ARCI3 Lamellar ichthyosis 5		17p13.1	<i>ALOXE3</i>	Epidermal lipoxygenase 3	Lipid enzyme and barrier defect	606545 607206
	ARCI4A Lamellar ichthyosis 2		2q35	<i>ABCA12</i>	ATP-binding cassette, subfamily A, member 12	Metabolism-transport defect	601277 607800
	ARCI4B Harlequin ichthyosis						242500 607800
	ARCI5 Lamellar ichthyosis 3		19p13.12	<i>CYP4F22</i>	Cytochrome P450, family 4, subfamily F, polypeptide 22	Lipid enzyme and barrier defect	604777
	ARCI6		5q33.3	<i>NIPAL4</i>	Ichthyin	Variable defects	612281 609383
	ARCI7	?	12p11.2-q13.1	?	?		
	ARCI8 Lamellar ichthyosis 4		10q23.31	<i>LIPN</i>	Lipase family, member N	Metabolic-barrier defect	613943 613924
	ARCI9 ARCI10		15q26.3 6p21.31	?	?		
				<i>PNPLA1</i>	Patatin-like phospholipase domain-containing protein 1	Metabolic-barrier defect	615023 615024 612121
Cyclic ichthyosis with epidermolytic hyperkeratosis	CIEHK	AD	12q13.13	<i>KRT1</i>	Keratin 1	Keratin defect	607602 139350
			17q21.2	<i>KRT10</i>	Keratin 10		607602 148080
Epidemolytic hyperkeratosis	EHK BCIE Brocq	AD	12q13.13	<i>KRT1</i>	Keratin 1	Keratin defect	113800 139350
		AD/AR	17q21.2	<i>KRT10</i>	Keratin 10		113800 148080

Erythrokeratoderma variabilis et progressiva	EKV Greither Mendes da Costa, Giroux-Barbeau	AD/AR	1p34.3	<i>GJB3</i>	Gap junction protein β3 (connexin 31)	Structural/transport defect	133200 603324
Gaucher disease	Gaucher types I–III	AR	1q22	<i>GBA</i>	Gap junction protein β4 (connexin 30.3) Acid β-glucosidase	133200 605425 230800 230900 231000 606463	
Hystrix-like ichthyosis with deafness	HID	AD	13q12.11	<i>GJB2</i>	Connexin 26 (gap junction protein β2) Keratin 2	Structural defect of gap junction channels Keratin defect	602540 121011
Ichthyosis, bullous type	Siemens	AD	12q13.13	<i>KRT2</i>	Keratin 1	Keratin defect	146800 600194
Ichthyosis hystrix, Curth-Macklin type	Curth-Macklin	AD	12q13.13	<i>KRT1</i>	Keratin 1	Keratin defect	146590 139350
Ichthyosis, leukocyte vacuoles, alopecia, and sclerosing cholangitis	Nisch	AR	3q28	<i>CLDN1</i>	Claudin 1	Barrier defect	607626 603718
Ichthyosis-prematurity syndrome		AR	9q34.11	<i>SLC27A4</i>	Solute carrier family 27, member 4	Metabolic-barrier defect	608649 604194
Ichthyosis vulgaris	AD/ Semidom	1q21.3		<i>FLG</i>	Flaggrin	Barrier defect	146700 135940
Ichthyosis, X-linked recessive	XLR	Xp22.31		<i>STS</i>	Steroid sulfatase	Metabolic-barrier defect	308100 300747
Keratitis-ichthyosis-deafness syndrome	KID	AD	13q12.11	<i>GJB2</i>	Connexin 26 (gap junction binding protein β2)	Structural defect of gap junction channels	148210 121011
Multiple sulfatase deficiency	Austin	AR	3p26.1	<i>SUMF1</i>	Sulfatase-modifying factor 1	Metabolic defect	272200 607939
Netherton syndrome		AR	5q32	<i>SPINK5</i>	LEKTI	Metabolic-barrier defect	256500 605010
Refsum disease		AR	10p13	<i>PHYH</i>	Phytanoyl-CoA 2-hydroxylase	Peroxisome defect	266500 602026
			6q23.3	<i>PEX7</i>	Peroxin-7		266500 601757
Sjögren-Larson syndrome		AR	17p11.2	<i>ALDH3A2</i>	Fatty aldehyde dehydrogenase	Lipid metabolism defect	270200 609523

? Unknown

**Table 10.5** Inheritable palmar-plantar keratodermas (PPKs)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Cerebral dysgenesis, neuropathy, ichthyosis, and keratoderma	CEDNIK	AR	22q11.21	<i>SNAP29</i>	Synaptosomal-associated protein, 29 kDa	Intracellular trafficking defect	609528 604202
Dilated cardiomyopathy with woolly hair and keratoderma	Carvajal	AR	6p24.3	<i>DSP</i>	Desmoplakin	Structural defect	605676 125647
Haim-Munk syndrome		AR	11q14.2	<i>CTSC (DPPI)</i>	Cathepsin C	Lysosomal defect	245010 602365
Keratosis palmoplantaris striata I (PPKS1)	Brinnauer-Fuhs-Siemens	AD	18q12.1	<i>DSG1</i>	Desmoglein 1	Structural defect	148700 125670
Keratosis palmoplantaris striata II (PPKS2)	Wachter	AD	6p24.3	<i>DSP</i>	Desmoplakin	Structural defect	612908 125647
Keratosis palmoplantaris striata III (PPKS3)		AD	12q13.13	<i>KRT1</i>	Keratin 1	Keratin defect	607654 139350
Knuckle pads, leukonychia, Bart-Pumphrey and sensorineural deafness	Bart-Pumphrey	AD	13q12.11	<i>GJB2</i>	Connexin 26 (gap junction protein β2)	Structural defect of gap junction channels	149200 121011
Mal de Meleda	Meleda	AR	8q24.3	<i>SLURP1</i>	Secreted leukocyte antigen-6 urokinase-type plasminogen activator-related protein 1	Structural defect	248300 606119
Oculodentodigital dysplasia		AD/AR	6q22.31	<i>GJA1</i>	Connexin 43 (gap junction protein α1)	Structural defect of gap junction channels	164200 121014
Pachyonychia congenita, type 1 (PC-1)	Jadassohn-Lewandowsky	AD	12q13.13	<i>KRT6A</i>	Keratin 6A	Keratin defect	167200 148041
Pachyonychia congenita, type 2 (PC-2)	Jackson-Lawler	AD	17q21.2	<i>KRT16</i>	Keratin 16		167200 148067
			12q13.13	<i>KRT6B</i>	Keratin 6B	Keratin defect	167210 148042
			17q21.2	<i>KRT17</i>	Keratin 17		167210 148069

PPK, epidermolytic	Vörner	AD	12q13.13 17q21.2	<i>KRT1</i> <i>KRT9</i>	Keratin 1 Keratin 9	Keratin defect	144200 139350 144200 607606
PPK, non-epidermolytic	Umma-Thost	AD	12q13.13 17q21.2	<i>KRT1</i> <i>KRT16</i>	Keratin 1 Keratin 16	Keratin defect	600962 139350 600962 148067
PPK with deafness		Mitochondrial	Mitochondrial	<i>MT-TS1</i> <i>GJB2</i>	Mitochondrial tRNA-silene 1 Connexin 26 (gap junction protein p2)	Cytochrome c oxidase defect Structural defect of gap junction channels	148350 590080 148350 121011
PPK with periodontitis	Papillon-Lefèvre PA LS	AR	11q14.2	<i>CTSC (DPPI)</i>	Cathepsin C	Lysosomal defect	245000 602365
PPK with woolly hair and cardiac abnormalities	Naxos	AR	17q21.2	<i>JUP (PKGB)</i>	Plakoglobin	Structural defect	601214 173325
Skin fragility-woolly hair syndrome	SFWHS	AR	6p24.3	<i>DSP</i>	Desmoplakin	Structural defect	607655 125647
Tyrosinemia type II	Richner-Hanhart	AR	16q22.2	<i>TAT</i>	Tyrosine aminotransferase	Enzyme defect	276600 613018
Vohwinkel syndrome, classic form	Vohwinkel (classic)	AD	13q12.11	<i>GJB2</i>	Connexin 26 (gap junction protein p2)	Structural defect of gap junction channels	124500 121011
Congenital deafness with keratopathy, dermia and constrictions of fingers and toes	Vohwinkel (ichthyotic)	AD	1q21.3	<i>LOR</i>	Loricrin	Cornified cell envelope defect	604117 152445
? Unknown							

**Table 10.6** Other inheritable disorders of keratinization

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Acral peeling skin syndrome		AR	15q15.2	<i>TGM5</i>	Transglutaminase 5	Structural defect	609796 603805
Acrokeratosis verruciformis	AKV Hopf	AD	12q24.11	<i>ATP2A2</i>	Sarcoplasmic/endoplasmic reticulum Ca <sup>2+</sup> ATPase 2 (SERCA2)	Calcium pump/cell adhesion defect	101900 108740
Benign chronic pemphigus (Hailey-Hailey disease)	Hailey-Hailey	AD	3q22.1	<i>ATP2C1</i>	Human secretory pathway Ca <sup>2+</sup> ATPase 1 (hSPCA1)	Calcium pump/cell adhesion defect	169600 604384
Cardiofaciocutaneous syndrome		AD/sporadic	12p12.1	<i>KRAS</i>	GTPase KRas	Signaling pathway defect	115150 190070
			7q34	<i>BRAF</i>	Serine/threonine-protein kinase B-raf		115150 164757
			15q22.31	<i>MAP2K1 (MEK1)</i>	Mitogen-activated protein kinase kinase 1		115150 176872
			19p13.3	<i>MAP2K2 (MEK2)</i>	Mitogen-activated protein kinase kinase 2		115150 601263
CDAGS syndrome	CAP	AR	22q12-q13	?	?	?	603116
Craniostenosis, anal anomalies, and porokeratosis							
Darier-White disease (keratosis follicularis)	DAR Darier	AD	12q24.11	<i>ATP2A2</i>	Sarcoplasmic/endoplasmic reticulum Ca <sup>2+</sup> ATPase 2 (SERCA2)	Calcium pump defect	124200 108740
Disseminated superficial actinic porokeratosis	Porokeratosis 3	AD	12q23.3	<i>SART3</i>	Squamous cell carcinoma antigen recognized by T cells	Transcription regulation defect	175900 611684
			12q24.11	<i>MVK</i>	Mevalonate kinase	Cholesterol biosynthesis defect	175900 251170
Erythrokeratoderma variabilis et progressiva	EKV Greither Mendes da Costa, Giroux-Barbeau	AD/AR	1p34.3	<i>GJB3</i>	Gap junction protein β3 (connexin 31)	Structural/transport defect	133200 603324
			1p34.3	<i>GJB4</i>	Gap junction protein β4 (connexin 30.3)		133200 605425
Hereditary benign intraepithelial dyskeratosis		AD	4q35	?	?	?	127600

Hyaline fibromatosis syndrome		AR	4q21.21	<i>ANTXR2 (CMG2)</i>	Anthrax toxin receptor 2	Structural defect	228600 608041
Keratolytic winter erythema	Oudtshoorn	AD	8p23-p22	?	?		148370
Keratosis follicularis spinulosa decalvans		XR	Xp22.11	<i>SAT1</i>	Spermidine/spermine N(1)-acetyltransferase	Metabolism defect	308800 313020
			Xp22.12-p22.11	<i>MBTPS2</i>	Membrane-bound transcription factor protease, site 2	Zinc metalloprotease defect	308800 300294
Noonan syndrome	NS1 Pterygium colli	AD	12q24.13	<i>PTPN11</i>	Protein tyrosine phosphatase, non-receptor type 11	Signaling pathway defect	163950 176876
NS2	AR	?	?	?	?		605275
NS3	AD	12p12.1	<i>KRAS</i>	GTPase KRas	Signaling pathway defect		609942 190070
NS4		2p22.1	<i>SOS1</i>	Son of sevenless homologue 1			610733
NS5		3p25.2	<i>RAF1</i>	v-raf-1 murine leukemia viral oncogene homologue 1			182530 611553 164760
NS6		1p13.2	<i>NRAS</i>	GTPase NRas			613224 164790 613706 164757
NS7		7q34	<i>BRAF</i>	Serine/threonine-protein kinase B-raf			
Restrictive dermopathy, lethal		AD	1q22	<i>LMNA</i>	Lamin A/C	Nuclear membrane defect	275210 150330
		AD/AR	1p34.2	<i>ZMPSTE24</i>	Zinc metalloproteinase STE24	Zinc metalloprotease defect	275210 606480
Seborrhea-like dermatitis with psoriasiform elements		AD	17q25.3	<i>ZNF750</i>	Zinc finger protein 750	?	610227 610226
White sponge nevus	Cannon	AD	12q13.13	<i>KRT4</i>	Keratin 4	Keratin defect	193900 123940
			17q21.2	<i>KRT13</i>	Keratin 13		193900 148065

? Unknown

## 10.6 Ectodermal Dysplasias and Other Inheritable Disorders of the Sweat Glands, Hair, Nails, and/or Teeth

Ectodermal dysplasias are a genetically heterogeneous group of disorders that, by classic definition, have defects in two or more of the following ectodermal components: sweat glands, hair, nails, and teeth. Skin manifestations are variable. These disorders have historically been subdivided by the presence or absence of sweat formation and mode of inheritance. As the molecular causes of these entities are better characterized, new classifications are emerging based on the molecular and functional defects [14]. The defects can be segregated into two broad main groups: (1) epithelial-mesenchymal interaction defects (e.g., signaling pathway and tissue developmental defects) and (2) structural defects (Table 10.7). There are many other miscellaneous defects that do not neatly fit into these groups and have a spectrum of clinical manifestations (Table 10.8). Some of the ectodermal dysplasias have frequent specific mutations, and focused sequencing of these regions can be used for screening purposes. Examples include p.R155 and p.R156 mutations in hypohidrotic ectodermal dysplasia (type 1); p.R243, p.R266, and p.R318 mutations in ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome (EEC type 3); and p.G11R and p.A88V mutations in hidrotic ectodermal dysplasia (type 2, Clouston).

## 10.7 Inheritable Connective Tissue Disorders

Hereditary connective tissue disorders are a genetically and clinically diverse group. Many connective tissue disorders—including lupus erythematosus, scleroderma, and dermatomyositis, for example—have susceptibility loci, and thus an inheritable component, but have complex genetics. Others, such as the cutis laxa, Ehlers-Danlos, and osteogenesis imperfecta disorders, have better characterized genotype-phenotype relationships. Somatic and mosaic defects can also cause connective tissue disorders (such as *AKT1* muta-

tions in Proteus syndrome and *PIK3CA* mutations in mosaic overgrowth syndrome [15]), but as these are rarely, if at all, inheritable, this group is not included here. In the category of connective tissue genodermatoses, genetic defects may result in alterations in quantity, distribution, or function of various components of connective tissue. Many of the disorders have mutations affecting the structure of collagen, but other mutations cause defects in other structural proteins and intracellular signaling molecules (Table 10.9). Most of these disorders require full-gene sequencing for mutation detection. Several disorders have high-frequency mutations and can be screened by mutation-specific PCR or focused sequencing. Examples include p.R1141X and del exons 23–29 in pseudoxanthoma elasticum and *HRAS* exon 2 mutations in Costello syndrome.

## 10.8 Inheritable Disorders of Pigmentation

Many genodermatoses have alterations in skin pigmentation; therefore, many of the entities listed here are shared with other categories (Table 10.10). There are many different pathways leading to alterations in pigmentation [16]. Melanin is the main pigment in the skin, generated by melanocytes, so it is not surprising that gene mutations resulting in defects in melanocyte development and/or melanin biosynthesis cause the majority of these disorders [17]. The pigmentary changes can be focal or diffuse. Lentigines, café-au-lait macules, and nevi as components of genodermatoses are also included here. Other causes of pigmentation can be due to defects in metabolism and/or distribution of heme and bile, among others. Full-gene sequence analysis is required for mutation detection in the majority of these disorders.

## 10.9 Inheritable Metabolic Disorders

There are a large number of metabolic disorders, some of which produce cutaneous manifestations (Table 10.11) [18]. The metabolic disorders,

**Table 10.7** Inheritable ectodermal dysplasias

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
<b>Group 1</b>							
<b>Hypohidrotic ectodermal dysplasia</b>	ECTD1 Christ-Siemens-Touraine	XLR	Xq13.1	<i>EDA</i>	Ectodysplasin A	TNF-R family defect	305100 300451
	ECTD10A	AD	2q12.3	<i>EDAR</i>	Ectodysplasin A receptor		129490 604095
	<b>ECTD10B</b>	AR	2q12.3	<i>EDAR</i>	Ectodysplasin A receptor		224900 604095
	ECTD11A	AD	1q42.3	<i>EDARADD</i>	EDAR-associated death domain		614940 606603
	<b>ECTD11B</b>	AR	1q42.3	<i>EDARADD</i>	EDAR-associated death domain		614941 606603
Hypohidrotic ectodermal dysplasia, with immunodeficiency		XLR	Xq28	<i>IKBKG (NEMO)</i>	Nuclear factor-κB essential modulator	Signaling pathway defect	300291 300248
		AD	14q13.2	<i>NFKBIA IKBA?</i>	Nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor-α		612132 164008 632132
Hypohidrotic ectodermal dysplasia, with immunodeficiency, osteopetrosis, and lymphedema		OLEDAID	XLR	<i>IKBKG (NEMO)</i>	Nuclear factor-κB essential modulator	Signaling pathway defect	300301 300248

(continued)

**Table 10.7** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Incontinentia pigmenti	IP2, Bloch-Sulzberger	XLD	Xq28	<i>IKBKG (NEMO)</i>	Nuclear factor-κB essential modulator	Signaling pathway defect	308300 300248
Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome	EEC1 EEC3	AD	7q11.2-q21.3 3q28	?	?		129900
Anchyoblepharon-ectodermal dysplasia-cleft lip/palate syndrome	AEC Hay-Wells			<i>TP63 (p63, TP73L)</i>	Tumor protein 63	Apoptosis defect	604292 603273
Acro-dermato-ungual-lacrimal-tooth syndrome	ADULT						103285 603273
Split hand-split foot malformation syndrome (not an ectodermal dysplasia)	SHFM4						605289 603273
Limb-mammary syndrome	LMS						603543 603273
Rapp-Hodgkin syndrome							129400
Ellis-van Creveld syndrome	AR	4p16.2	<i>EVC</i>	<i>EVC</i>		Cardiac development defect	603273 225500
		4p16.2	<i>EVC2</i>	<i>EVC2</i> (limbin)			604831 225500
Weyers acrofacial dysostosis	Curry-Hall	AD	4p16.2	<i>EVC</i>	<i>EVC</i>		607261 193530
				<i>EVC2</i>	<i>EVC2</i> (limbin)		604831 193530
Tricho-dento-osseous syndrome	AD	17q21.33	<i>DLX3</i>	<i>DLX3</i>	Distal-less homeobox 3	Bone formation defect	607261 190320
Tooth and nail syndrome	ECTD3 Witkop	AD	4p16.2	<i>MSX1</i>	Muscle segment homeobox 1	Developmental defect	600525 189500 142983
Group 2							
Cleft lip/palate-ectodermal dysplasia syndrome	Zlotogora-Ogur	AR	11q23.3	<i>PVRL1</i>	Poliovirus receptor- related 1 (Nectin-1)	Structural defect	225060 600644

Ectodermal dysplasia-skin fragility syndrome	McGrath	AR	1q32.1	<i>PKP1</i>	Plakophilin-1	Structural defect	604536 601975
Ectodermal dysplasia, ectrodactyly, and macular dystrophy syndrome	EEM	AR	16q22.1	<i>CDH3</i>	Cadherin-3 (P-cadherin)	Adhesion defect	225280 114021
<b>Hidrotic ectodermal dysplasia</b>	<b>ECTD2</b> Clouston	<b>AD</b>	<b>13q12.11</b>	<b><i>GJB6</i></b>	Connexin 30 (gap junction protein β6)	Gap junction defect	129500 604418
Odonto-onycho-dermal dysplasia	ODDD	AR	2q35	<i>WNT10A</i>	Wingless-type MMTV integration site family, member 10	Signaling molecule	257980 606268
Schöpf-Schulz-Passarge syndrome		AR	2q35	<i>WNT10A</i>			224750 606268
Rosselli-Gulinetti syndrome			?	?	?		225000
Other							
Ectodermal dysplasia, pure hair/nail type	ECTD4	AR	12q13.13	<i>KRT85</i>	Keratin 85	Keratin defect	602032 602767

Groups 1 and 2 based on classification by Priolo et al. [14]. Bold print indicates most common disorders  
 ? Unknown

**Table 10.8** Other inheritable hair and nail disorders

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Acrodermatitis enteropathica, zinc deficiency type	AEZ	AR	8q24.3	<i>SLC39A4</i>	Solute carrier family 39, member 4	Zinc transport defect	201100 607059
Alopecia universalis congenita	ALUNC	AR	8p21.3	<i>HR</i>	Hairless	Zinc finger transcription factor defect	203655 602302
Argininosuccinic aciduria		AR	7q11.21	<i>ASL</i>	Argininosuccinate lyase	Metabolic defect	207900 608310
Atrichia with papular lesions	APL	AR	8p21.3	<i>HR</i>	Hairless	Zinc finger transcription factor defect	209500 602302
Björnstad syndrome	BJS	AR	2q35	<i>BCS1L</i>	BSC1-like	Mitochondrial membrane	262000 603647
Cartilage-hair hypoplasia	CHH McKusick	AR	9p13.3	<i>RMRP</i>	RNA component of mitochondrial RNA-processing endoribonuclease	Ribonuclease defect	250250 157660
Congenital hypotrichosis with juvenile macular dystrophy	HJMD	AR	16q22.1	<i>CDH3</i>	Cadherin 3	Adhesion defect	601553 114021
Giant axonal neuropathy	GAN1	AR	16q23.2	<i>GAN</i>	Gigaxonin	Trafficking defect	256850 605379
Hypotrichosis simplex	HYPT1	AD	18p11.22	<i>APCDD1</i>	APC, downregulated by, 1	Signaling pathway defect	605389 607479
Scalp type 1	HYPT2	AD	6p21.33	<i>CDSN</i>	Corneodesmosin	Structural defect	146520 602593
Scalp type 2	HYPT3	AD	12q13.13	<i>KRT74</i>	Keratin 74	Keratin defect	613981 602593
Marie Unna 1	HYPT4	AD	8p21.3	<i>HR</i>	Hairless	Zinc finger transcription factor defect	146550 602302
Marie Unna 2	HYPT5	AD	1p21.1-q21.3	?	?		612841
localized	HYPT6	AR	18q12.1	<i>DSG4</i>	Desmoglein 4	Structural defect	607903 607892
HYPT7	LAH2 Mari	AR	3q27.2	<i>LIPH</i>	Lipase H	Complex defect	604379 607365
HYPT8	LAH3	AR	13q14.2	<i>LPAR6</i>	Lysophosphatidic acid receptor 6	Nucleotide receptor defect	278150 609239
HYPT9	AR	10q11.23-q22.3	?	?	?		614237
HYPT10	AR	7p22.3-p21.3	?	?	?		614238

Hypotrichosis-lymphedema-telangiectasia syndrome	HLTS	AD/AR	20q13.33	<i>SOX18</i>	Sex-determining region Y-box 18	Complex defect	607823 601618
Laron syndrome	Pituitary dwarfism 2	AR	5p13-p12	<i>GHR</i>	Growth hormone receptor	Growth defect	262500 600946
Menkes syndrome	MNK	XLR	Xq21.1	<i>ATP7A</i>	ATPase, Cu <sup>2+</sup> -transporting ( $\alpha$ -polypeptide)	Copper transport defect	309400 300011
Monilethrix		AD	12q13.13	<i>KRT81</i>	Keratin 81	Keratin/structural defect	158000 602153
		AD	12q13.13	<i>KRT83</i>	Keratin 83		158000 602765
		AD	12q13.13	<i>KRT86</i>	Keratin 86		158000 601928
Propiomelanocortin deficiency		AR	2p23.3	<i>POMC</i>	Propiomelanocortin	Melanogenesis defect	609734 176830
Skin/hair/eye pigmentation, variation 2		AR	16q24.3	<i>MC1R</i>	Melanocortin-1 receptor	Melanin synthesis variation	266300 155555
T-cell immunodeficiency, congenital alopecia, and nail dystrophy		AR	17q11.2	<i>FOXN1</i>	Forkhead box N1	T-cell development defect	601705 600838
Trichothiodystrophy	Tay Photosensitive	AR	2q14.3	<i>ERCC3 (XPB)</i>	Excision repair cross-complimenting 3	DNA repair defect	601675 133510
			6q25.3	<i>GTF2H5</i>	General transcription factor IIIH, polypeptide 5		601675 608780
				<i>ERCC2 (XPD)</i>	Excision repair cross-complimenting 2		601675 126340
Non-photosensitive		AR	7p14.1	<i>MPLKIP</i>	Chrom 7 open reading frame		234050 609188
Vitamin D-dependent rickets Type 2A	VDDR, Type 2A	AR	12q13.11	<i>VDR</i>	Vitamin D receptor	Metabolic defect	277440 601769
Hypertrichosis							
Cantu syndrome		AR	12p12.1	<i>ABCC9</i>	ATP-binding cassette C9	Transporter defect	239850 601439
Congenital generalized hypertrichosis	HTC2	XLD	Xq27.1	?	?		307150
Hypertrichosis universalis congenita	HTC1 Ambras	AD	8q22	?	?		145701

(continued)

**Table 10.8** (continued)

? Unknown

**Table 10.9** Inheritable connective tissue disorders

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Amyloidosis, Finnish type		AD	9q33.2	<i>GSN</i>	Gelsolin	Actin cleavage defect	105120
Hereditary gelsolin amyloidosis		AR	20q13.12	<i>SLC2A10</i> ( <i>GLUT10</i> )	Solute carrier family 2, member 10	Transport defect	137350
Arterial tortuosity syndrome				<i>LEMD3</i> ( <i>MANI</i> )	LEM domain-containing 3		208050
Buschke-Ollendorff syndrome		AD	12q14.3			Nuclear membrane protein defect	606145
Congenital disorder of glycosylation type Ia	CDG1A Jaeken	AR	16p13.2	<i>PMM2</i>	Phosphomannomutase 2	Glycosylation defect	166700
Costello syndrome		AD	11p15.5	<i>HRAS</i> ( <i>p21</i> )	GTPase HRas	Signal pathway defect	607844
Faciocutaneoskeletal syndrome	ADCL1	AD	7q11.23	<i>ELN</i>	Elastin	Structural defect	212065
Cutis laxa autosomal dominant				<i>FBLN5</i>	Fibulin-5	Structural defect	601785
ADCL2	AD	14q32.12				Structural defect	190020
Cutis laxa type I, autosomal recessive	ARCL1A	AR	14q32.12	<i>FBLN5</i>	Fibulin-5	Structural defect	123700
ARCL1B				<i>EFEMP2</i> ( <i>FBLN4</i> )	EGF-containing fibulin-like extracellular matrix protein 2	Structural defect	130160
ARCL1C Urban-Rifkin- Davis		11q13.1		<i>LTBP4</i>	Latent transforming growth factor-β-binding factor 4	Structural defect	614434
ARCL2A Debre		12q24.31		<i>ATP6VOA2</i>	ATPase, H + transporting, lysosomal	Proton pump defect	604580
ARCL2B		17q25.3		<i>PYCR1</i>	Pyrroline-5-carboxylate reductase 1	Protein synthesis defect	219200
ARCL3A De Barsy		10q24.1		<i>ALDH18A1</i>	Aldehyde dehydrogenase 18	Protein synthesis defect	611716
ARCL3B De Barsy B		17q25.3		<i>PYCR1</i>	Pyrroline-5-carboxylate reductase 1	Protein synthesis defect	612940
Occipital horn syndrome	X-linked cutis laxa		Xq21.1	<i>ATP7A</i>	ATPase, Cu <sup>2+</sup> -transporting (α-polypeptide)	Transport defect	179035
Cutis laxa, neonatal marfanoid type		AD	7q31.1	<i>LAMB1</i>	Laminin polypeptide (subunit β1)	Structural defect	304150
							300011
							150240

(continued)

**Table 10.9** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Ehlers-Danlos, type I (gravis)	EDS1	AD	9q34.3	<i>COL5A1</i>	Collagen V ( $\alpha 1$ chain)	Structural defect	130000 120215
			2q32.2	<i>COL5A2</i>	Collagen V ( $\alpha 2$ chain)		130000 120190
			17q21.33	<i>COL1A1</i>	Collagen I ( $\alpha 1$ chain)		130000 120150
Ehlers-Danlos II (mitis)	EDS2	AD	9q34.3	<i>COL5A1</i>	Collagen V ( $\alpha 1$ chain)		130010 120215
			2q32.2	<i>COL5A2</i>	Collagen V ( $\alpha 2$ chain)		130010 120190
Ehlers-Danlos III (benign hypermobile)	EDS3	AD	6p21.33	<i>TNXB</i>	Tenascin X		130020 600985
			2q32.2	<i>COL3A1</i>	Collagen III ( $\alpha 1$ chain)		130020 120180
Ehlers-Danlos IV (echymotic, arterial)	EDS4 Sack-Barabas	AD	2q32.2	<i>COL3A1</i>	Collagen III ( $\alpha 1$ chain)		130050 120180
Ehlers-Danlos VIA (kyphoscoliotic)	EDS6A	AR	1p36.22	<i>PLOD</i>	Lysyl hydroxylase		225400 153454
Ehlers-Danlos VIIA (arthrochalasia multiplex)	EDS7A	AD	17q21.33	<i>COL1A1</i>	Collagen I ( $\alpha 1$ chain)		130060 120150
Ehlers-Danlos VIIB (arthrochalasia multiplex)	EDS7B	AD	7q21.3	<i>COL1A2</i>	Collagen I ( $\alpha 2$ chain)		130060 120160
Ehlers-Danlos VIIIC (dermatoparaxis)	EDS7C	AR	5q35.3	<i>ADAMTS2</i>	Procollagen I N-peptidase		225410 604539
Ehlers-Danlos VIII (periodontitis)	EDS8	AD	12p13	?	?		130080
Ehlers-Danlos, cardiac valvular form	EDS (other)	AR	7q21.3	<i>COL1A2</i>	Collagen I ( $\alpha 2$ chain)	Structural defect	225320 120160
Ehlers-Danlos, progeroid form	EDS (other)	AR	5q35.3	<i>B4GALT7</i>	Galactosyltransferase I		130070 604327
Ehlers-Danlos, periventricular nodular heterotopia	EDS (other)	XLD	Xq28	<i>FLNA</i>	Filamin A		300537 300017
Ehlers-Danlos-like syndrome due to tenascin X deficiency	EDS (other)	AR	6p21.33	<i>TNXB</i>	Tenascin X		606408 600985
Fabry disease			Xq22.1	<i>GLA</i>	$\alpha$ -galactosidase A	Metabolic defect	301500 300644
Focal dermal hypoplasia	Goltz	XLD	Xp11.23	<i>PORCN</i>	Porcupine homologue	Signaling pathway defect	305600 300651

GM1-gangliosidosis	GM1 I	AR	3p22.3	<i>GLB1</i>	Galactosidase, $\beta$ 1		Metabolic defect	230500 611458
	GM1 II							230600 611458
	GM1 III							230650 611458
Hutchinson-Gilford progeria syndrome	HGPS	AD/AR	1q22	<i>LMNA</i>	Lamin A/C		Structural defect	176670 150330
Hyaline fibromatosis syndrome		AR	4q21.21	<i>ANTXR2 (CMG2)</i>	Anthrax toxin receptor 2		Structural defect	228600 608041
Johanson-Blizzard syndrome	JBS	AR	15q15.2	<i>UBRI</i>	Ubiquitin-protein ligase E3 component N-recognin 1		Metabolic defect	243800 605981
Lipoid proteinosis	Urbach-Wiethe	AR	1q21.3	<i>ECM1</i>	Extracellular matrix protein 1		Structural defect	247100 602201
Loeys-Dietz syndrome	LDS1A, Furlong	AD	9q22.33	<i>TGFBR1</i>	TGF- $\beta$ receptor 1		Signalling pathway defect	609192 190181
	LDS1B		3p24.1	<i>TGFBR2</i>	TGF- $\beta$ receptor 2			610168 190182
	LDS2A		9q22.33	<i>TGFBR1</i>	TGF- $\beta$ receptor 1			608967 190181
	LDS2B, Marfan type II		3p24.1	<i>TGFBR2</i>	TGF- $\beta$ receptor 2			610380 190182
Marfan syndrome type 1		AD	13q21.1	<i>FBNI</i>	Fibrillin		Structural defect	154700 134797
Mandibuloacral dysplasia with type A lipodystrophy	MADA	AR	1q22	<i>LMNA</i>	Lamin A/C		Structural defect	248370 150330
Mandibuloacral dysplasia with type B lipodystrophy	MADB	AR	1p34.2	<i>ZMPSTE24</i>	Zinc metalloproteinase ST1E24		Structural defect	608612 606480
MASS syndrome	OCTD	AD	15q21.1	<i>FBNI</i>	Fibrillin 1		Structural defect	604308 134797
Overlap connective tissue disease								
Melnick-Needles syndrome	MNS	XLD	Xp28	<i>FLNA</i>	Filamin A		Structural defect	309350 300017
Menkes syndrome	MNK	XLR	Xq21.1	<i>ATP7A</i>	ATPase, Cu <sup>2+</sup> -transporting ( $\alpha$ -polypeptide)		Transport defect	309400 300011
Microphthalmia syndrome 7 (microphthalmia with linear skin defects)	MIDAS	XLD	Xp22.2	<i>HCCS</i>	Holocytochrome c synthase		Oxidation-cytochrome c synthase defect	309801 300056

(continued)

**Table 10.9** (continued)

Disorder	Inh. pattern	Other names	Location	Gene(s)	Protein(s)	Functional category	OMIM
Mucolipidosis		II $\alpha/\beta$ I-cell dz	AR 12q23.2	<i>GNPTAB</i>	GlcNAc-phosphotransferase $\alpha/\beta$	Lysosomal defect	252500 607840
		III $\alpha/\beta$ Pseudo-Hurler III $\gamma$	AR 12q23.2	<i>GNPTAB</i>	GlcNAc-phosphotransferase $\alpha/\beta$	Lysosomal defect	252600 607840
		AR 16p13.3		<i>GNPTG</i>	GlcNAc-phosphotransferase $\gamma$	Lysosomal defect	252605 607838
Multiple pterygium syndrome, lethal type	AR	2q37.1		<i>CHRNNG</i>	Cholinergic receptor, nicotinic $\gamma$	Acetylcholine receptor defect	253290 265000
		2q31.1		<i>CHRNA1</i>	Cholinergic receptor, nicotinic $\alpha 1$		253290 100690
		2q37.1		<i>CHRND</i>	Cholinergic receptor, nicotinic $\delta$		253290 100720
Osteogenesis imperfecta, progressive	POH	AD	20q13.32 (imprinting)	<i>GNAS</i>	GNAS complex	Signalling pathway defect	166350 139320
Osteogenesis imperfecta	OI I	AD	17q21.33	<i>COLIA1</i>	Collagen I ( $\alpha 1$ chain)	Structural defect	166200 120150
			7q21.3	<i>COLIA2</i>	Collagen I ( $\alpha 2$ chain)		199200
OI II Vrolik	AD	17q21.33		<i>COLIA1</i>	Collagen I ( $\alpha 1$ chain)		120160 166210 120150
			7q21.3	<i>COLIA2</i>	Collagen I ( $\alpha 2$ chain)		166210
OI III	AD	17q21.33		<i>COLIA1</i>	Collagen I ( $\alpha 1$ chain)		120160 259420 120150
			7q21.3	<i>COLIA2</i>	Collagen I ( $\alpha 2$ chain)		259420
OI IV	AD	17q21.33		<i>COLIA1</i>	Collagen I ( $\alpha 1$ chain)		120160 166220 120150
			7q21.3	<i>COLIA2</i>	Collagen I ( $\alpha 2$ chain)		166220 120150
OI V	AD	11p15.5		<i>IFTM5</i>	Interferon-induced transmembrane protein 5	Signaling pathway defect	610967 614757
OI VII	AR	3p22.3		<i>CRTAP</i>	Cartilage-associated protein		610682 605497
OI XI	AR	17q21.2		<i>FKBP10</i>	FK506 binding protein		610968 607063

Popliteal pterygium syndrome		AD	1q32.2	<i>IRF6</i>	Interferon regulatory factor 6	Transcription factor defect	119500 607199
Van der Woude syndrome		AD	1q32.2	<i>IRF6</i>	Interferon regulatory factor 6	Structural defect	119300 607199
Primary hypertrophic osteoarthropathy (pachydermoperiostosis)	PHOAR1 Touraine-Solente-Gole PHOAR2	AR	4q34.1	<i>HPGD</i>	15-hydroxyprostaglandin dehydrogenase	Prostaglandin metabolism defect	259100 601688
Pseudoxanthoma elasticum	PXE Gronblad-Strandberg	AR	3q22.1-q22.2	<i>SLCO2A1</i>	Solute carrier organic anion transporter	Prostaglandin transport defect	614441 601460
Pseudoxanthoma elasticum-like disorder with multiple coagulation factor deficiencies		AR	16p13.11	<i>ABCC6</i>	Multidrug resistance-associated protein 6 (ATP-binding cassette, subfamily C, member 6)	Mineralization defect	264800 603234
Restrictive dermopathy, lethal		AD	2p11.2	<i>GGCX</i>	$\gamma$ -glutamyl carboxylase	Bone and coagulation defect	610842 137167
Shprintzen-Goldberg craniostenosis syndrome	SGS	AD	1q22	<i>LMNA</i>	Lamin A/C	Structural defect	275210 150330
Stiff skin syndrome		AD	1p34.2	<i>ZMPSTE24</i>	Zinc metalloproteinase STE24	Zinc metalloprotease defect	275210 606480
Torg-Winchester syndrome	Nao	AD	1p36.33	<i>SKI</i>	v-SKI avian sarcoma viral oncogene homologue	Signaling pathway defect	182212 164780
Torticollis, keloids, cryptorchidism, and renal dysplasia	TKCR Goemime	AR	15q21.1	<i>FBNI</i>	Fibrillin 1	Structural defect	184900 134797
Ullrich congenital muscular dystrophy		XLD	16q12.2	<i> MMP2</i>	Matrix metalloproteinase 2	Structural defect	259600 120360
		Xq28	?	?			314300
		AR/AD	21q22.3	<i>COL6A1</i>	Collagen VI, $\alpha 1$ chain	Structural defect	254090 120220
			21q22.3	<i>COL6A2</i>	Collagen VI, $\alpha 2$ chain		254090 120240
			2q37.3	<i>COL6A3</i>	Collagen VI, $\alpha 3$ chain		254090 120250

(continued)

**Table 10.9** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Weill-Marchesani syndrome, autosomal dominant	WMS1	AR	19p13.2	<i>ADAMTS10</i>	A disintegrin-like and metalloproteinase with thrombospondin 10	Protease defect	277600 608990
	WMS2	AD	15q21.1	<i>FBNI</i>	Fibrillin 1	Structural defect	608328 134797
	WMS3	AR	14q24.3	<i>LTPP2</i>	Latent transforming growth factor $\beta$ binding protein 2	Signaling pathway defect	614819 602091
Williams syndrome	WS Williams-Beuren	AD	7q11.23 deletion	?	?		194050
Wrinkly skin syndrome		AR	12q24.31	<i>ATP6VOA2</i>	ATPase, H <sup>+</sup> transporting, lysosomal	Proton pump defect	278250 611716
				? Unknown			

**Table 10.10** Inheritable disorders of pigmentation

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
<b>Hyperpigmentation</b>							
Dermatopathia pigmentosa reticularis	AD	17q21.2	<i>KRT14</i>	Keratin 14	Keratin defect	125595	
Dowling-Degos disease	AD	12q13.13	<i>KRT5</i>	Keratin 5	Keratin defect	148066	
Incontinentia pigmenti	IP2, Bloch-Sulzberger	XLD	<i>IKBKG</i> ( <i>NEMO</i> )	Nuclear factor-κB essential modulator	Signaling pathway defect	179850	
McCune-Albright syndrome	AD lethal Somatic mosaicism	20q13.32	<i>GNAS</i>	GNAS complex	Signaling pathway defect	148940	
Naegeli syndrome	Naegeli-Franceschetti-Jadassohn	AD	<i>KRT14</i>	Keratin 14	Keratin defect	308300	
Reticulate pigmentary disorder, with systemic manifestations, Partington type	Partington	XLR	Xp22-p21	?	?	Signaling pathway defect	300248
<b>Hypo- and depigmentation</b>							
Chédiak-Higashi syndrome	AR	1q42.3	<i>LYST</i>	Lysosomal trafficking regulator	Lysosomal defect	214500	
Griscelli syndrome	GS1	AR	<i>MYO5A</i>	Myosin Va	Myosin defect	606897	
			<i>RAB27A</i>	RAS-associated protein RAB27A	Signaling pathway defect	214450	
	GS2	15q21.3				160777	
	PADD					607624	
	GS3	2q37.3	<i>MLPH</i>	Melanophilin	Melanosome defect	603868	
						609227	
						606526	

(continued)

**Table 10.10** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OIMM
Hermansky-Pudlak	HPS1	AR	10q24.2	<i>HPS1</i>	HPS1 (BLOC-3 component)	Organelle development and trafficking defect	203300 604982
	HPS2		5q14.1	<i>AP3B1</i>	Adaptor protein 3, $\beta 1$ subunit		608233 603401
	HPS3		3q24	<i>HPS3</i>	HPS3		614072 606118
	HPS4		22q12.1	<i>HPS4</i>	HPS4		614073 606682
	HPS5		11p15.1	<i>HPS5</i>	HPS5		614074 607521
	HPS6		10q24.32	<i>HPS6</i>	HPS6		614075 607522
	HPS7		6p22.3	<i>DTNBP1</i>	Dystrobrevin binding protein 1		614076 607145
	HPS8		19q13.32	<i>BLOC1S3</i>	Biogenesis of lysosome-related organelles 1,53		614077 609762
	HPS9		15q21.1	<i>BLOC1S6</i> ( <i>PLDN</i> )	Biogenesis of lysosome-related organelles 1,56		614171 604310
Ocular albinism	Nettleship-Falls	XLR	Xp22.2	<i>GPR143</i>	G-protein-coupled receptor 143	Melanosome defect	300500 300808
Oculocutaneous albinism	OCA1A	AR	11q14.3	<i>TYR (absent)</i>	Tyrosinase	Melanocyte development defect	203100 606933
	OCA1B	AR	11q14.3	<i>TYR (decreased)</i>	Tyrosinase		606952 606633
	OCA2	AR	15q12-q13	<i>OCA2</i>	P protein	Melanosome defect	203200 611409
	OCA3	AR	9p23	<i>TYRP1</i>	Tyrosine-related protein 1	Melanocyte development defect	203290 115501
	OCA4	AR	5p13.2	<i>SLC45A2</i> ( <i>MATP</i> )	Solute carrier family 45		606574 606202
	OCA5	AR	10q22.2-q22.3	<i>C10orf71</i>	Chromosome 10 ORF 11		615179 614537

Tietz albinism-deafness syndrome	Tietz	AD	3p14-p13	<i>MITF</i>	Microphthalmia-associated transcription factor	Melanocyte development defect	103500 156845
<b>Localized</b>							
ABCD syndrome		AR	13q22.3	<i>EDNRB</i>	Endothelin receptor, type B	Complex defect	600501 131244
Albinism-deafness syndrome	Ziprkowski-Mangolis	XLR	Xq24-q26	?	?		300700
Piebald trait (piebaldism)		AD	4q12	<i>KIT</i>	v-KIT Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue (stem cell factor receptor)	Melanogenesis defect	172800 164920
Waardenburg syndrome	Type I WS1	AD	8q11.21	<i>SNAI2 (SLUG)</i>	Snail homologue of Drosophila 2	Transcription factor development defect	172800 602150
	Type IIA WS2A	AD	2q36.1	<i>PAX3</i>	Paired box gene 3	Melanocyte development defect	193500 606597
	Type IIB WS2B	Variable	3p14-p13	<i>MITF</i>	Microphthalmia-associated transcription factor	Melanocyte development defect	193510 156845
	Type IIC WS2C	Variable	1p21-p13.3	?	?		600193
	Type IID WS2D	AR	8p23	?	?		606662
	Type IIE WS2E	AD	8q11.21	<i>SNAI2 (SLUG)</i>	Snail homologue of Drosophila 2	Transcription factor development defect	608890 602150
	Type III WS3	AD/AR/contiguous gene syndrome	22q13.1	<i>SOX10</i>	SRY-box 10	Melanocyte development defect	611584 602229
	Klein-Waardenburg	AD/AR	2q36.1	<i>PAX3</i>	Paired box gene 3	Melanocyte development defect	148820 606597
	Type IVA WS4A	Waardenburg-Shah	13q22.3	<i>EDNRB</i>	Endothelin receptor, type B	Complex defect	277580 131244
	Type IVB WS4B	AD/AR	20q13.32	<i>EDN3</i>	Endothelin-3	Complex defect	613265 131242
	Type IVC WS4C	AD	22q13.1	<i>SOX10</i>	SRY-box 10	Melanocyte development defect	613266 602229

(continued)

**Table 10.10** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Yemenite-deaf-blind-hypopigmentation syndrome		AD	?	?	?		601706
Dyschromatoses							
Dyschromatosis	Dohi	AD	1q21.3	<i>ADAR</i>	Adenosine deaminase RNA specific	Complex defect	127400 146620
		AD	6q24.2-q25.2	?	?		127500
Other							
Achalasia-addisonianism-alacrima syndrome	AAAS	AR	12q13.13	<i>AAAS</i>	<i>AAAS</i> (aladin)	Neural development defect	231550 605378
Adrenal hypoplasia, congenital	Allgrove	XL	Xp21.2	<i>NROB1</i>	Nuclear receptor subfamily 0, group B1	Adrenal gland development defect	300200 300473
Hypoadrenocorticism, familial	Addison	AR	?	?	?		240200
Angelman syndrome							
	Isolated cases		15q11-q13	<i>UBE3A, others (continuous genes)</i>	Ubiquitin-protein ligase E3S	Complex defects	105530 601623
	Paternal uniparental disomy		Xq28	<i>MECP2</i>	Methyl-CpG-binding protein 2		105530 300005
Prader-Willi syndrome	PWS		15q11-q13	<i>SNRPN, NDN, others (continuous genes)</i>	Small nuclear ribonucleoprotein poly peptide N Necdin	Complex defects	176270 182279 602117
Carney complex	NAME LAMB	CNC1 CNC2	AD	<i>PRKAR1A</i>	Protein kinase A, type 1 (α subunit)	Signaling pathway defect	160980 188830
			2p16	?	?		605244
Carney complex variant		AD	17p13.1	<i>MYH8</i>	Myosin, heavy chain 8	Myosin defect	608837
Crouzon syndrome with acanthosis nigricans		AD	4p16.3	<i>FGFR3</i>	Fibroblast growth factor receptor 3		160741 612247
Cystinosis, nephropathic	CTNS	AR	17p13.2	<i>CTNS</i>	Cystinosin	Lysosomal storage defect	134934 219800 606272

Fanconi anemia	<i>FANCA</i>	AR	16q24.3	<i>FANCA</i>	<i>FANCA</i>	DNA repair defect	227650
	Estren-Dameshek	XL	Xp22.2	<i>FANCB</i>	<i>FANCB</i>		607139
	<i>FANCB</i>						300514
							300515
	<i>FANCC</i>	AR	9q22.32	<i>FANCC</i>	<i>FANCC</i>		227645
							613899
							605724
	<i>FANCD1</i>		13q13.1	<i>BRCA2</i>	<i>BRCA2</i>		600185
							227646
	<i>FANCD2</i>		3p25.3	<i>FANCD2</i>	<i>FANCD2</i>		613884
							600901
	<i>FANCE</i>		6p21.31	<i>FANCE</i>	<i>FANCE</i>		613976
							603467
	<i>FANCF</i>		11p14.2	<i>FANCF</i>	<i>FANCF</i>		613897
							614082
	<i>FANCG</i>		9p13.3	<i>FANCG</i> ( <i>XRCC9</i> )	<i>FANCG</i>		602956
							609053
	<i>FANCI</i>		15q26.1	<i>FANCI</i>	<i>FANCI</i>		611360
							609054
	<i>FANCI</i>		17q23.2	<i>BRIPI</i>	<i>BRCA1 interacting protein 1</i>		605382
							614083
	<i>FANCL</i>		2p16.1	<i>FANCL (PHF9)</i>	<i>FANCL</i>		608111
							614087
	<i>FANCM</i>		14q21.2	<i>FANCM</i>	<i>FANCM</i>		609644
							610832
	<i>FANCN</i>						610355
							613390
	<i>FANCO</i>		16p12.2	<i>PALB2</i> ( <i>FANCN</i> )	Partner and localizer of <i>BRCA2</i>		602774
							613951
	<i>FANCP</i>		17q22	<i>RAD51C</i>	<i>RAD51</i>		613278
			16p13.3	<i>SLX4</i>	<i>SLX4</i>		

(continued)

**Table 10.10** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Hemochromatosis	HFE	AR	6p21.3	<i>HFE</i>	HFE	Iron metabolism-homeostasis defect	235200 613609
<b>HFE2A</b>		AR	1q21.1	<i>HFE2 (HJV)</i>	Hemojuvelin		602390 608374
<b>HFE2B</b>			19q13.12	<i>HAMP</i>	Hepcidin antimicrobial peptide		613313 606464
<b>HFE3</b>		AR	7q22.1	<i>TFR2</i>	Transferring receptor 2		604250 604720
<b>HFE4</b>		AD	2q32.2	<i>SLC40A1</i>	Solute carrier family 40 (ferroportin 1)		606069 604653
<b>LEOPARD syndrome</b>	Type 1	AD	12q24.13	<i>PTPN11</i>	Protein tyrosine phosphatase, non-receptor type 11	Signaling pathway defect	151100 176876
	Type 2		3p25.2	<i>RAF1</i>	v-raf-1 murine leukemia viral oncogene homologue 1	Signaling pathway defect	611554 164760
	Type 3		7q34	<i>BRAF</i>	Serine/threonine-protein kinase B-raf	Signaling pathway defect	613707 164757
Lipodystrophy, congenital generalized	CGL1 Berardinelli-Seip	AR	9q34.3	<i>AGPAT2</i>	1-Acylglycerol-3-phosphate O-acyl transferase-2	Complex defect	608594 603100
	<b>CGL2</b>	AR	11q12.3	<i>BSCL2</i>	BSCL2 (Seipin)	Lipogenesis defect	269700 606158
	<b>CGL3</b>		7q31.2	<i>CAV1</i>	Caveolin 1	Caveolae defect	612326 601047
	<b>CGL4</b>		17q21.2	<i>PTRF</i>	RNA polymerase I and transcript release factor (cavin)	Caveolae defect	613327 603198
Lipodystrophy, "acquired" partial	APLD Barraquer-Simons	Complex	19p13.3	<i>LMNB2</i>	Lamin B2	Complex defect	608709 150341

Lipodystrophy, familial partial	FPLD1 Kobberling	AD	?	?	?	?	608600
	FPLD2 Dunnigan	1q22	<i>LMNA</i>	Lamin A/C	Structural defect	151660 150330	
	FPLD3	3p25.2	<i>PPARG</i>	Peroxisome- proliferator-activated receptor $\gamma$	Peroxisome- metabolic defect	604367 601487	
	FPLD4	15q26.1	<i>PLIN1</i>	Perilipin 1	Lipid storage defect	613877 170290	
	FPLD5	3p25.3	<i>CIDE C</i>	Cell death-inducing DFFA-like effector C	Adipocyte development defect	615238 612120	
Von Recklinghausen	AD	17q11.2	<i>NF1</i>	Neurofibromin 1	Signaling pathway defect	162200 613113	
Neurofibromatosis, type I	AD	22q12.2	<i>NF2</i>	Neurofibromin 2 (merlin)	Transmembrane signaling defect	101000 607379	
Neurofibromatosis, type II	Gardner Wishart	AD	<i>NF1</i>	Neurofibromin 1	Signaling pathway defect	162210 613113	
Neurofibromatosis, familial spinal	FSNF	17q11.2	<i>NF1</i>	Neurofibromin 1	Signaling pathway defect	613113 601321	
Neurofibromatosis- Noonan syndrome	NFNS	AD	<i>NF1</i>	Neurofibromin 1	Signaling pathway defect	613113 613113	
Peutz-Jeghers syndrome	Hamartomatous intestinal polyposis	AD	19p13.3	<i>STK11</i>	Serine/threonine- protein kinase 11	175200 602216	
Phenylketonuria	PKU	AR	12q23.2	<i>PAH</i>	Phenylalanine hydroxylase	261600 612349	
Susceptibility to autoimmune disease 1	AD	1p31.3	<i>FOXD3 (AISI)</i>	Forkhead Box D3	Transcription development defect	607836 611539	
Watson syndrome	AD	17q11.2	<i>NF1</i>	Neurofibromin 1	Signaling pathway defect	193520 613113	

Unknown



Hyperlipoproteinemia, type IV (familial hypertriglyceridemia)	HLP type IV	AD	21q11.2	<i>LPL</i>	Lipase, member 1	144600 145750 609252
Cerebrotendinous xanthomatosis (CTX)		AR	11q23.3	<i>APOA5</i>	Apolipoprotein A-V	144600 145750 606368
Hypercholesterolemia, autosomal recessive	ARH	AR	2q35	<i>CYP27A1</i>	Cytochrome P450, subfamily 27A, polypeptide 1 (sterol-27-hydroxylase)	213700 606530
Hypoalphalipoproteinemia		AD	1p36.11	<i>LDLRAP1</i> ( <i>ARH</i> )	LDL receptor adaptor protein 1	603813 605747
		AD	11q23.3	<i>APOA1</i>	Apolipoproteinemia A-1	604091 107680
Sitosterolemia			9q31.1	<i>ABCA1</i>	ATP-binding cassette-1, subfamily A, member 1	604091 600046
		AR	2p21	<i>ABCG5</i>	ATP-binding cassette, subfamily G, member 5	210250 605459
Tangier disease	HDL deficiency I	AR	2p21	<i>ABCG8</i>	ATP-binding cassette, subfamily G, member 8	210250 605460
Transporter defects		AR	9q31.1	<i>ABCA1</i>	ATP-binding cassette-1, subfamily A, member 1	205400 600046
Hartnup disorder	Hartnup	AR	5p15.33	<i>SLC6A19</i>	Solute carrier family 6, member 19 (AA transport)	234500 608893
Hemochromatosis (HFE)	HFE	AR	6p21.3	<i>HFE</i>	HFE	235200 613609
	HFE2A	AR	1q21.1	<i>HFE2</i> ( <i>HJV</i> )	Hemojuvelin	602390 608374
	HFE2B	?	19q13.12	<i>HAMP</i>	Hepcidin antimicrobial peptide	613313 606464
	HFE3	AR	7q22.1	<i>TFR2</i>	Transferring receptor 2	604250 604720
	HFE4	AD	2q32.2	<i>SLC40A1</i>	Solute carrier family 40 (ferroportin 1)	606069 604653
Wilson disease	Wilson	AR	13q14.3	<i>ATP7B</i>	Cu <sup>2+</sup> -transporting P-type ATPase 7B	277900 606882

(continued)

**Table 10.11** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Enzyme defects							
Alkaptonuria	AKU	AR	3q13.33	HGD	Homogentisate 1,2 dioxygenase	Enzymatic defect	203500 607474
Biotinidase deficiency	BTD	AR	3p25.1	BTD	Biotinidase		253260 609019
Fabry disease		XLR/XLD	Xq22.1	GLA	$\alpha$ -galactosidase A		301500 300644
Fucosidosis		AR	1p26.11	FUCAL	$\alpha$ -L-fucosidase		230000
Farber lipogranulomatosis	Farber Ceramidase deficiency	AR	8p22	ASAHI	N-acylsphingosine amidohydrolase 1		228000
Gaucher disease	Gaucher, types I-III	AR	1q22	GBA	Acid $\beta$ -glucosidase		230800 230900
Hereditary angioedema	Type I/II	AD	11q21.2	SERPING1 (C1INH)	Complement component 1 inhibitor		106100 606860
	Type III	AD (females only)	5q35.3	F12	Coagulation factor XII		610618 610619
Holocarboxylase synthetase deficiency	HLCS deficiency	AR	21q22.13	HLCS	Holocarboxylase synthetase		253270 609018
Homocystinuria due to cystathione $\beta$ -synthase deficiency		AR	21q22.3	CBS	Cystathione $\beta$ -synthase		236200 613381
Hyperphenylalaninemia, BH4 deficient	Type A	AR	11q23.1	PTS	6-pyruvoyltetrahydropterin synthase		261640 612719
	Type C		4p15.32	QDPR	Quinoid dihydropteridine reductase		261630 612676
Niemann-Pick disease	Type A	AR	11p15.4	SMPDI	Acid sphingomyelinase		257200 607608
Phenylketonuria	PKU Folling	AR	12q23.2	PAH	Phenylalanine hydroxylase		261600 612349
Prolidase deficiency		AR	19q13.11	PEPD	Prolidase		170100 613230

? Unknown

including classic inborn errors of metabolism, were some of the first to be associated with specific genetic mutations. The gene mutations responsible for these disorders result in accumulation of metabolic products. This may occur through defects in the breakdown, storage, or excretion of these products. Detection of metabolic components or enzyme activity in the urine or plasma can aid in diagnosis. Molecular testing is confirmatory and, in most cases, requires full-gene sequencing.

## 10.10 Miscellaneous Disorders

The members of this final group of disorders have a fairly consistent cutaneous component but do not quite fit into the other more classic categories. As expected, this group is biologically and genetically diverse (Table 10.12). Some, like the immunodeficiencies, indirectly have skin disease through chronic infections.

## 10.11 Practical Issues of Testing

The diagnosis, testing, and management of the genodermatoses patient require a collaborative effort between the patient, the patient's family, the dermatologist, the dermatopathologist, the molecular laboratory director, and the genetic counselor. Diagnosis can be suspected by the clinical presentation, family history, and/or biopsy results, but confirmation usually requires genetic analysis. Most laboratories prefer a whole blood specimen for analysis. Some laboratories can accept oral/buccal swabs as a substitute. Prenatal testing of chorionic villus sampling or amniotic fluid is also available for most genodermatoses. The turnaround time for most mutational analyses is 2–6 weeks.

Commercial laboratories continue to add more and more tests and panels to their menus, and academic centers constantly add and drop esoteric tests based on research interests of faculty members. At the time of this writing, the website [www.genetests.org](http://www.genetests.org) had 600 laboratories registered and is an effective site for identifying

options when looking for a laboratory to perform a specific genetic test [5]. Using this website, one can search by disorder, clinical features, gene, protein, laboratory, or location. Links to individual laboratories are also available for specifics on testing methods and cost [19, 20].

### 10.11.1 Testing Strategy

Some genodermatoses are the result of specific, localized, and easily identifiable genetic defects, while others are caused by mutations spanning all exons of one gene or multiple genes. Molecular defects range from point mutations to large genetic duplications or deletions. All of these possibilities factor into test design. Disorders with characteristic point mutations or hotspots can be diagnosed fairly quickly and inexpensively by targeted sequencing or routine PCR amplification. Disorders with a heterogeneous array of mutations or more complex mutations may require direct sequencing of the entire gene or genes or may require separate analysis for large deletions or duplications, as these are not easily detected by sequence analysis. Many disorders fall somewhere in between, and testing strategies must weigh the relative increment in assay sensitivity achieved by covering more mutation possibilities with the increased labor and cost of more complex testing. A common strategy is beginning with hotspot analysis and then, if negative, reflexing to full-gene sequencing and then deletion/duplication analysis. Deletion/duplication analysis is often performed by array comparative genomic hybridization (aCGH) or multiplex ligation-dependent probe amplification (MLPA).

As with many other areas of molecular diagnostics, there has been a recent surge in the use of massively parallel (next-gen) sequencing for genodermatoses mutational analysis. Next-gen sequencing is primarily used in the research setting, but more recently, some commercial laboratories have begun to add versions of these assays to their test menus. This technology has the advantage of generating massive amounts of data points in a relatively short period of time and at a

**Table 10.12** Miscellaneous inheritable disorders with prominent cutaneous manifestations

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Immunodeficiencies							
Autoimmune polyendocrine syndrome	APS1 APECED	AR	21q22.3	AIRE	Autoimmune regulator	Transcription factor defect	240300 607358
Bare lymphocyte syndrome	BLS I	AR	6p21.32	TAP2	Transporter, ATP-binding cassette 2	Antigen presentation (MHC class I) defect	604571 170261
		6p21.32	TAP1	Transporter, ATP-binding cassette 1	TAP binding protein		604571 170260
BLS II		AR	1q21.3	RFX5	Regulatory factor X 5	Antigen presentation (MHC class II) defect	601962 209920
		13q13.3	RFXAP	Regulatory factor X-associated protein			601863 209920
		16p13.13	MHC2TA	MHC class II transactivator			600005 209920
Chronic granulomatous disease	CGD, X-linked	XLR	Xp11.4	CYBB	Regulatory factor X, ankyrin repeat containing	Phagocytic defect	209920 603200
	CGD, AR, cyt-B-neg	AR	16q24.3	CYBA	Cytochrome b <sub>245</sub> α		300481 233690
	CGD, AR, cyt-B-pos type I	AR	7q11.23	NCFL	Neutrophil cytosolic factor 1		608508 233700
	CGD, AR, cyt-B-pos type II	AR	1q25.3	NCFL2	Neutrophil cytosolic factor 2		608512 233710
	CGD, AR, cyt-B-pos type III	AR	22q12.3	NCFL4	Neutrophil cytosolic factor 4		613960 601488
Congenital disorder of glycosylation, type IIc	CDG2C LAD2 Rambam-Hasharon	AR	11p11.2	SLC35G1 C1	Solute carrier family 35, member C1	Glycosylation defect	266265 605881
Donohue syndrome	Leprechaunism	AR	19p13.2	INSR	Insulin receptor	Metabolic defect	246200 147670

Hyperimmunoglobulin E recurrent infection syndrome	HIES, AD Job	AD	17q21.2	<i>STAT3</i>	Signal transducer and activator of transcription 3	147060 102382
	HIES, AR	AR	9p24.3	<i>DOCK8</i>	Dedicator of cytokinesis	243700 611432
Immune dysregulation, polyendocrinopathy, and enteropathy, X-linked	PEX	XLR	Xp11.23	<i>FOX P3</i>	Forkhead box P3	304790 300292
Leukocyte adhesion deficiency	LAD1	AR	21q22.3	<i>ITGB2</i>	$\beta$ 2-integrin	Neutrophil defect 116920
	LAD3 LAD1-variant		11q13.1	<i>FERMT3</i>	Fermitin family 3	600065 612840 607901
LIG4 syndrome		AR	13q33.3	<i>LIG4</i>	DNA ligase 4	DNA repair defect 606593 601837
Nijmegen breakage syndrome	NBS AT-2 Seemanova II	AR	8q21.3	<i>NBN</i>	Nibrin	DNA repair defect 251260 602667
Omenn syndrome		AR	11p12	<i>RAG1</i>	Recombination activating gene 1	Recombination (B/T development) 603554 179615
			11p12	<i>RAG2</i>	Recombination activating gene 2	defect 603554 179616
			10p13	<i>DCLRE1C (ARTEMIS)</i>	DNA cross-link repair 1C	603554 605988
Pineal hyperplasia, insulin-resistant diabetes mellitus, and somatic abnormalities	Rabson-Mendenhall	AR	19p13.2	<i>INSR</i>	Insulin receptor	Metabolic defect 262190 147670
Tyrosine kinase 2 deficiency		AR	19p13.2	<i>TYK2</i>	Tyrosine kinase 2	Cytokine defect 611521 176941
WHIM syndrome		AD	2q22.1	<i>CXCR4</i>	Chemokine (C-X-C) receptor 4	Cytokine defect 193670 162643
Wiskott-Aldrich syndrome	WAS1	XLR	Xp11.23	<i>WAS</i>	WAS protein	Cytoskeleton defect 301000 300392
	WAS2	AR	2q31.1	<i>WIPF1</i>	WAS/WASL-interacting protein 1	Cytoskeleton defect 614493 602357

(continued)

**Table 10.12** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Periodic fever disorders							
Periodic fever, familial	PFH Hibernal FMF	AD AR/ AD	12p13.31 16p13.3	<i>TNFRSF1A</i> <i>MEFV</i>	Tumor necrosis factor receptor superfamily 1A Familial Mediterranean fever (pyrin)	Cytokine-signaling defect	142680 191190
Familial Mediterranean fever							249100
Hyper-IgD syndrome	HIDS	AR	12q24.11	<i>MVK</i>	Mevalonate kinase	Cholesterol biosynthesis defect	134610 608107
CINCA syndrome	CINCA	AD	1q44	<i>NLRP3</i> ( <i>CIAST1</i> )	NLR family, pyrin domain 3 (cryopyrin)	Cytokine-signaling defect	260920 251170
Familial cold autoinflammatory syndrome	FCAS1	AD	1q44	<i>NLRP3</i> ( <i>CIAST1</i> )	NLR family, pyrin domain 3 (cryopyrin)	Cytokine-signaling defect	607115 606416
	FCAS2	AD	19q13.42	<i>NLRP12</i>	NLR family, pyrin domain 12		120100
Muckle-Wells syndrome		AD	1q44	<i>NLRP3</i> ( <i>CIAST1</i> )	NLR family, pyrin domain 3 (cryopyrin)		606416 611762
Pyogenic sterile arthritis, pyoderma gangrenosum and acne	PAPA	AD	15q24.3	<i>PSTPIP1</i>	Proline/serine/threonine phosphatase-interacting protein 1	Signaling pathway defect	191900 606416 604416
other							603347
Acromesomelic dysplasia, Maroteaux type	AMDM	AR	9p13.3	<i>NPR2</i>	Natriuretic peptide receptor B	Signaling pathway defect	602375 108961
Adrenoleukodystrophy	ALD Siemerling-Creutzfeldt	XLR	Xq28	<i>ABCD1</i>	ATP-binding cassette D1	Peroxisome defect	300100 300371
Aicardi-Goutières syndrome	AGS1 Cree AGS2	AR/ AD AR	3p21.31 13q14.3	<i>TREX1</i> <i>RNASEH2B</i>	3-prime repair exonuclease 1 Ribonuclease H2B	DNA repair defect	225750 606609
	AGS3	AR	11q13.1	<i>RNASEH2C</i>	Ribonuclease H2C	Ribonuclease defect	610181 610326
	AGS4	AR	19p13.2	<i>RNASEH2A</i>	Ribonuclease H2A		610329 610330
	AGS5	AR	20q11.23	<i>SAMHD1</i>	SAM domain and HD domain protein 1	Monocyte defect	612952 606754
	AGS6	AR	1q21.3	<i>ADAR</i>	Adenosine deaminase, RNA specific	Transcription defect	615010 146920

Aspartylglucosaminuria	AGA	AR	4q34.3	AGA	Aspartylglucosaminidase	Metabolic defect	208400 613228
Autoimmune lymphoproliferative syndrome	ALPS1A Canale-Smith ALPS1B	AD AD	10q23.31 1q24.3	FAS <i>FASLG</i>	Tumor necrosis factor receptor 6 (Fas)	Apoptosis defect	601859 134637
	ALPS2A	AD	2q33.1	<i>CASP10</i>	Tumor necrosis factor ligand 6 (Fas ligand)		601859 134638
	ALPS 4	AD	1p13.2	<i>NRAS</i>	Caspase 10		603909 601762
Blau syndrome	Jabs Blau	AD AR	16q12.1 10q24.2	<i>NOD2</i> ( <i>CARD15</i> ) <i>CPN1</i>	Nucleotide-binding oligomerization domain protein 2 Carboxypeptidase N 1	Apoptosis defect Peptide regulation defect	186580 605956
Carboxypeptidase N deficiency	CADASIL	AD	19p13.2	<i>NOTCH3</i>	NOTCH 3	Signaling pathway defect	212070 603103
Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy		AR	19p12	<i>CRLF1</i>	Cytokine receptor-like factor 1	Cytokine defect	125310 600276
Cold-induced sweating syndrome			11q13.3	<i>CLCF1</i>	Cardiotrophin-like cytokine factor 1		272430 604237
Cutis gyrata syndrome	Beare-Stevenson	AD	10q26.13	<i>FGFR2</i>	Fibroblast growth factor receptor 2	Fibroblast development defect	272430 607672
Familial isolated deficiency of vitamin E	VED Friedreich-like ataxia	AR	8q12.3	<i>TPPA</i>	Tocopherol transfer protein α	Vit. E homeostasis defect	123790 176943
Glucocorticoid deficiency	GCCD1	AR	18p11.21	<i>MC2R</i> ( <i>ACTHR</i> )	Melanocortin 2 receptor	Glucocorticoid defect	202200 607397
	GCCD2	AR	21q22.11	<i>MRAP</i>	Melanocortin 2 receptor accessory protein		607398 699196
	GCCD3	?	8q11.2-q13.2	?	?	Nicotinamide nucleotide transhydrogenase	
	GCCD4	AR	5p12	<i>NNT</i>		Mitochondrial defect	614736 607878

(continued)

**Table 10.12** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Hereditary sensory and autonomic neuropathy	HSAN1A	AD	9q22.31	<i>SPTLC1</i>	Serine palmitoyltransferase, long-chain base 1	Sphingolipid biosynthesis defect	162400
	HSAN1C	AD	14q24.3	<i>SPTLC2</i>	Serine palmitoyltransferase, long-chain base 2		605712
	HSN1D	AD	14q22.1	<i>ATL1</i>	Atlastin GTPase 1	Axon formation defect	613708
	HSN1E	AD	19p13.2	<i>DNM1T1</i>	DNA methyltransferase 1	Methylation defect	614116
	HSAN2A Morvan	AR	12p13.33	<i>WNK1 (HSN2)</i>	Protein kinase, lysine deficient	Complex defect	126375
	HSAN2B	AR	5p15.1	<i>FAM134B</i>	Family with sequence similarity 134B	Complex defect	201300
	HSN2C	AR	2q37.3	<i>KIF1A</i>	Kinesin 1A	Synaptic vesicle defect	605232
	HSAN3 Riley-Day	AR	9q31.3	<i>IKBKAP</i>	Inhibitor of kappa light polypeptide gene enhancer in B-cells kinase 1	Complex defect	613114
	HSAN4	AR	1q23.1	<i>NTRK1</i>	Neurotrophic tyrosine kinase receptor, type 1	Neuroregulation defect	601255
	HSAN5	AR	1p13.2	<i>NGFB</i>	Nerve growth factor β	Neural growth defect	223900
	HSAN6	AR	6p12.1	<i>DSTR</i>	Dystonin	Structural defect	603722
	HFTC Teutschlaender	AR	2q24.3	<i>GALNT3</i>	GalNAc transferase 3	Glycosylation defect	255800
			12p13.32	<i>FGF23</i>	Fibroblast growth factor 23	Complex defect	191315
			13q13.1	<i>KL</i>	Klotho		608654
						Complex defect	162060
							614653
							113810
							211900
							601756
							211900
							603380
							211900
							604824
							242900
							606622
							269920
							604322
							609242
							104170

Hyperphosphatemic familial tumoral calcinosis  
Immunosseous dysplasia, Schimke type  
Infantile sialic acid storage disorder (ISSD)  
Kanzaki disease

Lesch-Nyhan syndrome	LNS	XLR	Xq26.2-q26.3	<i>HPRT</i>	Hypoxanthine guanine phosphoribosyltransferase	Purine salvage defect	300322 308000
Lowe oculocerebral syndrome	OCRL	XLR	Xq25-q26	<i>OCRL</i>	OCRL	Metabolic defect	309000 300535
$\alpha$ -mannosidosis B, lysosomal Mucopolysaccharidosis	MANSA	AR	19p13.2	<i>MAN2B1</i>	$\alpha$ -mannosidase 2B1	Lysosomal storage defect	248500 609458
MPS3A Sanfilippo A	MPS3A	AR	17q25.3	<i>SGSH</i>	N-sulfoglucosamine sulfhydrolase	Lysosomal storage disease	252900 605270
MPS3B Sanfilippo B	MPS3B	AR	17q21.2	<i>NAGLU</i>	N-acetyl glucosaminidase $\alpha$		252920 609701
MPS3C Sanfilippo C	MPS3C	AR	8p11.21	<i>HGSNAT</i>	Heparin- $\alpha$ -glucosaminidase		252930 610453
Myotonic dystrophy 1	DM1	AD	19q13.32	<i>DMPK</i>	Dystrophia myotonica protein kinase	Tri nucleotide repeat	160900 605377
Steinert DM2 Ricker DM2	Steinert	AD	ZNF9	Zinc finger protein 9	Zinc finger protein 9	RNA binding defect	602668 116955
Orofaciodigital syndrome OFD51	OFD51	XLD	Xp22.2	<i>OFDI</i>	Oral-facial-digital 1	Complex defect	311200 300170
Papillon-Lefèvre syndrome	Papillon-Lefèvre	AR	3q22.3	<i>PCCB</i>	Propionyl-CoA carboxylase $\beta$	Metabolic defect	606054 232050
Propionic acidemia		13q32.3	<i>PCCA</i>	Propionyl-CoA carboxylase $\alpha$			606054 232000
Pseudohypoparathyroidism PHP 1A Albright	PHP 1A Albright	AD	20q13.32	<i>GNAS</i>	GNAS complex	Signaling pathway defect	103580 135320
Pseudopseudohypoparathyroidism		AD	20q13.32	<i>GNAS</i>	GNAS complex	Signaling pathway defect	612463 139320
RAPADILINO syndrome		AR	8q24.3	<i>RECQL</i>	RECQL protein-like 4	DNA helicase defect	266280 603780
Retinal vasculopathy with cerebral leukodystrophy	RVCL	AD	3p21.31	<i>TREX1</i>	3-prime repair exonuclease 1	DNA repair defect	192315 606609
Sarcoidosis, early onset	EOS	AD	16q12	<i>NOD2</i> ( <i>CARD15</i> )	Nucleotide-binding oligomerization domain protein 2	Complex defect	609464 605956

(continued)

**Table 10.12** (continued)

Disorder	Other names	Inh. pattern	Location	Gene(s)	Protein(s)	Functional category	OMIM
Simpson-Golabi-Behmel syndrome	SGBS1	XLR	Xq26.2	<i>GPC3</i>	Glypican 3	Signaling pathway defect	312870
	SGBS2	XLR	Xp22.2	<i>OFDI</i>	Oral-facial-digital 1	Complex defect	300037
Sotos syndrome	Sotos1	AD	5q35.2-q35.3	<i>NSDI</i>	Nuclear receptor-binding Su-var protein 1	Complex defect	300209
	Sotos2	AD	19p13.2	<i>NFIX</i>	Nuclear factor I/X	Transcription defect	300170
Weaver syndrome	WVS	AD	7q36.1	<i>EZH2</i>	Enhancer of zeste 2	Transcription defect	601573

? Unknown

relatively low cost per base. These features have made the sequencing of entire genes or multiple genes economically feasible. Details of this technology are beyond the scope of this text, and the interested reader is directed to reviews on the topic [21–23]. In short, next-gen sequencing involves simultaneously amplifying numerous and overlapping fragments of DNA, comparing the overlapping regions to determine the DNA sequences, and comparing the sequences with existing human databases. There are many variations of this technology. The signal outputs can be based on luminescence, fluctuations in ion concentration, or fluorescence. Sequencing can be performed on coding regions only (whole exome) or the entire genome (whole genome). Sequencing of RNA transcripts (whole transcriptome) can also be performed. This latter strategy does not yet have clear utility for diagnosis of the genodermatoses but could potentially be used to better characterize these disorders. While there is definitely an excitement about next-gen sequencing for diagnosis of the genodermatoses and other applications, there are still some limitations to widespread use. This technology continues to have technical limitations. One limitation is the accuracy (or inaccuracy) of sequencing highly repetitive sequences (ranging from several bases to several million bases), which account for almost half of the human genome. Another is sequencing stretches of DNA with high or low GC content (guanine/cytosine content, or GC bias), which affect the efficiency of PCR and thus data capture [24]. Beyond the technical limitations, there continue to be interpretive and bioinformatic challenges with the immense generation of sequence data, and there continue to be struggles with lagging appropriate reimbursement, as is the case with most new technologies.

## 10.11.2 Interpretation

Regardless of testing method, the results of mutational analyses can be broadly categorized as follows: (1) *normal* (no mutation identified), (2) *abnormal* (mutation identified), and (3) *indeterminate* (sequencing variant identified but

unknown significance). When the patient's gene sequence does not deviate from a normal reference or if variants are identified that have been previously shown to be biologically irrelevant, the result of the genetic test is normal. Of course, the genetic analysis is only one data point. The patient's clinical features and family history may clearly point to an inheritable disorder, and in these cases, the possibility of a mutation in another gene, perhaps a modifier gene or gene with a product of similar function, should be entertained. If a mutation is identified and it has previously been characterized as functionally relevant and associated with the observed phenotype, then the result of the genetic test is abnormal. In many cases, however, a genetic variant of unknown significance is identified, and causality can be difficult to prove. If the variant is detected among multiple affected family members, there is support for causality. Often times, however, these data are not available. For example, using whole-exome sequencing, on average, across all inheritable disorders, there is currently an estimated 25–33 % yield for obtaining a definitive mutational analysis result. Most interpretations are indeterminate (variants of unknown significance). These statistics will undoubtedly improve as further *in vitro* and *in vivo* studies determine the functional impact of specific genetic variants and the genetic databases become more populated.

## 10.11.3 Cost and CPT Coding

Regarding costs of molecular testing, there is extreme variability, as some laboratories charge a straight fee, others bill insurance providers, and others do not charge at all (usually for research purposes or compassionate care). The cost of performing a molecular test, in general, is reflected in the test complexity. The main variable impacting complexity is the size of the DNA real estate required for sequencing. This size increases (as does cost) with increased dispersement of mutations over the gene and increased overall size of the gene. Focused or small gene testing can usually be performed for under \$500 (e.g., *CDKN2A*). Testing for a wide array of potential mutations

within large genes (e.g., *COL7A1*) can cost \$3000 or more. An additional significant factor on cost is interpretation. As the cost of sequencing continues to drop, the cost of interpretation continues to rise [25]. This is an era of massive data generation. Statisticians and experts in bioinformatics are needed to review data and compare the data to updated mutational databases. This task becomes more complex with larger genes, which potentially lead to the detection of more genetic variants, each of which requires an interpretation. Data must be stored and laboratories must stay current, each adding to the overall cost of testing.

Current procedural terminology (CPT) coding was recently updated for molecular methods, attempting to standardize reimbursement for laboratories' efforts [26]. Reimbursement for CPT codes loosely reflects test complexity. Beginning in 2013, previously employed "stacking" techniques (i.e., listing of a CPT code for every extraction, amplification, etc., which can vary widely among laboratories) have been replaced by CPT codes designated for specific genes and/or methods. These CPT codes encompass cell lysis, nucleic acid stabilization, extraction, digestion, amplification, and detection procedures. Microdissection codes (88380/88381) and professional interpretation codes (26 modifiers) should be reported separately when applicable. CPT codes are updated annually.

There are now three tiers of CPT codes for molecular testing. Tier 1 contains genes with high-volume testing (e.g., *PTEN*) and uses codes in the 81200s and 81300s. There are only a handful of genes listed in Tier 1 and even fewer for the genodermatoses (Table 10.13). Tier 1 codes are preferred when available since these are high-volume tests with well-characterized and fairly standard methodologies. A larger list of genes resides in Tier 2 (Table 10.14). In general, these are more rare genetic disorders. Tier 2 coding is based on gene and method with levels 1–9 (88400–88408), lumped based on complexity of the tests for specific genes. If a molecular test or gene is not listed in Tiers 1 or 2, 81479 should be used. This is the unlisted molecular procedure code. Because populating Tiers 1 and 2 is still in its infancy, 81479 is currently the most commonly used code. Unfortunately, it is

**Table 10.13** Tier 1 CPT codes for genodermatoses testing

Tier 1 CPT code	Gene	Methods/comments
81201	<i>APC</i>	Full-gene sequencing
81202		Detection of known variant
81203		Duplication/deletion analysis
81209	<i>BLM</i>	Multiplex PCR variant analysis
81242	<i>FANCC</i>	Fanconi anemia, common variant
81251	<i>GBA</i>	Gaucher disease, common variants
81252	<i>GJB2</i>	Full-gene sequencing
81253		Detection of known variant
81254	<i>GJB6</i>	Sequencing and/or PCR amplification
81256	<i>HFE</i>	PCR amplification for hemochromatosis
81292	<i>MLH1</i>	Full-gene sequencing
81293		Detection of known variant
81294		Duplication/deletion analysis
81295	<i>MSH2</i>	Full-gene sequencing
81296		Detection of known variant
81297		Duplication/deletion analysis
81298	<i>MSH6</i>	Full-gene sequencing
81299		Detection of known variant
81300		Duplication/deletion analysis
81317	<i>PMS2</i>	Full-gene sequencing
81318		Detection of known variant
81319		Duplication/deletion analysis
81321	<i>PTEN</i>	Full-gene sequencing
81322		Detection of known variant
81323		Duplication/deletion analysis
81330	<i>SMPD1</i>	Niemann-Pick disease type A carrier screening, variable methods
81331	15q	MLPA for Angelman/Prader-Willi uniparental disomy, variable methods

Abbreviation: MLPA multiplex ligation-dependent probe amplification

currently a challenge to get reimbursed by insurance companies for 81479, as the corresponding test may be considered experimental. Because of the challenges surrounding 81479, many laboratories are faced with time-consuming appeals to insurance companies and/or directly passing on costs of these tests to patients or practices. The goal is to eventually move all 81479 genes and procedures

**Table 10.14** Tier 2 CPT codes for genodermatosis testing

Level	Tier 2 CPT code	Genes	Primary methods	Cost <sup>a</sup>
1	81400	<i>SHOC2</i>	Single variant detection by restriction enzyme digestion or melting curve analysis	\$
2	81401	<i>APOB, AR, CBS, FGFR3, H19, KCNQ1OT1, MUTYH, MT-TS1, NOD2</i>	2–10 SNPs, 1 methylated variant, 1 somatic variant	
3	81402	15q (uniparental disomy)	>10 SNPs	
4	81403	<i>FGFR3, HRAS, KRAS, MT-TS1</i>	Single exon sequencing, >10 amplicons by multiplex PCR, mutation scanning or dup/del analysis of 2–5 exons	
5	81404	<i>CDKN2A, FGFR2, FGFR3, HRAS, MEN1, RAF1, STK11, TYR</i>	2–5 exons by sequencing, mutation scanning or dup/del analysis of 6–10 exons	
6	81405	<i>ABCD1, AR, BCS1L, ENG, FH, GLA, KRAS, LDLR, MEN1, NF2, NSD1, RET, SHOC2, SMAD4, STAT3, STK11, SURF1, TSC1</i>	6–10 exons by sequencing, mutation scanning or dup/del analysis of 11–25 exons	
7	81406	<i>ATP7B, BRAF, CBS, COL6A2, CREBBP, DSP, ENG, LDLR, MAP2K1, MAP2K2, MUTYH, NF2, NOTCH3, NSD1, PTPN11, RAF1, SMAD4, SOS1, TSC1, TSC2, UBE3A, WAS</i>	11–25 exons by sequencing, mutation scanning or dup/del analysis of 26–50 exons	
8	81407	<i>COL6A1, COL6A2, COL6A3, CREBBP, TSC2</i>	26–50 exons by sequencing, mutation scanning or dup/del analysis of >50 exons	
9	81408	<i>ATM, COL1A1, COL1A2</i>	>50 exons by sequencing	\$\$\$

Abbreviation: SNP single nucleotide polymorphism

<sup>a</sup>\$<\$1000; \$\$\$>\$3000

to Tier 2 and eventually to Tier 1. Given the constantly changing data on diseases, hotspot mutations, and preferred testing methods, however, this will be an arduous, ongoing process.

#### <sup>1</sup>CPT coding

Current Tier 1 and Tier 2 CPT codes provided in Table 10.13 and Table 10.14, 81479, which is the unlisted molecular procedure code, for all others

<sup>1</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [26]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

## 10.12 Summary

The diagnosis, testing, and management of the genodermatosis patient require a collaborative effort between the patient, the patient's family, and the entire care team. Diagnosis can be suspected by the clinical presentation, family history, and/or biopsy results, but confirmation often requires genetic analysis. Molecular studies offer the added benefits of providing prenatal information, identifying a family signature mutation for screening carriers, and potentially more accurately predicting disease course by determining the specific mutational variant. Mutational analysis is usually performed by direct sequencing in conjunction with other complementary assays for identifying large duplications and deletions.

High-throughput (next-gen) sequencing is emerging as a rapid, cost-effective, and preferred method to screen multiple candidate genes for a single disorder or for a differential diagnosis spanning multiple genodermatoses.

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## Contents

11.1	<b>Introduction</b> .....	314
11.2	<b>Assay Design and Testing Strategies</b> ....	316
11.3	<b>Clinical Molecular Infectious Disease Testing</b> .....	318
11.4	<b>Viruses</b> .....	319
11.5	<b>Viral Infections Associated with Neoplasia</b> .....	320
11.5.1	Human Papillomavirus (HPV).....	320
11.5.2	Human Herpesvirus 8 (HHV-8).....	322
11.5.3	Merkel Cell Polyomavirus (MCV or MCPyV).....	323
11.6	<b>Herpesvirus</b> .....	324
11.7	<b>Fungi</b> .....	324
11.8	<b>Parasites</b> .....	326
11.8.1	Leishmania.....	326
11.9	<b>Bacteria</b> .....	327
11.9.1	Mycobacteria.....	327
11.9.2	Rickettsia.....	330
11.9.3	Lyme Disease.....	331
11.9.4	Syphilis .....	331
11.9.5	Bartonella.....	332
11.9.6	Cutaneous Anthrax.....	333
11.10	<b>Drug Resistance Testing</b> .....	334
11.10.1	Methicillin-Resistant Staphylococcus aureus .....	334
11.11	<b>Genetic Factors That Influence Susceptibility/Resistance to Infectious Agents</b> .....	335
11.12	<b>Practical Considerations</b> .....	335
11.12.1	External Controls (Positive, Negative, and No-Template) .....	336
11.12.2	Sensitivity Control .....	337
11.12.3	Internal Control.....	337
11.12.4	Inhibition Control.....	337
11.13	<b>Summary</b> .....	337
	<b>References</b> .....	338

## Key Points

1. All pathogenic organisms, including bacteria, viruses, fungi, and parasites, have nucleic acid genomes, which can be targeted by highly sensitive and specific molecular assays.
2. Molecular testing for infectious agents has advantages over traditional culture techniques, including the ability to detect nonviable organisms (allowing more flexibility for specimen type), superior sensitivity, and a more rapid turnaround time.
3. Assay design for molecular infectious disease testing can be highly species specific or able to detect multiple different pathogens simultaneously.
4. Clinical molecular assays have been designed and optimized for the identification of organisms either from a primary specimen (blood, tissue, etc.) or from organisms isolated in culture. The assay performance characteristics required for each approach are very different.

5. Appropriate assay controls are particularly important for ensuring accurate molecular infectious disease test results. Negative test results due to the absence of an organism are indistinguishable from a failed molecular enzymatic reaction if appropriate controls are not used.
6. Several viral infections, including HPV, HHV-8, and MCV, are associated with cutaneous tumors. Molecular testing for these viruses may be helpful for tumor diagnosis in specific clinical settings.
7. Molecular assays can be particularly helpful in the diagnosis of uncommon pathogens and for identification of pathogens that cause similar clinical and histomorphologic presentations, including deep fungal, mycobacterial, and some parasitic infections.
8. All known mechanisms of antibiotic drug resistance involve a change in the infectious organism's nucleic acids, which can be detected by nucleic acid-based tests.
9. In the future, analysis of germline genetic variants may be used to provide information about an individual's sensitivity or resistance to specific infectious organisms.

disease monitoring and for determining an infectious organism's drug resistance/susceptibility. In addition to their clinical utility, molecular assays are heavily relied upon for epidemiologic studies, such as outbreak investigations, and monitoring for drug-resistant strains. They are also useful for environmental and industrial monitoring such as food and water safety testing, reservoir and vector surveys, and bioterrorism surveillance.

Historically, pathogenic organisms have been identified by culture of a lesion, serologic methods, and/or microscopic examination of tissue samples with or without histochemical and immunohistochemical stains. Over the last two decades, however, assays for many clinical infectious disease targets have transitioned from standard culture and biochemical techniques to molecular methods. Initial targets for clinical molecular testing were predominantly viruses, primarily because culture-based and serologic methods performed relatively poorly and were time consuming. Molecular testing methods are now rapidly becoming the method of choice even for organisms that are readily cultured. This change has been driven by the significant advantages of molecular testing over traditional culture and direct detection methods (outlined in Table 11.1). Moving forward, molecular testing will also likely play a prominent role for emerging infectious diseases, as these assays can be developed and validated much more quickly than the process of establishing optimal culture conditions.

## 11.1 Introduction

All organisms, including bacteria, viruses, protozoa, fungi, and parasites, have nucleic acid genomes. The specific composition of their genomes is highly variable, composed of single- or double-stranded RNA or DNA that is arranged in a linear or circular structure. Regardless, molecular methods are well suited for detecting these organisms by specifically targeting their nucleic acid sequences. The exquisite sensitivity and specificity of molecular methods for detecting and differentiating infectious organisms have led to their use in a number of applications. In clinical medicine, molecular infectious disease testing is useful not only for identifying organisms but also for

One of the most significant advantages of molecular testing is that viability and/or growth of an organism is not required for testing. This allows molecular testing to be performed for a variety of pathogens for which diagnosis by traditional methods has been difficult or impossible due to complex nutrient requirements and/or slow growth cycles of the organism. Molecular approaches also allow testing to be performed on a broader array of specimen types. For example, formalin and other cellular fixatives generally render organisms nonviable for culture, but their nucleic acids are still present and suitable for identification by molecular testing. Additionally, the lack of a viability requirement provides for a safer testing environment, as laboratorians are

**Table 11.1** Advantages and disadvantages of using molecular techniques for infectious disease applications

Advantages
<p><i>Speed and throughput:</i> molecular methods are faster than traditional culture methods, allowing for shorter turnaround times and for more samples to be tested in a shorter period of time. Because molecular testing generally does not require a large amount of laboratory space and the testing is relatively automated molecular methods are amenable to high-volume/high-throughput testing</p>
<p><i>No requirement for organism viability:</i> this is particularly important in a reference lab setting where the conditions during transport (temperature, time, etc.) are variable. Despite the best efforts, transport conditions may not be suitable to maintain viability of the organism</p>
<p><i>Suitable for all types of organisms:</i> bacteria, virus, fungi, and parasites all have nucleic acid genomes that can be targeted for identification. Complex nutrient requirements and/or slow growth cycles can make some of these organisms very difficult or impossible to culture in the laboratory</p>
<p><i>Suitable for many specimen types:</i> molecular methods can be performed on a variety of specimens from which culture is not an option. These include fixed specimens such as formalin-fixed paraffin-embedded tissues and liquid cytology specimens</p>
<p><i>High sensitivity:</i> molecular tests generally have a superior limit of detection compared to direct detection methods such as special stains and immunohistochemistry</p>
<p><i>High specificity:</i> well-designed molecular assays are highly specific and generate very few false-positive results. Culture and direct detection methods are more subjective (interpreting how the organism “looks”), which can lead to false-positive results</p>
<p><i>Laboratory safety:</i> molecular methods reduce the exposure of laboratory employees to viable infectious agents</p>
Disadvantages
<p>Molecular assays only detect the specific organism(s) for which they are designed, thus requiring a clinical suspicion for a specific organism</p>
<p>Nonviable organisms in a specimen will yield a positive result. Risk for false-positive results from contaminating, nonviable organisms</p>
<p>Potential for false positives due to carryover contamination. Improvements in methodologies, techniques, and use of controls have greatly reduced the risk of false positives due to carryover contamination. Although unlikely, carryover contamination should not be discounted if a positive result does not correlate with the clinical presentation</p>

not exposed to viable infectious agents. Finally, the ability to perform diagnostic testing on nonviable organisms often contributes to the superior sensitivity (fewer false-negative results) of molecular methods compared to traditional methods. Although the improved sensitivity is partly due to the ability of molecular methods to detect very low number of organisms (superior limit of detection), it is also due to the fact that viability of the organism does not need to be maintained from collection and transport of the specimen through the entire testing process. This is especially true in a reference lab setting where transport conditions such as temperature and time can be highly variable and may not be suitable to maintain viability of the organism.

Additional benefits of molecular methods to patient care include more accurate and more rapid test results. Molecular methods can be performed in hours, compared to days or weeks for

standard culture methods. Accurate and rapid diagnosis allows for timely patient treatment and potentially improved clinical outcomes. In addition, reduction in the use of antibiotics that are ineffective for the infecting pathogen may help decrease or at least stabilize the emergence of resistant strains.

Additional benefits of molecular approaches to the testing laboratory include streamlined transport and testing of specimens, with no requirement for special handling to maintain viability. Once in the lab, DNA extraction and molecular testing for a variety of different organisms can be performed using very similar, if not identical, methods and conditions. This is in contrast to traditional diagnostic methods that require variable culture conditions (temperature, nutrients, and oxygen concentration) for different organisms. Because molecular methods are rapid and more streamlined, and generally require less physical

space for testing, laboratories using molecular approaches can support much higher testing volumes than those using traditional culture-based methods. For example, consider the time, personnel, and laboratory space requirements for analysis of 90 clinical samples for an infectious pathogen. For culture methodologies, this requires inoculation of media; incubation under specific conditions for at least 12–18 h, sometimes up to weeks; isolation of the suspicious organism and further incubation time; Gram and/or other staining with microscopic review; and biochemical testing for identification. For 90 clinical specimens, this could easily take at least several days to a week depending on the organism(s) of interest. In contrast, DNA extraction, amplification by PCR or other methods, and analysis of 90 clinical specimens can easily be performed by one technologist in less than one 8-h shift, resulting in a savings of both hands-on and total time for testing. The reduced labor requirement translates into more cost-effective, time-efficient testing.

Although very attractive, molecular methods do have some disadvantages. One is the risk for contamination within the laboratory, potentially resulting in false-positive results. In general, most clinical labs use appropriate controls and sufficient environmental barriers to moderate this risk. A second disadvantage of molecular testing is that they only detect the specific organism(s) for which the assay is designed. Thus, molecular testing generally requires a clinical suspicion for a specific organism, rather than the more shotgun approach of culture. Culture, in contrast, has the potential to detect virtually any pathogen, assuming the culture conditions are permissible for growth of the organism.

Furthermore, the ability to identify nonviable organisms, which is a significant advantage for molecular methods, is also one of its primary disadvantages. Nonviable organisms in a specimen will yield a positive result. False-positive results may be generated from a cutaneous specimen with contaminating, nonviable organisms. To overcome this, assays that target mRNA transcripts from the pathogen can be used, with the assumption that the production of mRNA indicates that the organism is transcriptionally active and therefore viable. Even if viable, however, the presence of an organism

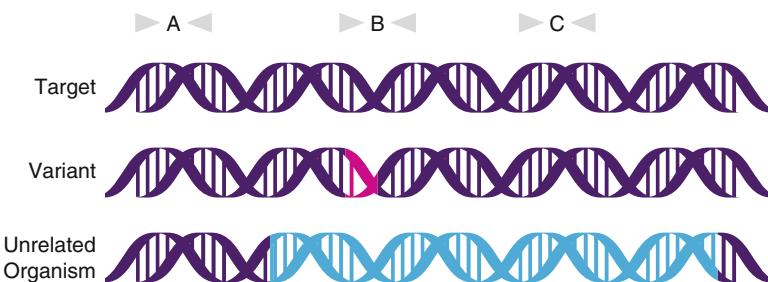
does not necessarily equate to infection. The skin is colonized by many organisms, some of which can cause opportunistic infections in the right setting. For this reason, some molecular assays must be quantitative or semiquantitative in order to accurately interpret their results.

This chapter focuses on molecular testing for cutaneous pathogens. Although molecular infectious disease testing is very common in many areas of the medical community, currently it is not commonly used by dermatologists and is rarely used by dermatopathologists. Because of the significant advantages of molecular methods over traditional pathogen detection strategies, molecular methods will become the preferred testing method. Molecular testing will be increasingly available on clinical test menus, and utilization of these assays by clinicians, including dermatologists, will continue to grow.

## 11.2 Assay Design and Testing Strategies

As discussed in Chap. 3, the performance characteristics of molecular assays are largely determined by the sequence of the primers/probes and the reaction conditions, primarily temperature and salt concentration. Compared to traditional culture methods, there are fewer variables that have the potential to affect the sensitivity and specificity of molecular assays. Because culture relies on viability and/or growth of the organism, its performance characteristics can be affected by variables such as specimen transport conditions (time, oxygen concentration, and temperature) and the presence of other organisms in the specimen that may out-compete the organism of interest for available nutrients.

Selection of the primer/probe sequence for any molecular assay is critical to achieve the desired sensitivity and specificity of the assay and can be extremely challenging. Some portions of bacterial genomes are highly conserved, limiting the amount of the genome that can be targeted for a highly specific assay (Fig. 11.1). Poorly designed assays can have cross-reactivity with other organisms, which can lead to false-positive results. Conversely, failure to account for genetic



**Fig. 11.1** Schematic of PCR primer design to detect infectious organisms. Represented are the genomes of an organism of interest (target), a variant of the organism of interest, and an unrelated organism. The purple represents sequence shared by all three organisms. The blue sequence is specific to the unrelated organism and is not present in the target or variant. The pink sequence in the variant is

the genetic alteration, which is not present in the target organism of interest. Primer set A would detect all three organisms but would not differentiate between them. Primer set B would be specific to the target organism, but would not detect the variant. Primer set C would detect both the target and variant without detecting the unrelated organism

**Table 11.2** Infectious disease testing strategies

Strategy	Method(s) <sup>a</sup>	Example
Sensitive and specific detection of one specific pathogen	Real-time PCR	MRSA
Simultaneous detection of multiple pathogens	Multiplex PCR with various detection strategies	Atypical mycobacteria
	PCR with sequencing	
Shotgun approach to detect many organisms that can cause similar clinical and histomorphologic presentations	PCR-RFLP	Fungi, parasites, mycobacteria
	PCR with sequencing	

<sup>a</sup>Note that many methods can be used. The methods listed are the most commonly used techniques in a clinical laboratory setting

polymorphisms within a species during assay design could lead to false-negative results. Novel mutations in the organism's genome can also result in false negatives, but this should be relatively uncommon with the possible exception of rapidly mutating viruses.

Molecular assays can be designed to be highly specific to one species or capable of detecting several different pathogens (Table 11.2). Two general testing strategies can be used for the simultaneous detection of multiple pathogens. One approach is to use multiple primers or probes that are specific to several different species in one

multiplex reaction. Individual organisms can be readily detected and differentiated by a variety of methods including electrophoresis or hybridization to a chip or bead array. An even broader testing strategy is the use of consensus primers that are capable of amplifying nucleic acids from a larger number of different species, followed by sequencing to identify the specific organism.

A multiplex or more comprehensive testing strategy is particularly useful when the clinical findings could be caused by several different organisms. For example, there are now several FDA-approved multiplex assays that simultaneously detect and differentiate multiple respiratory viruses, including Influenza A, respiratory syncytial virus (RSV), Rhinovirus, and Adenovirus. This allows rapid identification of the causative agent as opposed to a "reflex if negative" approach where additional testing is performed if the first is negative, which can be highly time consuming. This strategy is also particularly appealing when there is a limited amount of the clinical specimen. A multiplex reaction allows the lab to do more analyses with less specimen. A multiplex strategy is also cost-effective since it uses a single amplification reaction to identify multiple potential pathogens, rather than multiple separate analyses. With continued advancements and the development of new molecular technologies, the number of organisms that can be identified simultaneously in a single reaction will continue to increase, further enhancing the speed and cost-effectiveness of molecular testing.

Clinical molecular assays have been designed and optimized either for the identification of organisms from a primary specimen (blood, tissue, etc.) or for identification of the organism after it has been isolated in culture. Clearly these are two very different testing strategies and the performance characteristics required for each approach are very different. Identification of organisms isolated by culture generally does not require an amplification strategy or a particularly sensitive limit of detection since large quantities of the organism are already present in culture. These assays also carry a low risk for cross-reactivity with other organisms since the pathogen has already been isolated from other organisms that may have been present in the original specimen. This testing strategy can improve the speed and accuracy of organism identification once it has been isolated in culture.

Many infectious disease assays for the detection of organisms in a primary specimen are amplification based, often with a real-time detection strategy. While the quantitative nature of these assays can be valuable, perhaps more importantly, this strategy reduces the risk for false positives due to contamination since the reaction vessels are never opened following the amplification reaction. Molecular infectious disease assays for dermatologic applications use a variety of techniques including *in situ* hybridization, PCR, real-time PCR, and sequencing. Assays for bacterial pathogens often target the area of the organism's genome that codes for ribosomal RNAs (rDNA, both 16S and 23S). This area of the genome contains both conserved/consensus sequences and sequences that are highly organism specific, allowing for identification of specific organisms using a variety of assay designs.

### 11.3 Clinical Molecular Infectious Disease Testing

Infectious disease testing is by far the largest and most profitable application of molecular diagnostics in clinical medicine. Because infectious

disease testing tends to be relatively high volume and profitable, much commercial attention has been paid to this area of molecular testing. FDA-approved molecular assays for common, high-volume infectious disease targets such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, human immunodeficiency virus (HIV), hepatitis C virus (HCV), human papillomavirus (HPV), as well as others are widely available from numerous laboratories and are considered standard of care. The availability of FDA-approved infectious disease kits allows for rapid implementation of testing into the laboratory, without the need for primer/probe design, optimization of reaction conditions, or full-scale validation of the analytical and clinical performance characteristics of the assay.

FDA-approved infectious disease assays use a variety of amplification techniques including polymerase chain reaction (PCR), transcription-mediated amplification (TMA), and branched DNA (bDNA). Many of the assays use a real-time detection method that allows rapid analysis in a closed tube format. Several platforms offer completely automated testing without requiring separate extraction and amplification reactions, greatly reducing the labor involved in testing. Some of these platforms allow high-volume, "walk away" testing, while others offer individual test reactions that have the potential to be used for point-of-care testing. The FDA provides a list of assays that have received approval or clearance, which is available at [www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm#microbial](http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm#microbial).

There are relatively few dermatologic applications for the molecular detection of common infectious organisms, and FDA-approved tests are rare. More frequently, dermatology-related molecular infectious disease testing is needed for uncommon organisms and/or when clinical and/or histopathologic findings are nonspecific. This testing is, not surprisingly, much more challenging. Uncommon cutaneous infections can be difficult to diagnose both clinically and histomorphologically, often due to nonspecific clinical presentations and histopathology, as well

as inexperience on the part of the clinician and/or pathologist. Sensitive and specific molecular tests could be invaluable in this setting. Most clinical molecular assays for uncommon infectious organisms are lab-developed tests that are available through a Clinical Lab Improvement Act (CLIA)-approved clinical laboratory. The Association for Molecular Pathology (AMP, [www.amp.org/](http://www.amp.org/)) maintains a searchable list of molecular infectious disease testing that is available from CLIA-certified labs ([www.amptest-directory.org/index.cfm](http://www.amptest-directory.org/index.cfm)). Because participation in this listing is voluntary, this list may not be comprehensive. Clinical testing for uncommon organisms, particularly from difficult specimens such as FFPE tissue samples, may only be available from academic institutions.

The Centers for Disease Control and Prevention (CDC) can also be a valuable resource for reference diagnostic testing for esoteric pathogens. Clinically suspicious specimens can be submitted by state public health laboratories and other federal agencies for analysis. Specimens from private healthcare providers and institutions must be submitted to a local state health department laboratory for appropriate processing. A searchable directory of testing services is available at [www.cdc.gov/laboratory/specimen-submission/list.html](http://www.cdc.gov/laboratory/specimen-submission/list.html).

#### **<sup>1</sup>CPT coding**

Molecular microbiology codes depend on method and organisms. 87149 (direct probe), 87150 (amplification), and 87153 (sequencing of 16S rRNA) are used when testing from culture material. 87470–87801 are used for primary source material, including blood and biopsy specimens. 87800 (direct probe) and 87801 (amplification) are for simultaneously testing multiple organisms with a single result; 87999 is the unlisted microbiology procedure code.

<sup>1</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

## **11.4 Viruses**

Viruses are obligate intracellular parasites. Viral infections can be diagnosed by a variety of methods including (1) clinical presentation (5th disease), (2) serologic testing (measles, mumps), (3) cultures (RSV), (4) characteristic histologic patterns/lesions (HPV condyloma), direct observation of viral cytopathic effect (HSV, Molluscum), and immunohistochemistry (EBV, HHV-8). Although these methods are often sufficient to make an accurate diagnosis, they do have limitations. Viral culture can be very challenging, requiring infection of live mammalian cultured cells with virus from a patient specimen. The virus must replicate within the cultured cells, which can take days. Detection of the virus in the cell culture is accomplished by identification of viral-specific cytopathic effects (CPEs) or by the use of labeled antibodies specific to viral antigens (often a direct fluorescent antibody [DFA]/direct immunofluorescence assay). These methods are laborious and time consuming and carry a significant risk for false-negative results. Serologic assays are useful for demonstrating infection of the host at some time; however, their use as diagnostic tools for current infections can be limited. Diagnostic testing for current infection by serologic methods requires either an accurate detection of pathogen-specific IgM antibodies or a significant increase in IgG antibody titer to an organism. Both of these approaches frequently suffer from limited sensitivities due to biologic and technical limitations.

Despite the fact that viral genomes are highly variable and may consist of single- or double-stranded DNA or RNA, the presence of nucleic acids in their genomes allows for the development of highly sensitive and specific molecular assays that are capable of identifying virtually all medically relevant viruses. Molecular methods are rapidly replacing traditional viral testing methods, particularly viral culture, and also can play important adjunctive and confirmatory testing roles. Compared to viral culture, molecular methods allow a more rapid turnaround time, have fewer false-negative results, and can be performed from a wide variety of specimen types.

Although molecular methods can very accurately determine the presence of viruses in clinical specimens, their diagnostic use is not without challenges. For example, members of the herpesvirus family, including EBV, CMV, and VZV, are viruses that remain latent in the host for long periods of time and can reactivate producing clinical symptoms. Detection of these viruses in clinical specimens can therefore represent primary infection, viral reactivation, or asymptomatic shedding of the virus. As with all laboratory testing, the results must be interpreted in the context of other clinical and laboratory data.

## 11.5 Viral Infections Associated with Neoplasia

A number of viral human pathogens are associated with an increased risk for neoplasia. These include human papillomavirus (HPV), human herpesvirus 8 (HHV-8), Merkel cell polyomavirus (MCV), human T-lymphotropic virus type 1 (HTLV-1), and Epstein-Barr virus (EBV). HPV, HHV-8, and MCV are discussed here in the setting of cutaneous neoplasms.

### 11.5.1 Human Papillomavirus (HPV)

HPV is a double-stranded DNA virus with over 100 types that infect humans. Transmission can occur from skin to skin contact as well as contact with fomites. Most infections resolve spontaneously without symptoms, but persistent infection can lead to clinical lesions. Sexually transmitted HPV types are divided into high- and low-risk types based on their associated risk with the development of cervical disease. Persistent infection with only high-risk HPVs is associated with cervical dysplasia and cancer. Clinical testing for high-risk HPV viral DNA is now incorporated into the cervical cytology screening guidelines along with the traditional Pap cytology screen.

In the skin and mucosa, persistent HPV infection is associated with a number of clinical presentations (Table 11.3). Low-risk types 1, 2, 3, 4,

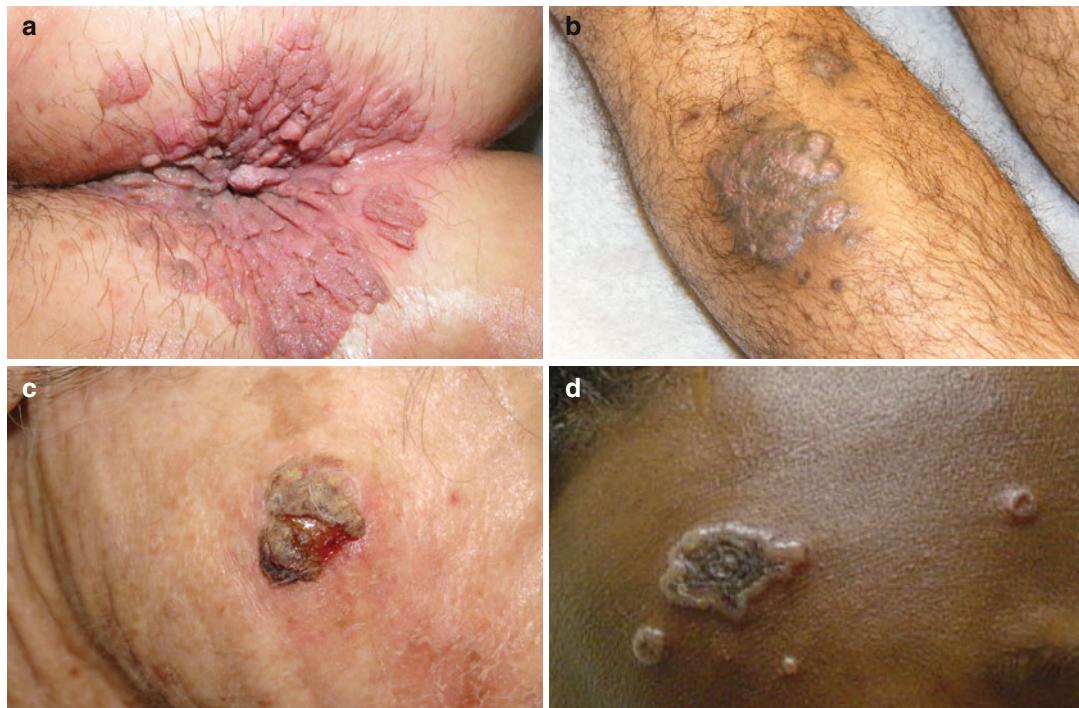
**Table 11.3** Clinical manifestations associated with HPV infection

Clinical manifestation	Most common HPV types	Other HPV types
Verruca vulgaris	1, 2, 4	7, 75, 76, 77
Butcher's warts	7	
Verruca plana	3, 10	
Verruca palmoplantaris	1, 4	45, 57, 60, 63, 65, 66
Condyloma acuminatum	6, 11	2, 16, 18, 30–33, 35, 39, 41–45, 51–56, 59
Giant condyloma of Buschke and Lowenstein	6, 11	16
Focal epithelial hyperplasia (Heck's disease)	13, 32	
Epidermodysplasia verruciformis	5, 8 <sup>a</sup>	3, 9, 10, 12, 14, 15, 17, 19–25, 36–38, more
Bowen's disease, Bowenoid papulosis, VIN 3	16, 18, 31, 33, 51	
SCC	5, 8, 16, 18, 31, 33, 51	

Abbreviation: VIN3 vulvar intraepithelial neoplasia grade 3 of 3

<sup>a</sup>Although infection with many low-risk types is seen, infection with types 5 and 8 is most commonly associated with malignant transformation

7, and 10 are associated with warts including common (verruca vulgaris), Butcher's, and palmoplantar types. Sexually transmitted low-risk types 6 and 11 are associated with condyloma acuminatum (Fig. 11.2a) and giant condyloma acuminatum of Buschke-Lowenstein. Low-risk types have also been associated with cutaneous squamous cell carcinoma (SCC) in immunocompromised individuals. This includes HIV-infected and organ transplant recipients as well as individuals with epidermodysplasia verruciformis (EDV, also called Lewandowsky-Lutz dysplasia). EDV is an extremely rare genodermatosis with an autosomal recessive inheritance pattern. The syndrome is caused by mutations in *EVER1* or *EVER2*, which results in an immunodeficiency that is characterized by a greatly



**Fig. 11.2** Clinical presentations of lesions related to select viruses. Condyloma acuminatum caused by HPV, frequently types 6 and 11, presenting as coalescing flat-topped and verrucous papules and plaques (**a**, perianal). Kaposi sarcoma associated with HHV-8 infection, presenting as multiple red-brown plaques and tumors on this HIV-positive male (**b**, lower extremity). Merkel cell carcinoma (*MCC*), associated with Merkel cell polyomavirus

(*MCV* or *MCPyV*) infection, presenting as a solitary violaceous ulcerated and crusted tumor on an elderly female (**c**, cheek/face). Disseminated herpes zoster caused by varicella zoster virus (VZV), presenting as multiple scattered vesicular papules and plaques, some with scalloped borders (**d**, forehead) (Select photos courtesy of Dr. Stephen Weis, Ft. Worth, TX)

increased susceptibility for HPV infections, particularly for low-risk types. Although patients are susceptible to many low-risk types, infections with types 5 and 8 carry a risk for SCC. High-risk HPV types are associated malignant transformation to SCC and various clinical presentations of *in situ* disease, including Bowen's disease, Bowenoid papulosis, vulvar intraepithelial neoplasia (VIN), and anal intraepithelial neoplasia (AIN).

HPV cannot be grown in culture and serologic methods are not useful for distinguishing between a past and present infection. Thus molecular assays to identify HPV nucleic acids are essentially the only reliable method for detection of the virus. A wide variety of molecular assays that target HPV have been developed. Molecular

HPV tests for cervical screening are widely available. There are currently four FDA-approved assays for detecting high-risk HPV in liquid-based cytology specimens, each of which uses a different molecular testing strategy: Hybrid Capture, Invader, transcription-mediated amplification, and PCR. There are also a number of assays available for research purposes or for clinical testing outside the United States.

In the skin, molecular HPV testing is generally limited to *in situ* hybridization (ISH), primarily with chromogenic detection (CISH). Immunohistochemical cocktails for HPV subtypes also exist but have not proven effective. ISH allows identification of HPV in relation to the pathologic lesion and may provide clues about the physical state of the virus. The presence of

nuclear punctate signals suggests that the virus is integrated into the human genome, while diffuse signals are more consistent with episomal virus. Although there are several commercially available HPV ISH assays, none are FDA approved, and there is no evidence that any have been sufficiently clinically validated. Commercially available assays include GenPoint HPV Probes (Dako, Glostrup, Denmark), Zytostain HPV Probes (Zytovision, Bremerhaven, Germany), PathoGene HPV Probes (Enzo Life Sciences, Plymouth Meeting, PA), and INFORM HPV (Ventana, Tucson, AZ). Some of these reagents may not be available in the United States. Each vendor offers one or more cocktails of probes specific to low-risk, high-risk, or a combination of low- and high-risk viral types. The available cocktails include the most common high- and low-risk types, but do not include less common types, resulting in a risk for false-negative reactions. ISH cannot identify the specific HPV type because probes to individual types, rather than cocktails, are generally not available.

The sensitivity and specificity of ISH-based assays can be problematic for diagnostic testing. ISH generally uses nucleic acid probes that are much larger than the primers and probes used in amplification-based assays, making it difficult to design HPV type-specific ISH probes that do not have sequence homology to closely related members of the virus family. Specificity can be compromised if the assay's hybridization conditions are not optimized and strictly maintained during testing. The limited sensitivity of HPV ISH-based assays may be due to the presence of low numbers of viral copies in the tissue of interest. In order to improve assay sensitivity, several of the available probe cocktails are now offered using a signal amplification ISH approach. HPV 6/11 ISH can be very useful for differentiating condylomas from seborrheic keratoses and skin tags, but suffers from fairly low sensitivity, especially in older, senescent lesions. High-risk HPV ISH may be useful with genital/perineal biopsies when the histologic differential diagnosis includes both reactive processes and high-grade HPV-driven lesions (Bowen's disease, vulvar intraepithelial neoplasia, etc.).

## <sup>2</sup>CPT coding

88365 (ISH, per probe), 87620 (direct probe), 87621 (amplification); 87999 (unlisted)

### 11.5.2 Human Herpesvirus 8 (HHV-8)

HHV-8, also called Kaposi sarcoma herpesvirus (KSHV), is a double-stranded DNA virus that infects endothelial cells and circulating mononuclear cells. Infection is associated with Kaposi sarcoma (KS), primary effusion lymphoma (PEL), and some types of multicentric Castleman's disease, primarily in immunocompromised, often HIV-infected, individuals. KS is a low-grade vascular proliferation that may involve the skin, mucosa, and viscera (see Fig. 11.2b). The classification of KS as a malignancy versus a reactive process remains controversial. It often presents at mucocutaneous sites and can evolve from early macules (patch stage) to plaque stage, which may subsequently develop into nodular and tumor stages. Individuals can have lesions of different stages at the same time.

The cellular origin of KS has been controversial because of varied protein marker expression on the spindle-shaped tumor cells. Ultrastructure and IHC studies demonstrated that KS cells have endothelial features of both lymphatic and vascular origin [2, 3]. In 2004, Hong and colleagues reported that HHV-8 infection of vascular endothelial cells in vitro induced expression of lymphatic endothelial markers [4]. It is now generally believed that HHV-8 infects circulating endothelial precursor cells and drives them toward a lymphatic lineage [3, 5].

Histologically, early patch-stage KS is characterized by abnormal vessels lined by thin endothelium dissecting the dermis. Extravasated red blood cells, inflammatory cells, including plasma cells, and hemosiderin deposits are frequently present. These early histologic changes can be subtle and may be missed on biopsy [5]. Plaque-stage KS demonstrates a proliferation of both spindle cells and vessels involving most of the dermis and

<sup>2</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

sometimes the subcutis. In the tumor stage, the vascular nature of the lesion is less obvious as the spindle cells form a mass, mimicking other soft tissue tumors. The differential diagnosis for KS can include hemangiomas, fibrohistiocytic tumors, as well as spindle cell melanoma, cutaneous leiomyosarcoma, and angiosarcoma. Immunohistochemical staining is positive for endothelial markers CD31 and CD34. IHC for the HHV-8 latency-associated nuclear antigen 1 protein (LANA-1 or LNA-1) has a characteristic salt-and-pepper nuclear pattern and is the most diagnostically helpful stain, with a sensitivity and specificity reported to be 99 and 100 %, respectively [6].

PCR for the detection of HHV-8 in formalin-fixed tissue specimens is available from a number of commercial and academic laboratories (see the Association for Molecular Pathology Test Directory). However, this test may yield a false-positive result due to contaminating mononuclear inflammatory cells that may harbor HHV-8, especially in HIV-positive patients [5]. In addition, HHV-8 may not be exclusively limited to KS and may be detected in some angiosarcomas, hemangiomas, and dermatofibromas [7]. Thus, while molecular testing may be useful for a select subset of cases, it does not have general clinical utility and offers few advantages over IHC.

#### <sup>3</sup>CPT coding

88342 (IHC); 87797 (direct probe), 87798 (amplification); 87999 (unlisted)

### 11.5.3 Merkel Cell Polyomavirus (MCV or MCPyV)

MCV is a double-stranded DNA virus. Infection is common, typically occurring in childhood or young adulthood. MCV is suspected to cause the majority of cases of Merkel cell carcinoma (MCC), a rare but aggressive tumor, most frequently seen in immunocompromised individ-

uals. The incidence of MCC in the United States is approximately 1,500 cases annually.

MCC is a neuroendocrine carcinoma of Merkel cells in the skin. It typically presents as an asymptomatic, solitary, erythematous, and rapidly growing nodule, often located on sun-exposed areas (see Fig. 11.2c). Histologically, the differential diagnosis can include small cell lung carcinoma (SCLC), lymphoma, and small cell melanoma. For clinical diagnosis, IHC can be helpful. MCC will stain for CK20 and other keratins (e.g., CAM 5.2), in a characteristic perinuclear dot-like pattern. It is also positive for neuron-specific enolase (NSE), but not for CK7 or thyroid transcription factor 1 (TTF-1) (positive in SCLC), leukocyte common antigen (LCA) (positive in lymphoma), and S100 (positive in small cell melanoma).

Molecular studies have been essential for identifying and understanding the pathogenesis of this virus. In fact, MCV is the first example of the use of next-generation sequencing to identify a human pathogen, using a technique called digital transcriptomic subtraction [8]. Molecular studies have demonstrated clonal integration of virus into the host genome and the oncogenic nature of viral genes, particularly LTA and small T antigen. The value of molecular testing for diagnosis or clinical management has yet to be established. Although some studies have demonstrated a high specificity of MCV PCR for MCC, others have demonstrated positivity in a variety of other tumor types [9–11]. The sensitivity of PCR for MCV detection is also questionable, with various studies demonstrating virus in 50–100 % of MCC cases [8, 12–14]. This variation may be due to differences in primer design and other technological issues. For now, molecular testing for MCV is limited to the research setting and is not clinically indicated.

#### <sup>4</sup>CPT coding

87797 (direct probe), 87798 (amplification); 87999 (unlisted)

<sup>3</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

<sup>4</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

## 11.6 Herpesvirus

The clinical distinction between herpetic dermatitis due to herpes simplex virus (HSV) and varicella zoster virus (VZV) is generally straightforward. However, there are cases where clinical differentiation can be difficult (see Fig. 11.2d) [15–17]. Clinical differentiation of VZV and HSV may be particularly difficult in early and/or recurrent disease, as well as in children and immunocompromised individuals who are more likely to have atypical presentations, including systemic forms.

The histopathologic features of VZV and HSV, if present, are identical, precluding accurate differentiation between the entities based on histomorphologic grounds. IHC is available to differentiate HSV from VZV in clinically ambiguous cases, although most IHC antibodies do not distinguish HSV-1 from HSV-2. Traditionally, these viruses have been detected by Tzanck smears or direct fluorescent antibody (DFA) staining of lesional scrapings or by viral culture. Tzanck smears do not distinguish between VZV and HSV infections, and DFA is less sensitive than nucleic acid amplification methods.

There are several reports describing the use of PCR to detect HSV-1, HSV-2, and VZV in cutaneous clinical specimens. These studies demonstrate that PCR has a superior sensitivity and specificity compared to traditional viral culture methods [18–20]. In addition to superior sensitivity and specificity, the benefits of PCR include a rapid turnaround time and the ability to perform testing on formalin-fixed paraffin-embedded (FFPE) tissues. Histologic diagnosis is dependent on the presence of characteristic viral cytopathic effect, which may be absent due to sampling (changes may be focal) or due to evolving lesions. Molecular testing may also be valuable for identification of patients with coinfections of VZV and HSV [21]. A recent publication comparing the clinical impression, histologic characterization, and PCR analysis of HSV-1, HSV-2, and VZV of 40 clinical cases highlights the utility of molecular testing for detecting and distinguishing these organisms [22]. In this study of highly selected cases, there was a discrepancy

between the clinical impression and histopathologic diagnosis in 19 of the 40 cases (48%). Twelve of the discrepant cases were not clinically suspicious for herpetic dermatitis. Histopathology demonstrated diagnostic viral cytopathic changes and PCR confirmed herpetic dermatitis (7 HSV-1, 1 HSV-2, 2 VZV, 2 mixed). The ability to perform PCR from the FFPE specimen enabled a definitive diagnosis without the collection of additional tissue samples from patients. Seven discrepant cases were clinically suspicious for herpetic dermatitis, but diagnostic viral cytopathic changes were absent. In three of these seven cases, PCR confirmed the clinical suspicion of herpetic dermatitis (1 HSV-1, 1 HSV-2, 1 VZV), and in four of the seven, PCR did not detect HSV or VZV, consistent with the histopathologic findings.

There are several clinical molecular diagnostic laboratories in the United States that offer PCR detection of HSV and VZV from FFPE samples. These tests are all considered lab-developed tests (LDTs), even though some may use kits that are FDA approved for other specimen types. Genetic regions targeted by molecular assays include the HSV envelope glycoprotein B gene (gB) and DNA polymerase 1 gene and the VZV ORF31 region [22–24].

### <sup>5</sup>CPT coding

87528 (HSV direct probe), 87529 (HSV amplification); 87797 (other herpesvirus direct probe), 87798 (other herpesvirus amplification); 87999 (unlisted)

## 11.7 Fungi

Standard methods for the detection of fungi include direct staining with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Gömöri methenamine silver (GMS) and culture

<sup>5</sup>. The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

methods. Serologic testing for some organisms is also available, but is not always useful due to limitations in sensitivity and specificity. Fungi grown in culture are typically identified by their characteristic macroscopic and microscopic morphologies. Similar to other organisms, sensitivity and specificity of culture testing are affected by the viability of the organism in transport to the lab and the experience and expertise of laboratory personnel. Culture can be time consuming and difficult as many fungi are rather slow growing and often require special culture conditions. For some fungal organisms, culture may take several weeks and can have a significant false-negative rate. Microscopy also lacks sensitivity and is unable to differentiate some genus and species.

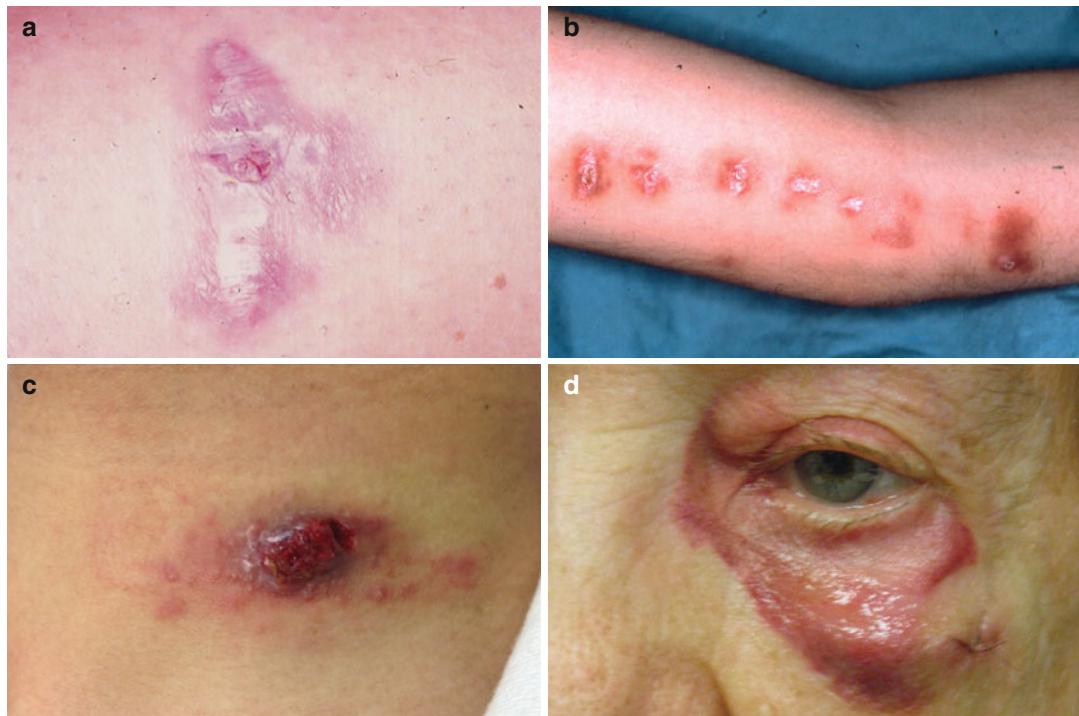
Molecular tests can be designed to be genus and/or species specific by using targeted primers and probes. However, cutaneous fungal and some bacterial (see Mycobacteria below) infections often present with clinical and/or histopathologic findings that are nonspecific. For this reason, a more comprehensive approach rather than an organism-specific approach may be required. Several methods have been described. PCR-RFLP (restriction fragment length polymorphism) uses consensus primers to amplify nucleic acids from multiple organisms, followed by enzymatic digestion of the PCR product using specific restriction endonucleases. The resulting fragments are then separated and sized by electrophoresis, generating species-specific patterns. PCR-RFLP is a labor-intensive process, and more recently, sequencing approaches have been developed to identify pathogens after amplification with consensus primers. Advances in sequencing methodologies have made it an automated and relatively inexpensive method that generates more detailed identification for species identification. A comprehensive approach requires a more sophisticated level of interpretation than an assay designed to give a yes/no answer for a single organism. Restriction patterns or sequences must be aligned to all of the known species and carefully interpreted. Confirmatory testing may be required. This type of testing is offered from FFPE samples by some academic institutions including the Microbiology Section

of the Molecular Diagnostics Laboratory at the University of Washington ([www.depts.washington.edu/molmicdx/mdx/available\\_tests.shtml](http://www.depts.washington.edu/molmicdx/mdx/available_tests.shtml)).

Dermatophytes, including *Trichophyton*, *Microsporum*, and *Epidermophyton*, are the main causes of the superficial mycoses and cause a variety of skin, hair, and nail infections. Diagnosis is typically made clinically with or without a potassium hydroxide (KOH) wet-mount prep and/or PAS stain. Cultures can be performed to identify the species. A large number of studies describing the use of molecular methods to detect and identify dermatophytes have been published over the last 20 years. Most of the molecular studies on dermatophytes have focused on the genes coding for the ribosomal RNA, including the transcribed and non-transcribed regions. These regions contain sufficient sequence variation to allow fungi differentiation. Despite the large number of published studies, only a few clinical laboratories, primarily in academic institutions, offer molecular assays for the detection of dermatophytes directly from clinical specimens, particularly from FFPE tissue specimens.

Sporotrichosis (also referred to as rose gardener's disease) is one of the deep mycoses, caused by the fungus *Sporothrix schenckii*. Lesions typically occur after introduction of the fungus into a minor skin abrasion/trauma. The organism may remain localized (Fig. 11.3a) or spread along lymphatic vessels in a sporotrichoid pattern (see Fig. 11.3b). Histopathologic diagnosis may be difficult due to the paucity of yeast in lesions. Several publications have described molecular methods for the detection of *S. schenckii*. These assays have targeted several different fungal genes including DNA topoisomerase II, chitin synthase, and the internal transcriber space in the rRNA gene [25–27]. Currently, a true clinical validation of a molecular assay has not been published, and the availability of molecular testing for this organism is questionable.

There are many other deep and systemic mycoses, including infections caused by *Aspergillus* species, *Blastomyces dermatitidis*, and *Coccidioides immitis*, to name a few (see Fig. 11.3c). Molecular assays for this group of infectious agents are not often necessary but have



**Fig. 11.3** Clinical presentations of select fungal and parasitic infections. *Sporothrix schenckii* infection (sporotrichosis) presenting as a single verrucous hyperkeratotic plaque (**a**, upper extremity). Erythematous nodules in a classic sporotrichoid pattern resulting from the spread of *Sporothrix schenckii* organisms along lymphatic vessels

(**b**, upper extremity). A deep fungal infection caused by *Coccidioides immitis* (coccidioidomycosis) presenting as an ulcerated nodule (**c**, trunk). Cutaneous leishmaniasis presenting as a large indurated plaque on an elderly man (**d**, periorbital) (Select photos courtesy of Dr. Alan Menter, Dallas, TX)

been described and are generally clinically available by PCR or other amplification strategies (see the Association for Molecular Pathology Test Directory). The available assays include both direct detection from clinical specimens and DNA probes for identification of cultured organisms. Not all labs accept FFPE tissue samples for testing.

#### <sup>6</sup>CPT coding

87797 (direct probe), 87798 (amplification);  
87999 (unlisted)

<sup>6</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

## 11.8 Parasites

### 11.8.1 Leishmania

Leishmania is a protozoal parasite that is transmitted by the bite of sand flies. Infection causes a spectrum of disease, including cutaneous (*L. tropica* and *L. mexicana*), mucocutaneous (*L. braziliensis*), and visceral (*L. donovani*) leishmaniasis. Historically, leishmaniasis has been relatively rare in the United States, but there has been a recent surge in cases, mostly among veterans returning from service in the Middle East and among civilians living in Texas. Typical acute lesions progress from a papule to a painless ulcerated and crusted nodule, which ultimately scars (see Fig. 11.3d). The more chronic phase consists of nodules and plaques. Clinical presentations are variable and can mimic other inflammatory and

neoplastic diseases [28]. Diagnosis of cutaneous leishmaniasis usually relies on identification of the Leishmania amastigotes either by direct visualization or by culture. On histopathology, the organisms are small, often within macrophages or aligning along the periphery of large vacuolar spaces, in the so-called “marquee” sign. The presence of a kinetoplast and lack of a capsule help differentiate Leishmania from other morphologically similar organisms (e.g., *Histoplasma capsulatum*). Giemsa staining is sometimes used to aid in microscopic detection. Direct visualization of the organism is more challenging in the chronic stages as tuberculoid granulomas form and the number of organisms in the tissue significantly decreases. The overall sensitivity of microscopic visualization of the parasite ranges from 16 to 74 % [29, 30].

Molecular techniques, primarily PCR-based, have been shown to have utility for diagnosis confirmation [28, 31–33]. PCR-based assays are more sensitive than histologic examination and allow for species identification, which is recommended for patient management. The Centers for Disease Control and Prevention (CDC) can perform both culture and a PCR with sequencing test for diagnostic confirmation.

#### <sup>7</sup>CPT coding

87797 (direct probe), 87798 (amplification);  
87999 (unlisted)

## 11.9 Bacteria

### 11.9.1 Mycobacteria

Mycobacteria are aerobic bacteria that have a characteristic waxy cell wall which contributes to their resilient nature. Cutaneous mycobacterial infections can present with a wide range of clinical manifestations and therefore may not be

suspected at initial presentation. The incidence of tuberculosis as well as nontuberculosis (or atypical mycobacterial) cutaneous infections has increased over the last two decades, primarily in immunocompromised individuals. Clinically and histologically, the granulomatous infiltrates caused by different mycobacterial species can be difficult to distinguish from each other, as well as from other infectious and noninfectious cutaneous diseases. The differential diagnosis may include sporotrichosis and other deep fungal infections, cutaneous leishmaniasis, granuloma annulare, and sarcoidosis, among others. Conventional techniques for the diagnosis of mycobacterial infections include demonstration of acid-fast bacilli (AFB) on smears or histologic material and isolation of the organism in culture, both of which lack robust sensitivity. Even when present on histology, AFB organisms can be scant and difficult to differentiate from water bath or stainer contaminants. Isolation of mycobacteria in culture, especially in cases with a low organism burden, can be difficult to impossible and takes weeks to generate a result. Clearly this is a field where molecular testing methods have the potential to significantly improve diagnostic accuracy and speed. Moreover, molecular testing can be performed using the FFPE skin biopsy, which is important since these infections are not always suspected, and therefore, cultures are not always performed at the time of biopsy. Many molecular assays have been described to detect and differentiate between several members of this genus. Most of the reported molecular assays for the detection of cutaneous infections have used amplification methods such as PCR, which are likely to be superior to direct detection methods such as in situ hybridization, particularly when low numbers of organisms are present. Common mycobacterial genetic targets for PCR amplification include 16S rDNA and the 16S rRNA internal transcribed spacer, although other targets have been described. Many early publications described the use of nested PCR to detect the often rare acid-fast organism, which can be problematic in a clinical lab setting because of the increased risk it presents for carryover contamination. More recent publications have utilized

<sup>7</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

real-time PCR, which is faster than other PCR approaches and significantly reduces the risk of contamination in the laboratory. Studies have also highlighted the importance of an internal control in order to avoid false-negative results, which are particularly common when analyzing nucleic acids isolated from formalin-fixed paraffin-embedded tissue specimens.

Several FDA-approved molecular assays are available for the detection of specific mycobacterial species either from clinical specimens or from culture isolates. Assays approved for testing from clinical specimens are primarily cleared for use with respiratory specimens. Clinical molecular assays for mycobacteria detection from cutaneous specimens are all considered lab developed. Because the clinical and histomorphologic presentation of skin infections with different species of mycobacteria can be identical, perhaps a more comprehensive approach, rather than testing for individual species, may be a more successful diagnostic approach. This approach typically involves PCR amplification using consensus primers, which amplify sequences from multiple mycobacterial species, followed by species identification by sequencing. Analysis of several different genetic targets, including the 16S ribosomal DNA, the 16S–23S rRNA internal transcribed spacer, and heat shock protein 65 (*hsp65*), may be needed for definitive species identification. Because this type of global approach requires a more sophisticated level of interpretation than an assay that is specific for a single organism, they are generally only offered by a few academic clinical labs.

### 11.9.1.1 *M. tuberculosis*

Although tuberculosis is primarily a pulmonary disease, a small number of cases are initially diagnosed by cutaneous presentations that are often difficult to distinguish from other granulomatous or suppurative diseases. Scrofuloderma is an extension of tuberculosis (or, less commonly, nontuberculosis mycobacterial infection) into the skin, most commonly from cervical lymph nodes (Fig. 11.4a). The gold standard for identification/diagnosis has been the demonstration of AFB and isolation of *M. tuberculosis* in culture. Over

the last several decades, molecular testing has been successfully introduced into clinical laboratories for identification of *M. tuberculosis* primarily for the detection of pulmonary disease. Molecular assays are fairly widely available for the detection of *M. tuberculosis* from clinical specimens, as well as from cultured isolates.

Although the sensitivity of PCR for the detection of *M. tuberculosis* varies by report, most studies conclude that the sensitivity of PCR-based methods is superior to AFB stains [34, 35]. In addition, molecular methods are more rapid than culture and allow specific identification of the species, which is not possible with AFB stains. Many published methods have been optimized for use with pulmonary related specimens, although work demonstrating the utility of molecular techniques to identify *M. tuberculosis* in FFPE tissues has been published [34].

### 11.9.1.2 *M. leprae*

*M. leprae* primarily affects the skin and peripheral nerves (see Fig. 11.4b). Leprosy (Hansen's disease) is rare in the United States. Most cases can be traced to travel to an endemic area, although some cases are acquired in the United States, likely through contact with infected armadillos in the Southern states [36]. Early diagnosis and treatment of *M. leprae* is critical because of the potential for serious neurological consequences of untreated infections. Diagnosis of *M. leprae* is challenging because of its rarity and the lack of serologic or skin tests and because *M. leprae* cannot be grown in culture [37]. The gold standard for the diagnosis of leprosy has been histology, with the demonstration of a granulomatous infiltrate, typically involving neurovascular bundles, and the identification of AFB within the infiltrate using special stains (e.g., Fite or Kinyoun's). The immunologic response by the host has a significant impact on the type of tissue reaction to the organisms, the number of organisms in the tissue, and therefore clinical course. This variability is captured in the broad clinical categories of leprosy (these have increased organism load from left to right): tuberculoid → borderline tuberculoid → borderline lepromatous → lepromatous. The categories with the lowest organism burden



**Fig. 11.4** Clinical presentations of select bacterial infections. Serofuloderma caused by *M. tuberculosis* infection of a 31-year-old female, presenting as a large erythematous and tender draining nodule (**a**, neck). *M. leprae* infection of a 47-year-old man presenting as multiple red-brown papules and plaques (**b**, lower extremities).

Cutaneous atypical mycobacterial infection caused by *M. kansasii*, presenting as multiple ulcerating nodules (**c**, lower extremity). A 51-year-old man with methicillin-resistant *Staphylococcus aureus* infection presenting as multiple crusted papules and ulcers (**d**, flank) (Select photos courtesy of Dr. Alan Menter, Dallas, TX)

(tuberculoid) are the most diagnostically challenging on biopsy. A number of publications have demonstrated the utility of standard PCR and real-time PCR as adjuncts to the clinical and histopathologic diagnosis of leprosy [35, 38–42]. These assays may be particularly helpful in cases with sparse bacilli and for monitoring response to therapy [30, 41, 42]. In general, these assays are more frequently available in countries with endemic disease. Resources for leprosy testing within the United States include the National Hansen's Disease Program, which is part of the US Department of Health and Human Services' Health Resources and Services Administration (HRSA) ([www.hrsa.gov/hansensdisease/index.html](http://www.hrsa.gov/hansensdisease/index.html)) and the Centers for Disease Control and Prevention (CDC) ([www.cdc.gov/nczved/divisions/dfbmd/diseases/hansens\\_disease/technical.html/](http://www.cdc.gov/nczved/divisions/dfbmd/diseases/hansens_disease/technical.html/)).

### 11.9.1.3 Other Atypical/ Nontuberculous Mycobacteria

Atypical mycobacteria, also referred to as nontuberculous mycobacteria (NTM), mycobacteria other than tuberculosis (MOTT), or environmental mycobacteria, include *M. marinum*, *M. ulcerans*, *M. kansasii*, and others. The number of organisms characterized and assigned to this genus has increased rapidly in recent years due to improved isolation and culture methods and now stands at over 100, several of which can cause cutaneous infections. The incidence of NTM infection has been increasing over the last two decades, in part due to the increased use of immunosuppressive agents [43]. These infections are often initiated by direct inoculation, that is, the introduction of the organism into an open wound or cutaneous abrasion. Because these are largely environmental infections, the patient's clinical history may

provide clues as to the most likely organism. For example, as the name suggests, *M. marinum* inhabits water. Historically, infection was linked to swimming pools (“swimming pool granuloma”), but now, infection is most commonly seen in fishermen, individuals with fish tanks (“fish tank granuloma”), and, more recently, clients of manicure/pedicure establishments. *M. marinum* is probably responsible for the majority of cutaneous atypical mycobacterial infections, particularly in immunocompetent hosts, although a number of other species can cause cutaneous disease [44–46]. *M. marinum* will often cause progressive cutaneous lesions, in a sporotrichoid pattern, clinically and histologically mimicking *Sporothrix* infections.

Infection with *M. kansasi* most commonly manifests as a pulmonary infection but can cause cutaneous lesions that result in a variety of clinical presentations including nodules, pustules, verrucous lesions, abscesses, and ulcers (see Fig. 11.4c) [47]. Rapid-growing nontuberculous mycobacteria, such as *M. chelonae*, *M. abscessus*, and *M. fortuitum*, can also cause cutaneous infections. These infections often present in immunocompromised individuals and are also frequently associated with surgical wounds or other nosocomial exposure [46, 48].

Cutaneous *M. ulcerans* infections occur in tropical areas, but are rare in the United States. Infection typically presents as a painless papule that evolves into an ulcer and may become necrotic [46, 48]. In Africa, the disease is called Buruli ulcer, while in Australia it is referred to as Bairnsdale ulcer. *M. scrofulaceum* infections commonly present as one-sided lymphadenitis affecting the submandibular and submaxillary regions. Infection is primarily through inhalation or ingestion of the organism and is more common in children. A number of other atypical mycobacteria can also cause opportunistic cutaneous infections including *M. gordonae* and *M. xenopi* [44, 45, 48].

The increase in the number of new species of NTM along with the general increase in NTM infections has resulted in diagnostic challenges. Identification of NTM by traditional culture and biochemical methods can be difficult and in some cases inadequate [48]. Clinical molecular assays for NTM detection from cutaneous specimens are all considered lab developed. Because the

clinical and histomorphologic presentation of skin infections with different species of NTM can be identical, a more comprehensive approach, rather than testing for individual species, is likely to be a more successful diagnostic approach. Analysis of several different genetic targets including the 16S ribosomal DNA, the 16S–23S rRNA internal transcribed spacer, and heat shock protein 65 (*hsp65*) may be needed for definitive species identification [30, 48].

#### <sup>8</sup>CPT coding

87550 (direct probe), 87551 (amplification);  
87555 (direct probe, tuberculosis specific),  
87556 (amplification, tuberculosis specific);  
87999 (unlisted)

### 11.9.2 Rickettsia

Rickettsia are Gram-negative obligate intracellular bacteria, which are categorized into 3 groups: spotted fever group, typhus group, and scrub typhus group. In the United States, Rocky Mountain spotted fever (RMSF, *R. rickettsii*) and rickettsialpox (*R. akari*) are the most commonly encountered in dermatology. These can pose a diagnostic challenge in non-endemic areas. RMSF is a tick-borne disease that occurs most commonly during summer months. The majority of the ~2,000 annual cases in the United States occur in North Carolina, Oklahoma, Arkansas, Tennessee, and Missouri. Rickettsialpox is endemic in New York City and is transmitted by mites. Diagnosis of a rickettsial infection is typically made based on clinical presentation and history. The eruption associated with RMSF may develop after systemic symptoms such as fever, headache, and muscle pain, but in some patients, it never develops. Cutaneous lesions consist of erythematous macules and/or papules, starting on the extremities and eventually moving to the trunk. With rickettsialpox, there is a characteris-

<sup>8</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

tic eschar at the site of the initial bite. Serologic test can be used for confirmation, although seroconversion may take 3–4 weeks. Histopathology is neither diagnostic nor specific for rickettsial infections. These infections are classified under the nebulous category of “lymphocytic vasculitis” and have variable degrees of perivascular lymphocytes, vascular fibrin, dermal edema, hemorrhage, and vacuolar change at the epidermal-dermal junction. The organisms reside in the endothelium of superficial vessels but are not visualized on routine sections. Select laboratories offer immunohistochemistry for *Rickettsia* species ([www.propathlab.com/antibodies-and-probes-available-immunohistochemistry-426/716](http://www.propathlab.com/antibodies-and-probes-available-immunohistochemistry-426/716)).

Several scientific studies have evaluated the use of molecular testing, specifically PCR, using DNA isolated from blood clots, serum, skin biopsies, and eschars. The published studies have targeted several different rickettsial genes and have shown varying degrees of success for confirming a diagnosis of spotted fever. Several studies have used a nested PCR approach to try to overcome the limited sensitivities originally reported. Currently, there are few clinical molecular assays available for detecting rickettsial infections, and the specimen of choice is typically blood. The CDC can perform real-time PCR and sequencing on blood and fresh tissue biopsies.

#### **<sup>9</sup>CPT coding**

87797 (direct probe), 87798 (amplification);  
87999 (unlisted)

### **11.9.3 Lyme Disease**

Lyme disease is caused by the spirochete *Borrelia burgdorferi*, which is transmitted to humans through the bite of infected *Ixodes* ticks (deer ticks, blacklegged ticks). The early/primary clinical stage is characterized by erythema migrans at the site of the tick bite, although the classic targe-

toid rash may not occur or be recognized in a significant percentage of cases. In addition, erythema migrans can have atypical appearances including uniformly erythematous lesions, lesions with a vesicular central region, or peripheral erythema, which may be difficult to distinguish from erythema annulare centrifugum, contact dermatitis, and arthropod bites [49]. Diagnosis of Lyme disease is typically based on clinical features and is often confirmed with serologic testing. In general, traditional laboratory and pathologic testing have a rather poor sensitivity. Skin biopsies often yield a typical gyrate erythema pattern with a tightly coat-sleeved perivascular lymphocytic infiltrate, similar to other gyrate erythemas. More exuberant pseudolymphomatous infiltrates may also occur. Silver staining (such as Warthin-Starry) of erythema migrans biopsies only detects spirochetes in 40 % of cases. Lyme immunohistochemistry may be performed but this assay is not widely available. Culture sensitivity ranges from 30 to 70 %, and serologic testing for IgM is positive in only 20 to 40 % of cases. A number of clinical labs offer molecular testing for *B. burgdorferi*, typically from blood, spinal fluid, or frozen tissue specimens, not FFPE skin. Although PCR assays are available, they are not recommended for first-line diagnostic use, but may be useful in select cases. Some state and local health departments as well as at least one national lab offer tick identification and testing for *Borrelia* species.

#### **<sup>10</sup>CPT coding**

87475 (direct probe), 87476 (amplification);  
87999 (unlisted)

### **11.9.4 Syphilis**

*Treponema pallidum*, the causative agent of syphilis, is a spirochete bacterium that in general cannot be cultured in the clinical labora-

<sup>9</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

<sup>10</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

tory. Primary, secondary, and tertiary syphilis all have cutaneous components. Primary syphilis is characterized by a painless ulcer, or chancre, usually on genital or perianal skin. Secondary syphilis occurs 1–2 months following the initial chancre and has a wide variety of clinical presentations including morbilliform, lichenoid, psoriasiform, pustular, and follicular (including alopecia) eruptions, to name a few. Tertiary syphilis occurs many years after the primary infection, and cutaneous manifestations include isolated nodular lesions or gummatous ulcers. Diagnosis of syphilis is typically made by dark-field microscopy (primary syphilis only) and serology. Dark-field microscopy requires a specialized microscope and a technician with experience and expertise in the procedure. Serologic testing, particularly in the primary stage, can generate false-negative results due to the lag time for antibody production. Skin biopsy in conjunction with immunohistochemistry for treponemes can be useful for diagnosis at all stages, but because the background histology is often variable and nonspecific and immunohistochemistry may not be performed when syphilis is not in the clinical differential diagnosis, many cases are missed. Moreover, the organism burden can be low in some secondary and tertiary lesions.

A large number of studies have described the use of molecular techniques, particularly PCR, for the detection of *T. pallidum*. A common bacterial genetic region targeted by molecular assays is the *PolA gene*, although other genetic targets including the *tpp47*, *bmp*, and *16SrRNA* genes have been described [50–54]. There are no FDA-approved nucleic acid tests for *T. pallidum*; all clinically available assays are considered lab-developed tests. Molecular assays are likely to be most helpful for congenital syphilis and neurosyphilis. They may also have utility in primary stage syphilis when traditional methods have limited sensitivity. The CDC offers molecular testing to detect *T. pallidum* in a variety of specimen types including FFPE tissues [55].

#### <sup>11</sup>CPT coding

87797 (direct probe), 87798 (amplification);  
87999 (unlisted)

### 11.9.5 *Bartonella*

*B. henselae*, *B. quintana*, and *B. bacilliformis* are the causative agents of cat scratch fever (cat scratch disease, CSD), trench fever, and Oroya fever (verruca peruana), respectively. CSD typically presents as a solitary skin papule or nodule, often but not always associated with a cat scratch, followed by regional lymphadenopathy and mild fever and malaise. Atypical cutaneous presentations (erythema nodosum, urticaria, pigmented purpura) have been described [56]. Most cases in immunocompetent individuals are self-limited and do not require antibiotic treatment. Diagnosis of CSD is often made based on clinical presentation and history as well as serology testing. Lymph node biopsies may show necrotizing granulomas and skin biopsies are often nonspecific. Silver stains (Warthin-Starry) or modified Gram stains may reveal bacilli. Culture of *Bartonella* species is difficult and time consuming and typically not performed [57].

*B. henselae* and *B. quintana* infections can also cause bacillary angiomatosis (BA), a vascular proliferative infectious disease that can involve the skin and other organs and is most frequently seen in immunodeficient patients. A recent study described three renal transplant patients who were diagnosed with BA after presenting to a clinic for skin care of organ transplant patients [58]. The patients presented with erythematous or violaceous nodules that on biopsy demonstrated a vascular proliferation, plump endothelial cells, and a background neutrophilic infiltrate. For two of the patients, *Bartonella* serology was negative, and the diagnosis was made by PCR from skin biopsy material.

<sup>11</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

*B. henselae* can be detected by PCR from skin and lymph node biopsies from patients with CSD and may be useful to confirm infections with atypical clinical presentations and inconclusive biopsy and culture findings. A recent report described detection of *B. henselae* by an RT-PCR assay using a swab of a skin papule, which is an attractive alternative to biopsy [59]. Several clinical molecular laboratories offer PCR-based testing for Bartonella species (see the Association for Molecular Pathology Test Directory). The CDC also offers PCR and sequencing from peripheral blood and fresh tissue biopsy specimens.

**<sup>12</sup>CPT coding**

87470 (direct probe), 87471 (amplification);  
87999 (unlisted)

### 11.9.6 Cutaneous Anthrax

Cutaneous anthrax is typically caused by the introduction of *Bacillus anthracis* endospores into open skin lesions. In non-bioterrorism settings, cutaneous anthrax is most commonly seen in individuals that handle animals and animal products. Occupations most at risk are farmers, wool sorters, and butchers. Animals, including domestic livestock, are infected through ingestion of pathogenic endospores from the soil. Humans become infected by direct skin exposure, inhalation, or ingestion of the organisms. The typical anthrax skin lesion is a painless pruritic papule that can resemble an insect bite. The lesion quickly forms a vesicle that undergoes central necrosis leaving a characteristic black eschar. Histologic examination of skin lesions demonstrates necrosis and massive edema with hemorrhage and a peculiarly sparse inflammatory infiltrate. Staining reveals Gram-positive bacilli in the exudate. At high power, the organisms have characteristic squared off ends and are

aligned in chains. The organisms may be sparse in partially treated lesions. Immunohistochemistry using monoclonal antibodies to *B. anthracis* may be helpful for diagnosis from formalin-fixed tissue specimens; however, it may require the use of at least two antibodies to be definitive and is not widely available [60]. Cultures from skin lesions are generally not useful because of a high false-negative rate [61].

A number of PCR-based assays for the detection of *B. anthracis* have been reported. The genetic targets of the reported assays include bacterial chromosomal sequences as well as the virulence plasmids pXO1 and pXO2, which code for toxin genes and genes required for capsule biosynthesis, respectively [62–67]. A real-time PCR assay for the detection of *B. anthracis* directly from clinical specimens was validated during the investigation into the 2001 bioterrorism-associated anthrax outbreak in the United States [68, 69]. That assay employed three genetic targets: each of the two virulence plasmids and one locus within the *B. anthracis* chromosome.

Currently the only FDA-approved molecular assay for *Bacillus anthracis* is the Joint Biological Agent Identification and Diagnostic System (JBAIDS) Anthrax Detection kit from BioFire Diagnostics Inc. (formerly Idaho Technology, Inc.). This system, which evolved from the manufacturer's ruggedized advanced pathogen identification device (R.A.P.I.D.), is a portable real-time PCR system that is used by the military for field testing of clinical and environmental specimens. Although clinical molecular testing for *B. anthracis* is not widely available, the Centers for Disease Control and Prevention (CDC) may be able to provide testing in some cases.

**<sup>13</sup>CPT coding:**

87797 (direct probe), 87798 (amplification);  
87999 (unlisted)

<sup>12</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

<sup>13</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

## 11.10 Drug Resistance Testing

There are several mechanisms by which microorganisms can develop resistance to therapeutic agents, including the production of enzymes that inactivate the therapeutic agent, alteration of the target of the therapeutic agent, and alteration of cellular uptake or export of the agent. All known mechanisms of drug resistance involve a change in the infectious organism's nucleic acids, either a change in sequence of their genome or acquisition of new genetic material. Traditional testing methods for sensitivity/resistance testing identify if growth of the organism is inhibited by the presence of the drug and thus are "functional" or "phenotypic" assays. In contrast, molecular methods identify the genotype of the organism, typically assessing for alterations in a single gene that is known to result in antibiotic resistance.

Growth inhibition assays are still commonly performed and have several key benefits over molecular approaches, including the ability to assess the sensitivity or resistance to multiple drugs simultaneously. Importantly, growth inhibition assays identify essentially all resistance to a specific antibiotic therapy, regardless of the mechanism. Conflicting results between phenotypic and molecular assays may result when an organism has acquired resistance through a mechanism other than the genetic target of the molecular test. Despite this limitation, molecular methods for the detection of antibiotic resistance continue to proliferate and have several advantages compared to traditional growth inhibition assays. Molecular testing is an attractive approach for detecting antibiotic-resistant organisms that cannot be grown or grow very slowly in the laboratory. Molecular methods have also become the test of choice when rapid and/or high-throughput detection of a resistant organism is required.

### 11.10.1 Methicillin-Resistant *Staphylococcus aureus*

*Staphylococcus aureus* (SA) and methicillin-resistant *Staphylococcus aureus* (MRSA) are common causes of skin and soft tissue infections.

*Staphylococcus aureus* infections have many clinical presentations, including but not limited to folliculitis, impetigo, scalded skin syndrome, and septicemia. Community-acquired MRSA most commonly presents as furunculosis, with an erythematous and edematous papule or nodule that may ulcerate and mimic a spider bite (see Fig. 11.4d). In medical facilities, MRSA causes life-threatening infections including surgical site infections, septicemia, and pneumonia. Although MRSA can be identified by traditional culture methods, molecular methods are preferable due to their superior turnaround time, which allows more effective patient management. Molecular testing is also more amenable to high-volume carrier screening, which can be used to prevent (or reduce) MRSA transmission.

Methicillin resistance is the result of alterations to the bacterial gene *mecA*, which codes the penicillin-binding protein. Alterations in *mecA* result in an altered penicillin-binding protein (PBP2a) to which oxacillin cannot bind and therefore has no effect. Molecular methods targeting the altered *mecA* gene can rapidly identify MRSA directly from clinical specimens. A disadvantage of MRSA nucleic acid amplification tests targeting *mecA* is that they will not detect novel resistance mechanisms such as those related to *mecC* or uncommon phenotypes such as borderline oxacillin resistance.

There are several FDA-approved molecular assays for MRSA (Table 11.4). Most of these assays allow direct detection of nasal colonization in an effort to prevent and control MRSA infections in healthcare settings. Several studies have demonstrated that active MRSA surveillance programs are effective at reducing nosocomial MRSA infections and result in cost savings [70–72].

There is one FDA-approved MRSA assay for the direct detection and differentiation of SA and MRSA from skin and soft tissue swabs, the Cepheid Xpert MRSA/SA SSTI assay. This assay uses primers and probes specific to three gene targets: staphylococcal protein A (*spa*), *mecA*, and the staphylococcal cassette chromosome (SCCmec) inserted into the *S. aureus* chromosomal *attB* insertion site. This testing strategy

allows identification of the presence of *SCCmec* cassette variants with *mecA* gene deletions, reducing the risk of false positives that occur in molecular tests that target only the *SCCmec* cassette [73, 74]. Wolk and colleagues compared the performance of the Xpert MRSA/SA SSTI assay to standard culture, identification and growth inhibition assays using 114 wound swab specimens [75]. The assay's sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the detection of MRSA were 97.1, 96.2, 91.9, and 98.7 %, respectively, and for the detection of *S. aureus* were 100, 96.6, 96.5, and 100 %, respectively. The somewhat reduced NPV for MRSA (91.9 %) was the result of three specimens reporting positive for the Xpert MRSA/SA SSTI assay, but negative by traditional methods. Although the reason for the discrepancies could not be definitively determined, it may be that the molecular assay is in fact superior to traditional methods and that the discrepant results represent false-negative cultures rather than false-positive molecular test results.

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**<sup>14</sup>CPT coding**

87641 (amplified probe); 87999 (unlisted)

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## 11.11 Genetic Factors That Influence Susceptibility/Resistance to Infectious Agents

An interesting area of molecular testing that has the potential for clinical use is the analysis of germline genetic variants that render individuals particularly sensitive or resistant to specific infectious agents. An increasing body of evidence suggests that inherited genetic factors control susceptibility to a large number of infectious agents. An extreme example is the rare genetic

syndrome Mendelian susceptibility to mycobacterial diseases (MSMD), which predisposes affected individuals to serious disseminated infection with low virulence mycobacteria, including the BCG vaccine [76]. Alterations in at least five genes have been implicated: *IFNGR1*, *IFNGR2*, *STAT1*, *IL12B*, and *IL12RB1*. Interestingly, affected individuals are resistant to infections by many other bacteria, fungi, and parasites.

Genetic variants associated with a more subtle risk of mycobacterial infections, particularly *M. leprae*, have also been described. These include variants in the genes *HLA-DR-DQ*, *NOD2*, *TNFA*, and *IL23* [77, 78]. Genetic risk factors for other organisms that cause cutaneous disease, including tubercular and atypical mycobacterial species, as well as specific fungal and parasitic organisms, have also been described [78]. In an era when full genome sequencing is becoming a reality, one can imagine that germline testing could be used to identify individuals with risk factors for infection with specific pathogenic organisms. The information could prove useful for disease prevention as well as for diagnosis and disease management.

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## 11.12 Practical Considerations

Because dermatology-associated molecular infectious disease testing is relatively uncommon, most of the available assays are lab developed, emphasizing the importance of a reliable laboratory that produces clear and accurate patient reports and is available for questions regarding test methods and individual test results.

To understand and/or compare the performance characteristics of molecular assays targeting various pathogens, review of the published literature is often a good starting point. As a word to the wise, for any given organism and molecular test, the published literature describing an assay's performance characteristics may be highly variable. These differences are likely multifactorial and include the specific region of the genome that is targeted by the assay and the design of the specific primer/probe used to detect the organism. A key and often overlooked

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<sup>14</sup>The CPT codes are from 2014 listings and are provided for reference only, not as a billing guide [1]. Recommended codes may vary depending on molecular targets and testing methods. These are updated annually. A reference for physician fee schedules can be found at [www.cms.gov](http://www.cms.gov).

**Table 11.4** FDA-approved real-time PCR assays for the detection of SA and MRSA

Manufacturer	Test	Method	Instrument	Organism(s) detected/identified	Acceptable sample
Becton Dickinson	GeneOhm MRSA Assay	Real-time PCR	Smart Cycler	MRSA	Nasal swab
Becton Dickinson	BD MAX MRSA Assay	Real-time PCR	BD MAX	MRSA	Nasal swab
bioMerieux	NucliSENS EasyQ MRSA	NASBA	NucliSENS	MRSA	Nasal swab
Cepheid	Xpert SA Nasal Complete	Real-time PCR	GeneXpert	SA	Nasal swab
				MRSA	
Cepheid	Xpert MRSA/SA SSTI	Real-time PCR	GeneXpert	SA	Skin and soft tissue swab
				MRSA	
Cepheid	Xpert MRSA/SA BC	Real-time PCR	GeneXpert	SA	Gram-positive blood culture
				MRSA	
Cepheid	Xpert MRSA	Real-time PCR	GeneXpert	MRSA	Nasal swab
Roche	LightCycler® MRSA Advanced Test	Real-time PCR	LightCycler	MRSA	Nasal swab

Abbreviation: NASBA nucleic acid sequence-based amplification

variable is the quality and type of specimens tested. For example, the performance characteristics of an HPV test that were determined by testing liquid-based cytology (Pap) specimens cannot unilaterally be applied to testing from FFPE tissue samples for the diagnosis of condyloma or Bowen's disease. A final consideration when reviewing literature describing performance characteristics of a molecular assay is whether appropriate controls were used to allow accurate interpretation of the molecular data.

Controls are extremely important for ensuring accurate test results in all situations, but especially in infectious disease applications. Most molecular assays for genetic and oncology applications test for the presence or absence of a specific genetic alteration. The result is either the presence of a wild-type sequence (negative for mutation) or altered sequence (positive for mutation). A failed enzymatic reaction generates neither and can be readily identified. In contrast, when using molecular assays that test for the presence or absence of a specific organism, a negative result due to absence of the organism is indistinguishable from a failed reaction if appropriate controls are not used. This is particularly important for assays that employ formalin-fixed paraffin-embedded

tissue specimens since the quality and quantity of nucleic acids isolated from these specimens can be highly variable. Controls ensure that a negative result is not due to poor quality of nucleic acids, or a failed enzymatic reaction, and also ensure that positive results are not due to carryover contamination. There are a variety of types of controls that can be used to ensure adequate analysis, and the terms used for these controls can be overlapping and confusing. The next sections present some general guidelines about controls that may be used in molecular testing.

### 11.12.1 External Controls (Positive, Negative, and No-Template)

These controls are run every time the test is performed and should be processed and tested in the exact same way as clinical specimens. These controls are useful for demonstrating that the reagents and equipment are functioning properly and that known specimens (positive and negative) give expected results. Sources for positive and negative controls include previously tested patient samples and commercially available cell lines or nucleic acids. The no-template control,

also called a blank control, contains all of the reaction constituents except for template nucleic acid. It is used to identify the presence of contaminating targets that may cause false-positive results.

### 11.12.2 Sensitivity Control

Sensitivity controls are needed for assays that require a specific level of analytical sensitivity (limit of detection). This control is required for quantitative assays such as viral load tests to ensure that the expected limit of detection of the assay is met during each test performance. It is also useful in non-quantitative assays to clarify the lowest amount of a specific analyte that can be detected by an assay.

### 11.12.3 Internal Control

Internal controls are typically human genes that can be targeted to ensure that several different aspects of testing are functioning appropriately. An internal control ensures that the DNA extraction process was successful and that the extracted nucleic acids are of sufficient quality and quantity to generate a test result. Internal controls also ensure that inhibitory substances are not present in the reaction and that equipment such as thermal cyclers are functional. Because internal controls do identify the presence of inhibitory substances, some may refer to them as inhibition controls. If possible, the primers/probes for this control should be multiplexed within the target amplification reaction to demonstrate validity of the results within each specific reaction vessel. The internal control should be designed to yield an amplification product that is longer/larger than the product generated by the target reaction to ensure that the nucleic acids within the specimen are capable of generating a product if the target is present. Internal controls that target human genes are preferred when sufficient human cellular material is used. If the specimen is acellular, an inhibition control may be more useful.

### 11.12.4 Inhibition Control

An inhibition control is a nucleic acid sequence that can be added to a clinical specimen prior to testing. For this reason it is sometimes referred to as a “spike” control. This control assesses for successful DNA extraction and for the presence of inhibitors in the reaction, but does not ensure that the quality or quantity of the nucleic acids in the test specimens is adequate. This control can be helpful for clinical specimens that may have no or very little human cellular material, such as cerebrospinal fluid (CSF).

## 11.13 Summary

Many infectious agents result in cutaneous lesions that have overlapping or nonspecific clinical symptoms and histopathology. Culture for many of these organisms is slow and laborious and generally has a relatively limited sensitivity. Serologic testing also has limitations in terms of sensitivity and timeliness. Organisms may be appreciated in histologic sections using special stains; however, stains such as AFB cannot effectively discriminate between different species and focal staining can be difficult to discriminate from artifact. Molecular assays have the potential to provide diagnostic information in these challenging cases.

Molecular assays can be designed to be highly specific to one species or capable of detecting several different pathogens. Assays that are capable of detecting multiple different organisms simultaneously are advantageous since their use is not limited by clinical suspicion. Two testing strategies can be used for this global detection approach. One strategy is to use consensus primers that are capable of amplifying nucleic acids from a number of different species, followed by sequencing to identify the specific species. This type of testing requires a fairly sophisticated interpretation and is offered by a few academic clinical laboratories. A second approach is to multiplex several primers or probes specific to several different species into a single reaction. Individual organisms can be readily detected and

differentiated by a variety of methods, including electrophoresis or hybridization to a chip or bead array. Future testing strategies may include designing panels for specific clinical differential diagnoses such as nonhealing cutaneous wounds, wounds with systemic findings, and recent travel to regions with endemic disease.

Moving forward, the availability of molecular assays to aid in the diagnosis of fastidious or uncommon infectious organisms will continue to increase. Potential additional applications of molecular testing include the identification of viral genomes in cutaneous neoplasms, identification of antimicrobial resistance, and identification of inherited germline variants that increase or decrease an individual's risk for infections by specific pathogens.

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## Contents

12.1	<b>Molecular Testing in Current Clinical Practice</b> .....	342
12.1.1	Clinically Significant Targets.....	344
12.1.2	New Technologies.....	345
12.2	<b>Looking Ahead</b> .....	346
12.2.1	Theranostics.....	346
12.2.2	Pharmacogenetics .....	347
12.3	<b>Summary</b> .....	352
	<b>References</b> .....	353

### Key Points

- Molecular diagnostics is a rapidly changing field. There is a constant influx of new technology platforms and new genetic targets with potential clinical utility.
- One of the biggest challenges for the field of molecular diagnostics is the translation of research findings into clinical practice.
- Despite the challenges, molecular diagnostics has already impacted many areas of dermatologic testing.
- Assessment of germline genetics can be useful for diagnosis and for identification of carriers of genodermatoses.
- Assessment of acquired somatic mutations can have diagnostic, prognostic, and/or therapeutic selection utilities for melanoma, cutaneous lymphoma, and cutaneous soft tissue tumor patients. The ability to provide therapeutic selection information sets molecular testing apart from other testing modalities and presents one of the greatest opportunities for molecular diagnostics.
- Molecular infectious disease testing of dermatology specimens provides significant advantages over traditional culture and serologic techniques and will play important roles in the discovery and tracking of emerging infectious diseases.
- Pharmacogenetic testing has the potential to impact the selection of a wide variety of

therapeutic agents used in the practice of dermatology, by identifying responders, nonresponders, and perhaps more importantly those that are at risk for adverse drug reactions, especially the most severe forms such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).

- Ultimately, molecular testing will likely play a role in the diagnosis and management of essentially all dermatology patients.

of clinical medicine—genetics, oncology, and infectious disease testing—with dermatologic applications in all three areas (Table 12.1). Moving forward, clinical testing in all three areas will continue to expand, although the applications within each discipline will likely shift (Fig. 12.1).

Germline genetic testing is now available for many inherited diseases with dermatologic presentations. These include genodermatoses associated with skin fragility, hyperkeratosis, and malignant skin tumors. Germline genetic assays are usually performed by DNA sequencing applications, which are transitioning from Sanger to next-generation sequencing platforms. From a technical standpoint, identification of sequence variants is generally straightforward. One of the greatest challenges for these types of assays is determining if a specific variant is disease causing or increases disease risk or is a benign polymorphism. An

## 12.1 Molecular Testing in Current Clinical Practice

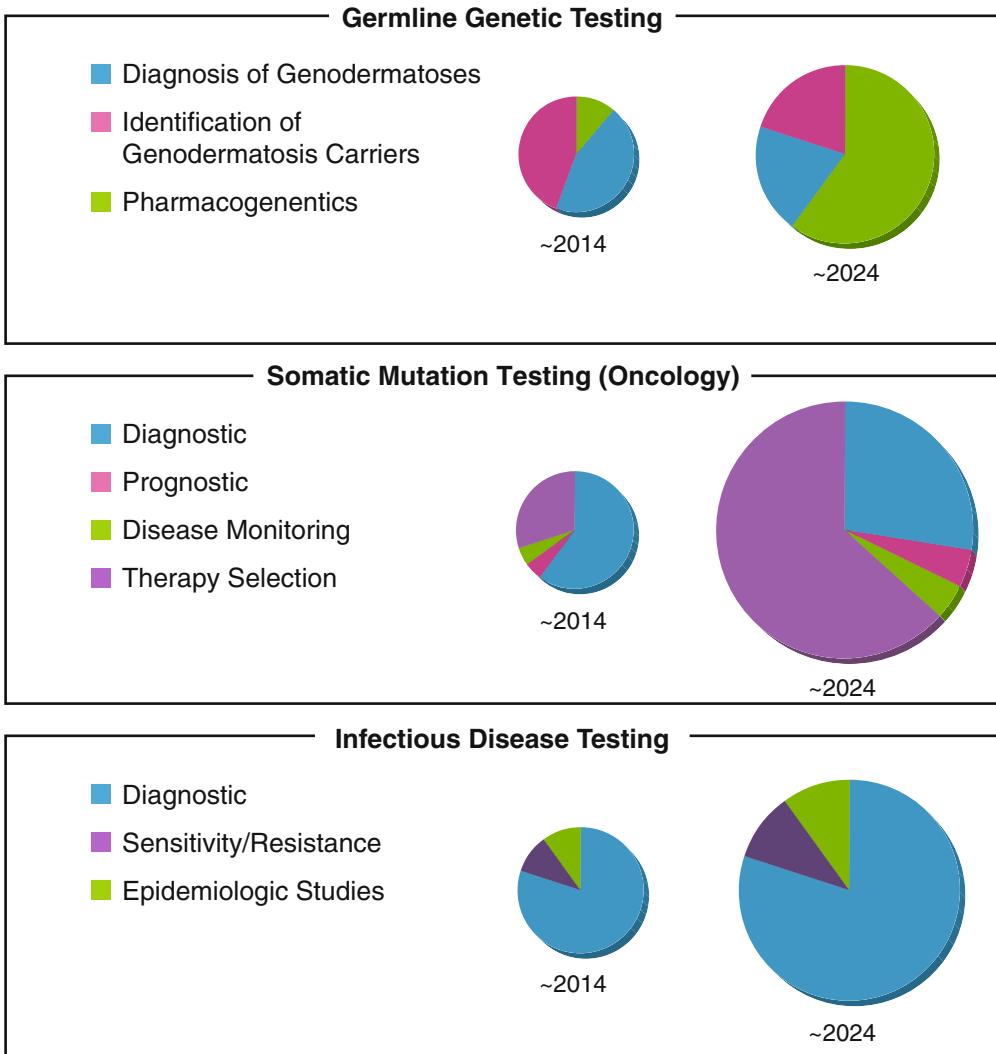
Molecular diagnostics is a rapidly changing and complex field. Despite the challenges, molecular diagnostics has already transformed three areas

**Table 12.1** Molecular diagnostic applications in dermatology: present and near future

Dermatologic condition	Chapter	Current testing	Near-future applications
Melanoma	4/5	FISH (d)  aCGH (d)  PCR-based mutational analysis (t)  <i>BRAF</i>  <i>NRAS</i>  <i>KIT</i>	High-throughput (next-gen) sequencing (d,p,t)  FISH and aCGH (p,t)  Gene expression profiling (d,p,t)
Mycosis fungoides and Sézary syndrome	6	PCR-based clonality (TCR/Ig) assays (d)	
Non-MF/SS cutaneous lymphoma	7	PCR-based clonality (TCR) assays (d,p)	
Soft tissue tumors	8	FISH and RT-PCR for translocation and amplification detection (d)	
Muir-Torre syndrome	9	Microsatellite instability assay (d)  Complete MMR gene sequencing (d)	High-throughput (next-gen) sequencing (d)
Genodermatoses	10	Complete gene sequencing (d)	High-throughput (next-gen) sequencing (d,p,t)  Gene expression profiling (d,p,t)
Infectious disease	11	Pathogen-specific gene sequencing (d)  Identification of drug-resistant strains (t)	<sup>a</sup> High-throughput (next-gen) sequencing (d, t)  <sup>a</sup> Gene expression profiling (d, t)
Others	1–12	Pharmacogenetic PCR-based gene variant analysis and gene sequencing (t)	High-throughput (next-gen) sequencing  Gene expression profiling

Abbreviations: *d* diagnosis, *p* prognosis, *t* guiding therapeutic decisions

<sup>a</sup>Includes applications for predicting organism drug resistance and pharmacogenetics



**Fig. 12.1** Current and future utilization of molecular diagnostic testing in dermatology practice. Note that the use of molecular testing in each of the three areas—genetics, oncology, and infectious disease—will increase. Somatic mutation detection, particularly for therapeutic selection purposes, has the greatest potential for expanding

over the next decade. Increased germline testing will likely be for pharmacogenetic applications. All areas of infectious disease testing, diagnostics, identifying resistant strains, and epidemiologic studies, particularly for emerging pathogens, will increase

emerging application for germline genetic testing is for the identification of variants that determine drug metabolism and response (see Sect. 12.2.2 below).

In oncology, somatic mutation detection has potential utility for diagnostic, prognostic, disease monitoring, and therapeutic decision making. One of the earliest diagnostic applications of molecular technologies, which is still in use today,

is the use of T-cell gene rearrangement studies for the diagnosis of mycosis fungoides and other T-cell leukemias and lymphomas. These assays are currently almost universally performed by PCR and electrophoresis, although next-generation sequencing applications are being developed. The assays can be technically challenging because of the complexity of the TCR genetic loci and because there is no standardization of

interpretation criteria. This combination can lead to wide lab-to-lab variation in TCR gene rearrangement results.

Molecular testing related to the diagnosis and treatment of melanoma provides a good example of the multiple potential utilities of nucleic acid testing. Molecular testing is now performed for all stages of management of the melanoma patient: assessment of risk through germline mutational analysis, diagnosis of melanoma by fluorescent *in situ* hybridization (FISH) and comparative genomic hybridization (CGH), assessment of biologic behavior of the tumor and patient prognosis, and determination of eligibility for targeted therapy by tumor mutational analysis. The number and availability of clinical molecular assays for therapeutic selection purposes are increasing as data demonstrating their ability to predict response to targeted therapies continue to be accumulated. Many of these therapies target the MAP kinase and PI3K/Akt/mTOR signaling pathways. The B-Raf<sup>V600E</sup> mutation is the most common mutation in melanoma, occurring most frequently in melanomas from intermittently sun-damaged skin [1]. The presence of B-Raf<sup>V600E</sup> mutations in melanoma tumors predicts response to three recently FDA-approved therapies for advanced melanoma patients: vemurafenib (B-Raf<sup>V600E</sup> inhibitor), dabrafenib (B-Raf<sup>V600E</sup> inhibitor), and trametinib (MEK inhibitor) [2–5]. Mutations in *NRAS*, which occur in a similar subset of melanomas as *BRAF* mutations, albeit at a lower frequency, may play a role in resistance mechanisms to B-Raf inhibitor therapy. Specific *KIT* mutations, which occur with a relatively high frequency in acral melanomas, primary mucosal melanomas, and melanomas from chronically sun-damaged skin, appear to predict response to small-molecule tyrosine kinase inhibitor therapies such as imatinib mesylate (Gleevec) and nilotinib (Tasigna) [6–8]. The use of molecular tests for drug selection will continue to expand as additional targets are identified and new therapies developed.

Infectious disease applications of molecular testing have expanded rapidly over the last two decades. Bacteria, viruses, protozoa, fungi, and parasites all have nucleic acid genomes that can be targeted for highly sensitive and specific

molecular assays. Molecular techniques are particularly well suited for testing for organisms that are difficult or impossible to culture (viruses) and/or for organisms for which culture presents a hazard to laboratory personnel (mycobacteria), as well as for specimens from which organisms are not viable for culture (formalin-fixed tissues). Molecular-based testing for infectious organisms often has superior performance characteristics (sensitivity and specificity) compared to standard culture and serology techniques. Additional advantages of molecular testing over traditional diagnostic methods include a more rapid turnaround time, more cost-effective testing, and the ability to support high-volume screening for drug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA). Moving forward, molecular testing will play important roles in the discovery, tracking, and diagnosis of emerging pathogens.

Molecular diagnostics has come a long way, baby. Table 12.1 describes molecular dermatologic testing in current practice and on the horizon. The use of molecular diagnostics for patient management has the potential to expand rapidly, but the field is faced with challenges. New molecular technologies and enhancements to current methods are rapidly being developed. One of the challenges for the field is to select and implement clinical testing methodologies that are reliable, accurate, and cost-effective and will remain relevant for a reasonable period of time. A second challenge is to weed through the numerous and frequent reports describing genetic targets with potential clinical utility in order to identify truly clinically relevant targets. This process of translating research findings into clinical practice is the greatest challenge facing the field of molecular diagnostics.

### 12.1.1 Clinically Significant Targets

Reports of genetic variants that appear to have clinical diagnostic, prognostic, or therapy selection utility are published monthly in scientific journals. Often these first studies have compared individuals with classic disease presentation to non-diseased individuals. When follow-up

studies are performed analyzing individuals with non-classic disease presentation to individuals with mimicking presentations, which is exactly the population for which testing is likely to have the greatest impact, often the strength of the findings fades. Translation of research findings into clinical practice requires sufficient data to fully understand the performance characteristics (sensitivity, specificity, etc.) of the test within the population(s) to be tested. These studies can be time consuming and expensive. Traditionally, academic researchers have had little incentive to perform these types of tedious studies. Academicians are acknowledged for publishing novel findings but receive little academic credit for doing larger-scale studies to either confirm or disprove the original findings. In vitro diagnostic (IVD) companies, which provide FDA-approved assays to clinical laboratories, are generally not able to perform much of this work because of the high frequency at which the initial findings cannot be reproduced resulting in zero return on the often significant efforts invested. Even when a particular genetic target is determined to have clinical significance, much additional work and data compilation are needed to understand the significance of all of the variants that occur. For example, mutations in *KIT* have been described in several tumor types, including melanoma and gastrointestinal stromal tumors (GISTs), and are an appealing target for tyrosine kinase inhibitor therapy. In melanoma, mutations in *KIT* exons 9, 11, 13, 17, and 18 have been described [8, 9]. However, only mutations in exons 11 and 13 appear to predict response to the tyrosine kinase inhibitor imatinib [8]. Similarly, initial research reported that *EGFR* mutations in lung cancers were associated with response to EGFR-targeted small-molecule therapies [10]. However, subsequent studies demonstrated that some mutations, such as *EGFR* exon 20 deletions, result in resistance to the same therapies [11]. These stories, like many others, take years to evolve and demonstrate the need for ongoing studies, as well as the need for clinicians and laboratory professionals to remain current with scientific literature.

The huge gap between initial identification of a novel genetic marker and accumulation of data

demonstrating clinical validity is often filled by biotech companies and other small businesses funded through government grants, private donations, and individual and small group investors. Moving forward, the questionable future of gene patents may make this work appear more risky and therefore less appealing to investors.

Although new molecular discoveries in medicine continue to be made at an ever-increasing rate, translation of these findings to clinical practice has lagged behind. The gap between “cutting edge” and clinically available diagnostic testing continues to widen. New strategies and incentives are needed to expedite the clinical validation studies required to bring the fruits of these new discoveries into clinical practice. An additional challenge, beyond the scope of this chapter, is the reluctance of third-party payers to reimburse for new and novel testing.

### 12.1.2 New Technologies

New technologies and new commercial companies offering new technologies appear and disappear on a regular basis. This is a real challenge for laboratories that want to be on the front edge of new technology but need to be certain that the technology and the company will still be in existence tomorrow. This problem is not unique to molecular diagnostics, but is shared by many areas of technology. How many different data storage devices and cell phones have you had over the last 5–10 years? The challenge for molecular diagnostic labs is that as opposed to the cost of a cell phone or a floppy disc (a what?), the cost of laboratory equipment for molecular diagnostics often reaches into the six-figure range.

Advances in technologies increase the speed, throughput, and automation of molecular testing. The ability to analyze multiple variants essentially simultaneously will continue to increase, which will allow the rapid generation of increased amounts of data in reduced amounts of time. This will continue to drive down the expense of molecular testing, making testing more cost-effective. It will also enhance the clinical utility of molecular tests by making results more rapidly

available and therefore more easily incorporated into patient management.

The trend toward increased interpretation of molecular data by computer algorithms will undoubtedly continue. Some of this is inevitable since a human could not accurately interpret the hundreds of thousands of data points now generated in a single experiment. However, it will remain essential that experts review some aspects of the actual data, not the just the software's interpretation of the data. Close review of data can reveal unexpected results that are not correctly interpreted by a computer algorithm. New or unexpected variants as well as technical problems can be identified by evaluating primary data. The increasing ease with which new methods can be performed and interpreted by a computer algorithm comes with risks that labs will offer testing for which they do not have the expertise to understand the subtle nuances that can affect the quality and/or interpretation of the data.

## 12.2 Looking Ahead

While new molecular tests to aid in diagnosis and prognosis will continue to increase and evolve, perhaps the greatest opportunity for molecular diagnostics relates to the potential for tailoring treatment to an individual patient, so-called personalized medicine. Personalized medicine calls for treatment strategies to be determined based on a variety of individual-specific factors, especially genetic makeup, rather than a “one size fits all” approach. Historically, therapies have been chosen largely based on statistical data. A patient with a specific disease would be given a therapy that was known to benefit the largest percentage of people with that diagnosis, regardless of the individual’s genetic makeup. Using this approach, many patients receive drugs from which they receive no benefit and/or from which they may have toxic side effects. Precious time is wasted before transferring the patient to an alternate therapy. The hope of personalized medicine is to “get it right the first time,” resulting in improved outcomes.

Both theranostic and pharmacogenetic testing can be used to help choose the right drug for the right patient. These two areas of molecular test-

ing have the potential to expand rapidly over the next decade. Although some may use these terms interchangeably, here the term “theranostics” refers to somatic alterations that predict response/resistance to targeted therapeutic agents, primarily in the oncology setting. Theranostic testing is generally used to identify patients who will respond to a specific agent or a specific group of targeted therapeutics, such as B-Raf inhibitors.

The term “pharmacogenetics” is used to describe germline variants, particularly in drug-metabolizing enzymes, that predict an individual’s ability to metabolize a large number of pharmaceutical agents. Pharmacogenetic testing can be used to determine how an individual will respond to one or more broad classes of drugs that are metabolized through the same enzymatic pathway. Response in this context includes not only responders and nonresponders but perhaps more importantly those that are at increased risk for adverse drug reactions. Ultimately, evaluation of both germline and somatic variants may be necessary to accurately determine the best therapy for an individual patient.

### 12.2.1 Theranostics

Decades of research and experience have led to morphologic, histologic, and protein expression (primarily by immunohistochemistry) criteria for the diagnosis of many tumor types. Molecular testing has the potential to unlock additional information about individual tumors that generally cannot be obtained through microscopic evaluation. Molecular testing has the potential to answer the question, “To what therapy(s) will this tumor respond?”

The concept of co-development of a therapeutic agent with a molecular test to predict response to the agent has existed for several decades. The field has struggled for a number of reasons, including the significant genetic diversity that exists, in both germline and somatic DNA sequence. With the sequencing of the human genome, and the genomes of several tumor types now complete, and with the recently gained practical experience of implementing several therapeutic agents into clinical practice, theranostic testing is on the brink of rapid expansion.

New technologies have allowed rapid accumulation of data to assess the effects of genetic diversity. These data are now shared across previously separate disciplines. Tissue- or organ-specific drug development is being replaced by the development of targeted therapies that may be beneficial for a subset of patients with a wide variety of tumor types. For example, early reports indicate that the B-Raf inhibitor dabrafenib (Tafinlar), which is used for melanoma, may have utility for a subset of patients with non-small cell lung cancer (NSCLC), Langerhans cell histiocytosis (LCH), thyroid cancer, and other diseases [12–14]. This new strategy has streamlined drug development resulting in the generation of thousands of compounds with potential therapeutic utility. The challenges now are to identify (1) which compounds are most effective and (2) what molecular assays best predict response.

The United States Food and Drug Administration (FDA) has strongly supported, if not insisted on, the development of companion diagnostics for new therapeutic agents. The path has been paved for drug and diagnostic assay to essentially go through the approval process in tandem, reducing the associated costs, particularly for companies that own both the drug and diagnostic. Companion diagnostics that accurately identify individuals likely to respond to novel therapies, allow for smaller, more targeted trials, which are less expensive, and are more likely to show dramatic effects of the drug in the targeted population. These assays have the potential to expedite the process of bringing oncology drugs to market. The FDA maintains a website that is a good resource for specific information about all of the FDA-cleared or FDA-approved nucleic acid-based companion diagnostics (available at [www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm](http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm)).

An ongoing challenge in this area will be that the co-development paradigm may result in the selection of a companion diagnostic before critical evaluation of the performance characteristics of all potential targets is complete. For example, EGFR-targeted monoclonal antibody therapies were originally FDA approved with an IHC companion diagnostic, which later was determined to be ineffective at identifying individuals likely to respond

to the therapies [15]. Additional challenges relate to the fact that many tumors are relatively uncommon. Once there is one FDA-approved test, other vendors may see little value in bringing another test to market, resulting in little to no competition. The owner of the test may have little incentive to improve upon the test, even as new data are accumulated. In this setting, lab-developed tests may have better performance characteristics than an FDA-approved assay. These issues will require ongoing discussions about how to ensure that the best testing is available to clinicians and patients.

### 12.2.2 Pharmacogenetics

Patient responses to drugs can be highly variable and at times unpredictable. For any given therapeutic agent, some patients respond well, some poorly, some adversely, and some not at all. Many factors influence drug response, including nongenetic factors, such as age, organ function, and underlying disease, and genetic factors, particularly genes encoding drug-metabolizing enzymes, drug transport proteins, and cellular receptors. While pharmacogenetic testing has not yet entered routine dermatologic practice, studies of several relevant therapies are ongoing and worth discussing.

*Pharmacogenetics*, a term that has been used for several decades, refers to how genetic differences affect drug response. *Pharmacogenomics* is a relatively new term, coined in connection with the human genome project. Pharmacogenomics refers to how the systematic identification of all human genes, their products, and intraindividual variation can be used both to predict the right treatment for individual patients and to design new drugs. There is no universally accepted distinction between pharmacogenetics and pharmacogenomics, and in practice the two terms are used interchangeably. Regardless of the term used, the ultimate goal is to be able to identify patients with an increased risk of adverse side effects or a decreased likelihood of response at standard dosage of drug.

Almost half a million serious injuries and hospitalizations due to adverse drug events (ADEs) were reported in 2010, and this number is likely a

**Table 12.2** Examples of drugs metabolized by different CYP enzymes

CYP2C9	CYP2C19	CYP2D6	CYP3A4/5
NSAIDs	Proton pump inhibitors	Beta-blockers	Calcium channel blockers
Ibuprofen	Omeprazole	Metoprolol	Amlodipine
Naproxen	Lansoprazole	Propafenone	Nisoldipine
Celecoxib		Timolol	Verapamil
Angiotensin receptor blockers		Antidepressants	Immunosuppressants
Irbesartan		Amitriptyline	Tacrolimus
Losartan		Clomipramine	Sirolimus
Warfarin		Antipsychotics	Statins
		Opioids	Lovastatin
			Macrolide antibiotics
			Erythromycin

significant underestimate [16]. ADEs cause significant morbidity and mortality, result in hospital admissions and prolonged stays, and generate significant expense for the healthcare system. Application of pharmacogenetic testing to routine clinical practice has the potential to significantly reduce the number of ADEs and may result in an overall cost savings, not only by avoiding hospitalizations and other expenses associated with ADEs, but also by avoiding the use of therapies from which a patient is likely to gain no benefit.

Pharmacogenetics has seemed to be on the brink of full-scale implementation for some years now. Although there have been some “close calls,” very few have made it to prime-time use. Therapies that are particularly appealing for developing pharmacogenetic testing include those with a narrow therapeutic range, high toxicity profile, and high cost. The lack of widespread success for pharmacogenetic testing to date relates to a number of factors that can be overcome.

- Complexity. Without a full understanding of human genetics and other variables involved in drug metabolism, it has been difficult to identify variants with the kind of discriminatory power needed for clinical testing. Although some highly penetrant single-gene traits have been identified, response to many drugs is likely more complex involving polymorphisms in multiple genes (polygenic). Genome sequencing continues to shed light on this area, but the large-scale studies and bioinformatic modeling still required remain challenging (see Sect. 12.2.2.4 below).

- Need for rapid turnaround time. When making a decision about which drug and/or what dose of drug to prescribe, a clinician may not be able to wait a week or more for a genetic test result. New technologies will facilitate rapid test results, potentially even at point of care in a dermatology office [17].

- Need for data demonstrating improved outcome and/or overall cost benefit of testing. Data must be generated that clearly demonstrate that performing the test results in improved outcome for patients and/or a clear cost benefit. Reimbursement companies have little interest in paying for a test for which this data is not readily available. This data can be difficult to compile. Not only are pharmacogenetic studies complex (see Sect. 12.2.2.4 below), but it can be difficult to accurately quantify the benefit of testing to individual patients and the expenses avoided by testing.

Many of the genes responsible for variations in drug response are liver enzymes that metabolize drugs and other molecules. One of the primary gene families involved in this process is the cytochrome p450 (CYP) gene family. There are at least 29 genes in this family, although *CYP2D6*, *CYP2C9*, *CYP2C19*, and *CYP3A4* are probably the most important in terms of drug metabolism [18]. Table 12.2 lists some common medications metabolized by the four major CYP enzymes. This list is an oversimplification since some medications can be metabolized by more than one CYP family member. The nomenclature associated with genetic

variants in the CYP gene family can be challenging. For example, there are over 30 *CYP2D6* alleles each with varying clinical significance. *CYP2D6\*1A* is the nomenclature for a wild-type *CYP2D6* allele, while the *CYP2D6\*1B* allele contains a specific single base substitution, *CYP2D6\*3A* contains a specific single base deletion, and *CYP2D6\*5* designates a gene deletion. Variant alleles are generally categorized as follows:

- Poor metabolizer: Low- or nonfunctioning enzyme. Drugs are processed much more slowly than normal, resulting in a higher effective dose than expected, greatly increasing the likelihood of side effects.
- Intermediate metabolizer: Reduced enzyme function. Drugs are processed more slowly than normal. Increased risk for side effects.
- Normal metabolizer: Normal functioning enzyme. Decreased risk for side effects. Increased chance of benefit from drug.
- Ultrarapid metabolizer: Rapidly functioning enzyme. Drug is processed and removed more quickly than normal reducing benefit from the drug. May benefit from increased dose of drug.

An important consideration for predicting drug response is that both genetic and nongenetic factors influence drug metabolism. A common source for altered or unexpected drug metabolism is concomitant therapies, which can result in drug interactions and competition within metabolic pathways. Tamoxifen provides an interesting example. Response to tamoxifen, an estrogen modulator used as an adjuvant treatment for women with estrogen receptor-positive breast cancer, is variable. Some women receive no benefit. One reason is genetic. The cytochrome p450 enzyme *CYP2D6* is the principal enzyme responsible for metabolizing tamoxifen. Genetic variants in *CYP2D6* that result in poor metabolism are found in 7–10 % of Caucasians. Women with these genetic variants are not able to metabolize tamoxifen to its active form and therefore receive no benefit from the drug. But genetics is not the full story. Approximately 30 % of women who take tamoxifen also take antidepressants to alleviate depression, anxiety, or hot flashes, which are a common side effect of tamoxifen therapy. Some antidepressants are strong inhibitors of *CYP2D6*,

preventing tamoxifen metabolism to its active form. Thus, even women with normal metabolizer genetics may receive no benefit from tamoxifen therapy if they are also taking an antidepressant medication [19–21].

Multiple pharmacogenetic studies are currently ongoing, and several pharmacogenetic consortiums have been formed to share data and to coordinate and focus research efforts. The Pharmacogenetics Research Network (PharmGKB) ([www.pharmgkb.org/](http://www.pharmgkb.org/)) is a good resource for remaining current in the field. PharmGKB is funded by the National Institutes of Health (NIH) and is charged with curating data regarding the effects of genetic variation on drug response. The database is searchable by disease and/or gene.

### 12.2.2.1 Biologics

Biologics are protein therapeutics that are typically administered parenterally. Several biologic agents are used in dermatology practice, primarily for the treatment of psoriasis (see Table 12.2). In general, these agents suppress immune function by targeting specific molecules important for immune stimulation [22, 23]. This rapidly growing class of drugs includes potent immunomodulators with several potential side effects, most notably infusion reactions and an increased risk for infections, often of the upper respiratory tract. Patients are also at increased risk of *Mycobacterium tuberculosis* (TB) infection, necessitating screening and monitoring testing.

Pharmacogenetic investigations involving these agents are ongoing and are primarily focused on identifying markers that identify responders/nonresponders. Although the data are still preliminary, some general themes are discussed here. Monoclonal antibodies are a common therapeutic approach for biologic agents (Table 12.3). The antigen-binding region (Fab') of the antibody (typically IgG) binds to a specific protein target such as tumor necrosis factor alpha (TNF- $\alpha$ ). Depending on the specific agent, both the soluble and membrane-bound forms of TNF- $\alpha$  may be recognized by the antibody. Binding of an antibody to the soluble form prevents it from binding to its receptors, blocking its biologic activities including cytokine production and

**Table 12.3** Characteristics of biologic agents

Biologic name	Trade name	Biologic type	Target
Infliximab	Remicade	Monoclonal antibody (mouse-human chimeric)	TNF- $\alpha$
Etanercept	Enbrel	Human fusion protein: TNF- $\alpha$ receptor 2 (TNF-R2) fused to IgG	TNF- $\alpha$
Adalimumab	Humira	Monoclonal antibody (human)	TNF- $\alpha$
Golimumab	Simponi	Monoclonal antibody (human)	TNF- $\alpha$
Certolizumab	Cimzia	PEGylated Fab' fragment of a humanized monoclonal antibody	TNF- $\alpha$
Alefacept	Amevive	Human fusion protein: LFA-3 fused to Fc portion of IgG1	CD2, T cells
Abatacept	Orencia	Human fusion protein: CTLA-4 fused to Fc region of IgG1	Blocks T-cell activation
Ustekinumab	Stelara	Monoclonal antibody (human)	Interleukins 12 and 23
Briakinumab	ABT-874	Monoclonal antibody (human)	Interleukins 12 and 23
Secukinumab	AIN457	Monoclonal antibody (human)	Interleukin 17A
Ixekizumab	LY2439821	Monoclonal antibody (human)	Interleukin 17A

inflammation. Binding of an antibody to membrane-bound TNF- $\alpha$  prevents it from interacting with TNF receptors. In addition to the physical inhibition, antibody binding to membrane-bound TNF- $\alpha$  may initiate suppression signals in the cell, decreasing cytokine production. Finally, antibody binding to membrane-bound TNF- $\alpha$  may target TNF- $\alpha$ -producing cells for destruction via either complement-dependent cytotoxicity (CDC) or antibody-dependent cell cytotoxicity (ADCC). ADCC is mediated by the constant region (Fc) of the antibody molecule, which is recognized by Fc receptors expressed on specialized immune cells including macrophages, mast cells, natural killer cells, follicular dendritic cells, neutrophils, and B lymphocytes. Agents that are composed of the Fab' region of antibody, without the Fc portion, are unlikely to exert effects through this mechanism.

Resistance to one biologic agent does not necessarily indicate resistance to others. However, a trial-and-error approach is not in the best interest of the patient or clinician. Continually switching therapeutic agents is inconvenient and wasteful. Pharmacogenetic studies hold the promise of being able to choose a therapy to which the individual patient is most likely to have the greatest response.

Variations in response to monoclonal antibodies that target TNF- $\alpha$  may be due to polymorphisms in TNF- $\alpha$  itself, its receptors, TNF receptor I (*TNFRSF1A*), and TNF receptor II (*TNFRSF1B*)

or in the Fc $\gamma$  receptor (Fc $\gamma$ R) [24–27]. Additional studies have evaluated polymorphisms in *IL23R*, *IL6*, and *HLA-Cw6* [27, 28]. On a side note, pharmacogenetic studies of methotrexate, also a psoriasis therapy, have identified candidate polymorphisms in several genes, including *TYMS* and *ABCC1* and *ABCG2*, which encode efflux transporters of methotrexate [29]. Although the initial studies are promising, larger studies are needed before any of these genetic markers can be implemented into clinical practice.

### 12.2.2.2 Melanoma Therapy

Dacarbazine and temozolomide (TMZ) are standard therapies for metastatic melanoma despite their limited clinical efficacy. These drugs are alkylating agents that primarily exert their effects via DNA damage that can lead to cell death. The DNA damage induced by these agents can be repaired by the enzyme O(6)-methylguanine DNA methyltransferase (MGMT). Several studies have investigated whether polymorphisms in the *MGMT* gene and/or expression levels of MGMT protein determine either response or toxicity in patients treated with TMZ or dacarbazine. The effect of drug-metabolizing variants on response to cancer treatments is often complex and can be somewhat paradoxical. MGMT provides a good example. High germline expression and/or activity of MGMT is likely to be associated with decreased toxicity such as myelosuppression, but

it is also likely to result in decreased responsiveness to therapy. Conversely, low expression and/or activity of the enzyme is likely to be associated with increased response to the therapy, but at the risk of increased toxicity. It is scientifically interesting, but on a practical level, very frustrating, that individuals who tolerate a therapy may receive little benefit, while individuals who cannot tolerate the therapy due to toxicity are exactly the population that would derive the greatest therapeutic benefit.

Germline *MGMT* expression, as assessed in peripheral blood mononuclear cells, appears to correlate with TMZ toxicity. One study of 93 melanoma patients who received TMZ demonstrated that leukopenia and thrombocytopenia were more prevalent among patients with low expression of *MGMT* [30]. Additional studies are needed to fully determine if germline *MGMT* expression can identify patients at greatest risk of toxicity and whether this information can be translated into improved patient management.

In addition to germline *MGMT* assessment, studies have investigated whether expression levels and/or methylation of the *MGMT* promoter in tumors predict response to TMZ. In glioblastoma, promoter methylation of *MGMT* correlates with survival of patients treated with TMZ. For melanoma, data are limited and controversial [31, 32]. Additional studies are required to fully understand the significance of *MGMT* promoter methylation for melanoma patients for whom alkylating agents are being considered.

*MGMT* provides a good example of the complexities associated with personalized medicine in the oncology setting. Similar to studies of other genetic variants, data suggest that both germline variations and somatic (tumor) alterations can affect the overall efficacy of a therapy [33]. Germline variants may more accurately identify individuals at risk of toxicity, while somatic alterations within tumors may more accurately identify individuals most likely to receive benefit from a therapy. Research in this area will hopefully lead to improved patient outcomes by reducing the use of therapies that are likely to be ineffective or to cause patient harm.

### 12.2.2.3 Immune-Mediated Adverse Drug Reactions

Hypersensitivity drug reactions are mediated by an inappropriate immune response. Most drugs are capable of causing a hypersensitivity reaction, although most are mild. Skin rashes, for example, can occur relatively frequently in patients receiving a variety of medications. The frequency, severity, and clinical manifestations of hypersensitivity reactions vary according to the drug, the disease being treated, and an individual's genetics. Drug-induced hypersensitivity reactions range from mild rashes to severe and life-threatening effects. Drug-induced hypersensitivity syndrome (DIHS), also called drug rash with eosinophilia and systemic symptoms (DRESS), is a severe drug hypersensitivity reaction characterized by fever, rash, and multi-organ failure. The drugs most frequently attributed to this syndrome include antiepileptics, antimicrobials including sulfonamides, and antiviral agents such as abacavir and nevirapine, as well as others. The aim of pharmacogenetic studies is to identify individuals likely to have a drug-induced hypersensitivity reaction so that alternate therapies can be considered.

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are two of the most severe adverse reactions caused by drugs. These syndromes are characterized by high fever, malaise, and blistering. SJS is associated with a mortality rate of 5–10 %. The more severe syndrome, TEN, has more extensive skin involvement and a mortality rate of approximately 30 %. It is debatable whether SJS and TEN are truly different entities or whether they represent different severities along a spectrum of the same syndrome. These hypersensitivity reactions are most frequently caused by carbamazepine (Tegretol), a commonly prescribed treatment for epilepsy, trigeminal neuralgia, and bipolar disorder. Approximately 5 % of the Asian population and 2–5 % of the Caucasian population are at increased risk for carbamazepine-induced SJS and TEN. The HLA-B\*1502 allele has been strongly linked to carbamazepine-induced SJS-TEN. HLA-B\*1502 is more common in Asians of Han Chinese descent and less prevalent (<2 %)

in populations of European descent, likely explaining, at least in part, the lower incidence of carbamazepine-induced SJS-TEN in populations of European descent. Studies have shown that testing for HLA variants and using alternate therapies for HLA-B\*1502-positive individuals is strongly associated with a decrease in the incidence of carbamazepine-induced SJS-TEN [34]. These results have led to a warning label on the drug indicating the need for genotyping for HLA-B\*1502 prior initiating treatment: “Patients testing positive for the allele should not be treated with Tegretol unless the benefit clearly outweighs the risk” [35]. This is a pharmacogenetic success story that acts as a model for identifying, validating, and implementing genetic testing to decrease the risk of ADEs. Recently, another HLA allele, HLA-A\*3101, was implicated in carbamazepine-induced hypersensitivity reactions in Northern European populations [36].

A number of HLA alleles have been identified as risk factors for drug hypersensitivity reactions. Perhaps the best studied is the association of the HLA-B\*5701 allele with severe hypersensitivity to abacavir, a nucleoside analog used to treat human immunodeficiency virus (HIV). This medication has also been relabeled with a warning that genetic testing is indicated to prevent adverse reactions. The association of HLA alleles with immune-mediated reactions should not be surprising given that the function of class I HLA antigens is to present intracellular peptides to the immune system. If the immune system determines that the presented peptide is abnormal or foreign, the peptide-presenting cell is targeted for destruction. Why these particular HLA alleles are associated with specific drug-induced immune-mediated adverse reactions is not entirely clear.

#### 12.2.2.4 Challenges

Pharmacogenetic studies are very challenging. One of the problems is evaluating a large enough number of individuals with every possible genotypic variation. Consider a monoclonal antibody therapy targeting TNF- $\alpha$  (of which there are at least four already in clinical use). Assume that there are two relevant polymorphic loci in both TNF- $\alpha$  (*TNFA*) and TNF receptor I (*TNFRSF1A*).

For each genetic variant locus, there are three possible genotypes (AA, BB, and AB). In this scenario there are two gene players, with two polymorphic sites for which there are three possible genotypes. Now take into account that humans are diploid and have two gene copies of each genetic variant. There are thousands of possible combinations, which precludes the inclusion of individuals with every possible genotype in any study design. In addition to genetic variation, study participants must be controlled for other biases such as age, health, concomitant diseases and medications, etc. Although challenging, pharmacogenetic studies are essential to ensure the safety and efficacy of the many pharmaceutical agents used currently and in the future.

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### 12.3 Summary

Molecular diagnostics has already impacted many areas of dermatologic testing, including testing for genetic disorders with dermatologic presentations, testing related to the diagnosis, prognosis, and therapy decision making for oncologic diseases such as melanoma and cutaneous lymphomas, and infectious disease testing. Molecular tests in these areas will continue to evolve and improve. The greatest growth potential for molecular diagnostics relates to the goal of personalized medicine.

In testing related to oncology, decades of research and experience have led to morphologic, histologic, and protein expression (primarily by immunohistochemistry) criteria that, in most cases, function well for diagnostic and prognostic purposes. Molecular tests have the potential to augment this testing by identifying somatic alterations in tumors that predict response to drugs (theranostics) as well as germline alterations that dictate drug metabolism (pharmacogenetics). Interestingly, it is likely that both types of tests may be required to accurately predict response to therapeutic agents.

Outside of oncology applications, pharmacogenetic testing will impact the selection of a wide variety of therapeutic agents used in the practice of dermatology. If successful, pharmacogenetic research will lead to clinical testing that will

decrease severe drug reactions such as SJS-TEM and will improve patient outcomes by allowing therapeutic selection based on the individual rather than statistical data.

Ultimately, molecular testing will likely play a role in the management of essentially all dermatologic patients, whether the purpose is for diagnosis, prognosis, or predicting response to specific therapeutic agents. The rapid advances in technologies and molecular targets create challenges for clinicians, pathologists, and other medical and laboratory specialists. Similar to other professions, gone are the days of learning the facts in school and applying that knowledge over a career of decades. Although a basic understanding of nucleic acids and molecular biology is essential, perhaps equally important is the need to keep pace with the rapid advances in the field of molecular diagnostics. Optimal patient management of the dermatologic patient will increasingly require a team approach including dermatologists and other caregivers, pathologists/dermatopathologists, laboratory specialists, and perhaps disease-specific experts.

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## Appendix 1: Internet Resources

### General Purpose Websites for Molecular Testing and Disease

American College of Medical Genetics (ACMG)

<http://www.acmg.net/>

American Society of Human Genetics

<http://www.ashg.org>

Association for Molecular Pathology (AMP)

<http://www.amp.org/>

Center for Disease Control and Prevention (CDC)

<http://www.cdc.gov/>

The Food and Drug Administration (FDA)

Nucleic acid-based tests

<http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm>

Companion diagnostics

<http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm>

National Comprehensive Cancer Network (NCCN)

<http://www.nccn.org/professionals/default.aspx>

National Institute of Health (NIH)

<http://nih.gov/>

### Clinical Trial Information

ClinicalTrials.gov

<http://clinicaltrials.gov/>

CollabRx

<http://www.collabrx.com/>

### Gene and Protein Databases

COSMIC database

<http://www.sanger.ac.uk/genetics/CGP/cosmic/>

HUGO gene nomenclature committee

<http://www.genenames.org/>

The Human Gene Mutation Database

<http://www.hgmd.cf.ac.uk/ac/index.php>

International HapMap Project

<http://hapmap.ncbi.nlm.nih.gov/>

The International Immunogenetics Information System

<http://www.imgt.org/>

International Society for Gastrointestinal Hereditary Tumours

<http://www.insight-group.org/>

Online Mendelian Inheritance in Man (OMIM)

<http://www.ncbi.nlm.nih.gov/omim>

UniProt (protein database)

<http://www.uniprot.org/>

### Melanoma Websites

Aim at Melanoma

<http://www.aimatmelanoma.org/en/index.html>

The Melanoma Genetics Consortium

<http://www.genomel.org/>

Prognosis determination

<http://www.melanomaprognosis.org/>

<http://www.melanomacalculator.com/>

### Molecular Testing and Select Commercial Laboratory Websites

Association for Molecular Pathology (AMP) Test Directory

<http://www.amptestdirectory.org/index.cfm>  
GENETests  
<http://www.genetests.org/>

**Select specific commercial laboratories and test provider websites**

<http://www.aruplab.com/>  
<http://www.genedx.com/>  
<http://www.invivoscribe.com/>  
<http://www.mayomedicallaboratories.com/>  
<http://preventiongenetics.com/>  
<http://www.promega.com/>  
<http://www.propath.com/>  
<http://www.vanderbilthealth.com/cancer/>

**Clinical Laboratory Regulations and Guidelines Websites**

College of American Pathologists (CAP)  
<http://www.cap.org>

Centers for Medicare and Medicaid Services (CMMS)  
<http://www.cms.gov/>  
[http://www.cms.gov/Regulations-and-Guidance.html](http://www.cms.gov/Regulations-and-Guidance/Regulations-and-Guidance.html)  
Clinical Laboratory Improvement Advisory Committee (CLIA)  
<https://www.cdc.gov/CLIA/default.aspx>  
Clinical Laboratory Standards Institute (CLSI)  
<http://www.clsi.org/>