Cancer Growth and Progression

Domenico Coppola *Editor*

Molecular Pathology and Diagnostics of Cancer

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Domenico Coppola
Editor

Molecular Pathology and Diagnostics of Cancer

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Editor
Domenico Coppola
Anatomic Pathology
Moffitt Cancer Center
University of South Florida
Tampa, FL, USA

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Preface

This volume is a comprehensive update on the most common molecular tests currently available to facilitate the diagnosis of challenging pathologic lesions. This book includes recent advances in the field of diagnostic techniques including, among others, fluorescence in situ hybridization (FISH), microarray, proteomics, mutational analysis, and others. The book is structured by organ sites (brain, thyroid and parathyroid, breast, pulmonary, esophago-gastric, hepatocellular, pancreatic, gastroentero-pancreatic/neuroendocrine, prostate, kidney and bladder, male genital, gynecologic and ovarian, soft tissue, pediatric, cutaneous, and lymphoid and myeloid neoplasms). This state-of-the-science reference will provide the practicing pathologists, oncologists, cancer researchers, postgraduates and students of cancer science with the latest information to help with the diagnosis of difficult lesions. In this era where personalized medicine is becoming a reality, we also provided, for each chapter, information about prognostic molecular tests able to predict patient prognosis and/or therapy response. The broad range of topics will make this text a source of easily accessible information on a variety of tumor types for practicing pathologists whether in academic or clinical practice.

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Chapter 1
Molecular Pathology and Diagnostics of Gliomas

Jesse Kresak and Yuan (Frank) Shan

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J. Kresak
University of South Florida, Tampa, FL, USA

Y. (Frank) Shan, M.D. (✉)
Department of Anatomic Pathology, Moffitt Cancer Center & Research Institute,
12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: Yuan.Shan@moffitt.org

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Abstract Gliomas are the most common primary malignancy in the central nervous system. With recent advancements in molecular pathology, pathologists now have additional tools for histologic analysis of these brain tumors. Genomic and proteomic studies may aid in the diagnosis of gliomas, as well as have utility in prognostication and response to treatment options. Presented are the most current molecular studies utilized in neuropathology of gliomas, as well as a glimpse to future potential biomarkers.

Keywords Gliomas • Genomics • Proteomics • IDH1 • p53 • 1p/19q • EGFR

Abbreviations

2HG	(R)-2-hydroxyglutarate
COBRA	Combined bisulfite restriction analysis
EGFR	Epidermal growth factor receptor
FISH	Fluorescent in situ hybridization
IDH	Isocitrate dehydrogenase
LOH	Loss of heterozygosity
MALDI-TOF	Matrix assisted laser desorption ionization—time of flight
MAPK	Mitogen-activated protein kinase
MGMT	O^6-methylguanine-DNA methyltransferase
miRNA	microRNA
MMP	Matrix metalloproteinase
MS	Mass spectroscopy
MS-MLPA	Methylation-specific multiplex ligation-dependent probe amplification
PTEN	Phosphatase and tensin homolog
RT-PCR	Reverse transcription-polymerase chain reaction
SELDI-TOF	Surface enhanced laser desorption ionization—time of flight
WHO	World Health Organization

1.1 Introduction

Tumors of the central nervous system account for approximately 1 % of tumors in the body. Glial tumors are the most frequent primary tumor of the central nervous system and are classified as astrocytic, oligodendroglial, mixed oligoastrocytic, or ependymal according to the criteria of the World Health Organization (WHO) [1]. Astrocytomas are the most frequent primary brain neoplasm and range in grade from I to IV based on morphology. Oligodendrogliomas and mixed oligoastrocytomas are graded as either II or III and ependymomas have varieties ranging from grades I to III (Table 1.1).

Table 1.1 Glial tumor grades

		I	II	III	IV
Astrocytomas	Pilocytic	X			
	Diffuse		X		
	Anaplastic			X	
	Glioblastoma				X
	Gliosarcoma				X
Oligodendroglial tumors	Oligodendroglioma		X		
	Anaplastic oligodendroglioma			X	
Mixed tumors	Oligoastrocytoma		X		
	Anaplastic oligoastrocytoma			X	
Ependymomas	Ependymoma		X		
	Anaplastic ependymoma			X	

Based on WHO Classification, 2007 [1]

Although pathologists can classify and stratify central nervous system tumors histologically, one cannot predict the clinical outcome of a particular tumor on morphology alone. Tumors within the same histopathologic entity may behave substantially different. For example, a glioblastoma, the most common and aggressive glial tumor, can be subclassified as primary or secondary, although they are histologically indistinguishable. Primary glioblastomas arise de novo, whereas secondary arise from a pre-existing lesser grade glioma. These two subtypes should be considered as two distinct entities having unique pathogenetic pathways with different behaviors and treatment targets.

Focus has shifted to the molecular level in order to further subclassify glial tumors. Molecular alterations leading to gliogenesis can be genetic or epigenetic, resulting in altered expression of genes and their protein products. Knowledge of the molecular changes in the various types and malignancy grades of gliomas has increased dramatically over the past decade. The goal of such research is to identify biomarkers with clinical utility in establishing diagnosis, predicting prognosis, guiding treatment choices, as well as providing new cellular therapeutic targets.

1.2 Genetic Profiling

1.2.1 IDH1/IDH2

Isocitrate dehydrogenases, IDH1 and IDH2, are homologous, NADP+−dependent cytoplasmic and mitochondrial enzymes, respectively. The role of these enzymes is the conversion of isocitrate to α-ketoglutarate with the simultaneous reduction of NADP+to NADPH. IDH1 has recently been discovered to be mutated in a vast majority of astrocytic, oligodendroglial, and oligoastrocytic gliomas (WHO grades II–III), as well as in secondary glioblastomas (WHO grade IV). IDH1 mutation is very rare in primary glioblastoma and is not involved in pilocytic astrocytomas.

The most common mutation is a heterozygous point mutation with substitution of arginine by histidine at codon 132 (R132H), located in the substrate binding site. This IDH1-R132 mutation has a reported frequency of 50–93 % [2, 3]. IDH2 gene mutations affecting the amino acid R172 are much less common than the IDH1 isoform, 3–5 %, yet have been identified in a small subset of gliomas that lack the typical IDH1 mutation with a predominance in oligodendrogliomas [3]. IDH1 and IDH2 genes appear to behave dominantly and are mutually exclusive.

The carcinogenic effect caused by the mutations of IDH1 and IDH2 is not fully understood but appears to be multifactorial. The product and byproduct of the reaction, α-ketoglutarate and NADPH, both defend against cellular oxidative stress. Thus, with decreased quantities of these compounds, the cell may be more susceptible to oxidative damage. In addition to the tumorogenetic property conferred by the inability to perform the conversion, it appears that the IDH mutation confers an enzymatic gain of function. With the IDH mutations, the cancer cell has the gained ability to convert α-ketoglutarate into (R)-2-hydroxyglutarate (2HG) [4]. This reaction will not only further decrease α-ketoglutarate stores, but will also reduce NADPH to NADP+, further increasing the cell's susceptibility to oxidative stress. The increased amount of 2HG in the brain has been associated with an increased risk of brain tumors in patients with an inborn error of 2HG metabolism [5]. Furthermore, there is an association between the IDH mutation and increased hypoxia-inducible factor-1α [6]. Hypoxia-inducible factor-1α is a transcription factor associated with carcinogenic processes, such as the upregulation of vascular endothelial growth factor, and thus promotes angiogenesis.

Mutations in IDH1 and IDH2 have also been observed in up to 22 % of acute myelogenous leukemias, which concurrently display a dramatic increase in 2HG, supporting the neoenzymatic activity [7].

IDH1 mutation has been shown to be a strong, independent prognostic biomarker not only in glioblastomas, but in diffuse gliomas of lesser grades as well [8]. There is no difference yet to be seen in terms of the point mutation, R132H versus others, regarding patient outcome [9]. While the IDH1 mutation conveys a better patient outcome, it does not predict a better response of the glioma to chemotherapy [8, 10]. In addition to its prognostic value, identification of IDH mutations could be used diagnostically. Analysis of IDH1/2 mutations could be utilized in the separation of primary and secondary glioblastomas or in the challenge of differentiating pilocytic astrocytoma from glioblastoma.

Recently, immunohistochemical staining using a specific antibody against mutant IDH1-R132H was developed, which can be applied to routine paraffin-embedded specimens. This has proved to be a tumor-specific marker differentiating reactive from neoplastic cells in grade II and III gliomas [11, 12]. In addition, this marker also identifies tumor cells in post-therapy specimens with extensive reactive gliosis. While this IHC stain is easy to use and convenient, it will neither detect the IDH mutations of other types, nor the IDH2 mutations of equal importance [11, 13]. Detection of IDH1/2 mutations can also be achieved through various polymerase chain reaction (PCR) techniques and direct sequencing.

1.2.2 MGMT Status

The MGMT (O^6-methylguanine-DNA methyltransferase) gene at 10q26 encodes for a DNA repair protein. In gliomas of different grades, the MGMT gene is silenced by promoter hypermethylation, impeding transcription, and thus resulting in a decreased expression of the MGMT protein. This epigenetic modification has been associated with an increased sensitivity to alkylating chemotherapy. In alkylating therapies such as temozolamide, a methyl group is added to the O^6-position of the nucleotide guanine, resulting in DNA damage and apoptosis [14]. A full-functioning MGMT would remove this methyl group; however, with reduced expression of the protein secondary to promoter hypermethylation, the cell has a decreased ability to repair alkylated DNA. Therefore, MGMT expression analysis can be used to predict which tumors may have a more favorable response to alkylating chemotherapeutic agents [15, 16]. Testing of MGMT can be applied to pediatric gliomas as well [17, 18]. MGMT promoter methylation has been found in up to 40 % of primary glioblastomas and 40–60 % of secondary glioblastomas. The aberration is also present in other diffuse gliomas, with preponderance of oligodendrogliomas at 60–93 % [19].

Although studies have shown that MGMT promoter methylation results in a significantly longer survival time for patients with glioblastoma treated with concomitant treatment of temozolomide and radiotherapy, there have been discordant reports regarding MGMT methylation as a predictor for increased survival in patients receiving radiotherapy alone [15, 20, 21]. However, in gliomas of lesser grades, there is a clear prognostic association between MGMT methylation status and sole radiotherapy [10, 22]. The underlying mechanism by which MGMT methylation would offer a favorable prognosis when not in relationship to chemotherapy is a bit more difficult to discern. As mentioned previously, gliomas often contain multiple molecular aberrancies and thus it may be the result of another molecular change, or the summation of several changes, that convey this prognostic significance to radiotherapy.

The most common method utilized to assess the MGMT promoter methylation status is a methylation-specific PCR analysis, which applies primers composed of differing quantities of CpG sites to allow differentiation between methylated and unmethylated DNA [23]. Methylation-specific pyrosequencing has also been employed with strong sensitivity [24]. Other DNA-based methods are available such as combined bisulfite restriction analysis (COBRA) and methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). Nonmethylation-specific methods have been tested such as immunohistochemical staining, Western blotting, and biochemical enzyme assays; however, they were found to be less reliable due to staining of non-neoplastic cells, which retain MGMT expression and thus give a false result [23].

1.2.3 p53

As far as current genomic data can reveal, p53 is the most frequent genetic alteration in cancer. p53 is a tumor suppressor protein encoded by the TP53 gene located on the short arm of chromosome 17. The innate role of p53 is genomic stability

achieved by cell cycle inhibition and induction of apoptosis. In activated states, p53 acts a transcription regulator leading to the upregulation of p21. The protein p21 is the stop protein responsible for binding to the cyclin-dependent kinase and inhibiting the cell from transitioning from the G1 to the S phase of cell division (see Entrez Gene). Thus, a mutated p53 will be unable to prevent cell replication resulting in uncontrolled tumor growth.

p53 is strongly associated with secondary glioblastomas and found significantly less frequently in primary glioblastomas [1].

The majority of mutations involving p53 lead to missense mutations, and there is a resultant accumulation of the protein in the nucleus of the cell. Therefore, immunohistochemical stains for p53 highlight the nuclei of tumor cells and are used as a surrogate marker for identifying cells affected by a mutation in this pathway. This methodology is more economical than PCR analysis; however, interpretation of the stain is not standardized, leading to ambiguity of interpretation [25].

The significance of the detection of p53 overexpression in gliomas is inconsistent. In terms of diagnosis, p53 would be a less favorable marker than others in distinguishing primary from secondary glioblastoma given that p53 overexpression has been reported in up to 25 % of primary glioblastomas [26]. As a prognostic marker, p53 has shown inconsistent results. While some reports indicate a shorter survival time for gliomas overexpressing p53, this finding has not been confirmed by several meta-analyses [25, 27].

1.2.4 1p/19q Deletion

The loss of chromosome arms 1p and 19q is an established genetic hallmark of oligodendroglial tumors [28]. This combined loss is detected in up to 80 % of oligodendrogliomas and up to 60 % in anaplastic oligodendrogliomas, with decreasing frequency in mixed oligoastrocytic tumors [28, 29].

The 1p/19q deletion has proven its use as a prognostic marker conveying a better response to therapy and a longer survival time [22, 29]. This co-deletion carries the same significance regardless of using chemotherapy, radiotherapy, or the combined and therefore cannot be used to guide treatment choice [22, 29]. Although there is a strong correlation between classic oligodendroglioma morphology (“fried eggs” and “chicken wire”) and the co-deletion, 1p/19q deletion cannot always be predicted by morphology alone [30].

It should be noted that deletions of the 1p arm other than the 1p/19q co-deletion have different prognostic implications. For example, a partial loss of 1p in an oligodendroglioma has been suggested to be a less favorable clinical outcome [31]. Therefore, the entire chromosomal arm should be evaluated to detect deletions of differing sizes.

Various techniques are available to detect 1p/19q deletions; however, fluorescent in situ hybridization (FISH) is often employed due to its technical ease. FISH can be used directly on formalin-fixed paraffin-embedded tissue and does not

require additional specimens from the patient. Another frequently utilized method is loss of heterozygosity (LOH), which is a PCR-based test that compares tumor DNA to the patient's "normal" DNA, usually from peripheral blood.

1.2.5 BRAF Duplication/Fusion

BRAF, which is part of the mitogen-activated protein kinase (MAPK) pathway and located at 7q34, is aberrantly activated in a majority of pilocytic astrocytomas. BRAF is most commonly activated by duplication of the gene with subsequent fusion of the BRAF genes. BRAF aberrancies exist in 60–80 % of pilocytic astrocytomas and are rarely seen in other gliomas [32]. This discrepancy can lend utility in the differentiation of pilocytic astrocytomas and low-grade gliomas [33]. A missense mutation of BRAF, V600E, has also been identified in a minority of extracerebellar pilocytic astrocytomas; however, this mutation is more frequently seen in gangliogliomas and pleomorphic xanthoastrocytomas.

Aside from diagnostic utility, BRAF analysis may prove useful in development of therapeutic options in patients with pilocytic astrocytoma. Studies have demonstrated that silencing and inhibition of BRAF or other constituents of the MAPK pathway inhibit growth of the tumor [34].

Detection of BRAF aberrancies is often accomplished through FISH analysis with probes specific to the KIAA1549 and BRAF genes. PCR has also been utilized; however, this method may not allow for detection of variants of this fusion. Immunohistochemistry is available for detection of the BRAF V600E mutation.

1.2.6 MSH6 Mutation

Unfortunately, high-grade gliomas all too often do not respond to alkylating chemotherapeutic treatments. In these treatment-resistant gliomas, MSH6, a mismatch repair gene, has often been found to be mutated. Studies point to the alkylating chemotherapeutic agents as a cause to the somatic mutations in the MSH6 gene [35, 36]. More research into the role of MSH6 and other mismatch repair genes may be helpful in identifying therapy-resistant tumors and provide for a specific target for further treatment.

1.3 Proteomics

Genetic profiling and proteomics fall along on the same spectrum of diagnostic armory; however, they differ in detection methodologies and in clinical utility. In some cases, proteomic data will validate the established genetic alterations, while in others, proteins have been identified with little knowledge of their originating genes.

There is a wide spectrum of proteomic methodologies, ranging from a simple Western blot to 2-dimension gel electrophoresis to advanced mass spectroscopy (MS) techniques. Various MS methods have been employed, including matrix assisted laser desorption ionization—time of flight (MALDI-TOF) MS, surface enhanced laser desorption ionization—time of flight (SELDI-TOF) MS, and nanoliquid chromatography with tandem mass spectroscopy, yielding a different set of tumor proteins than provided by standard immunohistochemistry or Western blotting. The differing results may be a reflection of protein molecular weight and/or protein abundance [37, 38].

As the elucidation of genetic alterations in gliomas advances, proteomic studies will continue to reveal altered proteins. Future research endeavors into the protein-protein interactions and network-analysis may prove useful in solidifying our understanding of proteomic study results.

1.3.1 EGFR

Epidermal growth factor receptor (EGFR) gene, located on chromosome 7p12, codes for EGFR protein, a member of the transmembrane tyrosine kinase receptor family. The EGFR gene is often amplified in primary glioblastomas, up to 40 % [1, 19], resulting in over-expression of the EGFR protein. EGFR plays a role in tumorigenesis by activating MAPK and PI3K-Akt pathways, leading to cell proliferation, decreased apoptosis [39], and angiogenesis and ease of invasion [40].

Aside from overexpression of wild-type EGFR, primary glioblastomas may also harbor mutated EGFR. The most frequent rearrangement leads to a variant of the EGFR gene known as EGFRvIII (delta EGFR) [40]. This mutation is characterized by an 801 base pair (267 amino acid) in-frame deletion of exons 2–7, resulting in a truncated extracellular domain with the inability to bind a ligand. However, the receptor retains ligand-independent constitutive activity and produces tonic activation of the pathway [41]. The EGFRvIII mutation is seen in half of the glioblastoma multiforme cases exhibiting EGFR amplification, yet can also be seen as a stand-alone mutation as well [40].

The presence of EGFR amplification or EGFRvIII mutation conveys high-grade malignancy and thus may have utility as a diagnostic and prognostic tool. As such, in cases of anaplastic gliomas with equivocal histology, the detection of EGFR/EGFRvIII lends support to a higher-grade malignancy with an associated poorer patient outcome. This prognostication correlates with the finding that EGFR mutations are more frequent in primary versus secondary glioblastomas, of which primary glioblastomas are associated with later onset and aggressive clinical behavior.

Therapeutically, the EGFR pathway has provided a new target for treatment with variable success. Glioblastomas with EGFR or EGFRvIII expression have been more responsive to tyrosine kinase inhibitors most prominently when PTEN is also expressed [41].

Detection of EGFR amplification is most frequently accomplished via FISH analysis. EGFRvIII analysis is usually done via reverse transcription-PCR (RT-PCR).

Table 1.2 Diagnostic molecular panels

	IDH1/2	**p53**	**EGFR**	**PTEN**
Primary glioblastoma vs.	–	–	+	+
Secondary glioblastoma	+	+	–	–
	IDH1/2	**BRAF**		
Pilocytic astrocytoma vs.	–	+		
Higher grade astrocytoma (except 1′ glioblastoma)	+	–		
	IDH1/2	**p53**	**EGFR**	
Astrocytoma vs.	+	+	Strong	
Reactive gliosis	–	+/–	Weak	

1.3.2 PTEN Alterations and 10q LOH

Phosphatase and tensin homolog (PTEN), located at 10q23, is a tumor suppressor gene with an integral role in opposing the PI3K-Akt pathway. In gliomas with a mutant PTEN gene, there is an associated increase in PI3K-Akt pathway signaling, which may contribute to the tumor's ability to invade and infiltrate. There have also been reports to support the theory that mutations in PTEN lead to activation of EGFR via a PI3K-Akt pathway demonstrating that PTEN plays a role in the angiogenesis of glioblastomas as well [42]. Mutations at the PTEN gene are found in 15–40 % of primary glioblastomas, but they are practically absent in secondary glioblastomas and other gliomas [43].

PTEN is often altered by a LOH at 10q. The LOH can occur in various sites aside from 10q23 (PTEN) such as 10q25, or 10q total loss.

PTEN mutations and 10q LOH both carry the same negative prognostication for high-grade gliomas [19]. LOH analysis or FISH can be used for evaluation.

1.3.3 Prohibitin

Prohibitin is a ubiquitous protein located at 17q21, the same location as the BRCA1 gene. Prohibitin has been found to be overexpressed in various tumor tissues, including breast, bladder, prostate, and thyroid. However, it should be noted that in gliomas there are reports of upregulation as well as downregulation of prohibitin [37, 38]. Although the exact mechanism that prohibitin may play in tumorigenesis remains unclear, given its mitochondrial location and chaperon responsibility, it is believed that the protein may have a regulatory role by modulating transcription [44]. Much more research into the mechanisms and prognostic and therapeutic values of this protein marker should be performed before detection of it is commonly employed in practice.

In summary, the knowledge gained thus far in the study of the genomics and proteomics of gliomas has allowed for both diagnostic, as well as prognostic tools when analyzing glial tissue (Tables 1.2 and 1.3). It is important to note that this is an emerging field and thus use of these evaluation methods should be used in clinical context and in reference to the rapid influx in the literature.

Table 1.3 Molecular alterations as prognostic markers

	Positive			Negative	
	IDH1/2	MGMT	1p19q	EGFR	PTEN
Primary glioblastoma		+		+	+
Secondary glioblastoma	+	+		Rare	
Anaplastic astrocytoma	+	+			
Diffuse astrocytoma	+	+			
Pilocytic astrocytoma		+			
Oligodendroglioma		++	++		
Anaplastic oligodendroglioma			+		
Mixed oligoastrocytoma			+/–		

1.3.4 Comprehensive Diagnostics

Gliomas are tumors characterized by multiple genetic alterations (see Fig. 1.1). Evidence is emerging to support an association between compounded molecular and genetic alterations found in gliomas. There is a strong correlation between the presence of IDH1 mutations and genetic mutations such as p53 and 1p/19q deletions. Recent evidence supports the idea that the IDH mutation is an early event occurring in a glial precursor cell and may lead to, or be prerequisite for, the loss of 1p/19q [10, 45, 46]. The presence of IDH1 mutation is also closely associated with p53 mutations and MGMT promoter methylation in gliomas [10]. Interestingly, there is an inverse correlation between IDH1 mutation and the amplification of EGFR. With IDH1 mutations being prevalent in secondary glioblastomas and EGFR amplification being predominant in primary glioblastomas, this inverse association lends support to the theory of primary and secondary glioblastomas being distinct entities [10]. Similarly, pediatric high-grade gliomas rarely exhibit an IDH mutation conveying a fundamental difference from the adult counterpart [47].

1.4 miRNA

The role of microRNAs (miRNAs) in tumorogenesis is a relatively new and exciting field of research. miRNAs are noncoding RNA segments that can bind mRNA to affect translation of genes. In this manner, miRNAs can regulate oncogenes and tumor suppressor genes and thus are associated with tumor growth, including cellular proliferation, angiogenesis, invasion, and apoptosis [48, 49]. However, it was only in 2005 that the first aberrant miRNA expression was report in gliomas [50, 51].

Utilizing Northern blots and microarrays, the miRNAs that were found by these studies to be upregulated in glioblastoma cell lines and tumors included miR-21 [51] and miR-221; those found to be downregulated included miR-128 and miR-181a, miR-181b, and miR-181c [50]. With the use of quantitative RT-PCR, Silber

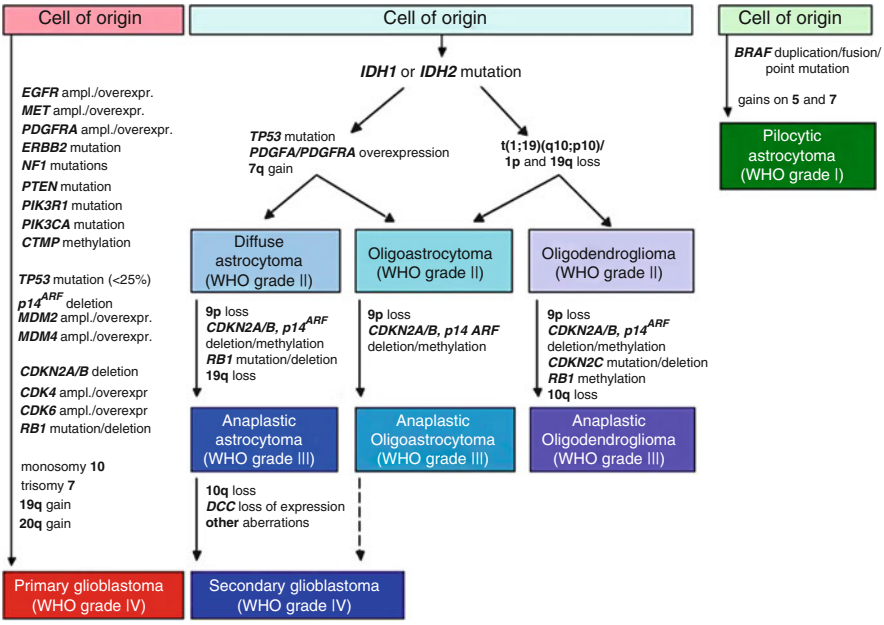

Fig. 1.1 Summary of most frequent molecular alterations in astrocytic, oligodendroglial, and oligoastrocytic gliomas. Primary glioblastomas, the most common gliomas in adults, show complex chromosomal, genetic, and epigenetic alterations targeting genes involved in important cellular pathways, namely the receptor tyrosin kinase/mitogen-activated protein kinase/phosphoinositol 3-kinase pathway (e.g. *EGFR, MET, PDGFRA, ERBB2, NF1, PTEN, PIK3R1, PI3KCA, CTMP*), the p53 pathway (e.g. TP53, *p14^{ARF}, MDM2, MDM4*), and the pRb1 pathway (e.g. *CDKN2A, CDKN2B, CDK4, CDK6, RBI*). In addition, primary glioblastomas frequently show monosomy 10, trisomy 7 and gains of 19q and 20p. Diffuse WHO grade II and III astrocytic, oligodendroglial and oligoastrocytic gliomas and secondary glioblastomas frequently carry mutations in *IDH1* or *IDH2*, suggesting that they share a common, yet to be defined cell of origin. Diffuse astrocytic gliomas often carry additional *TP53* mutations, while oligodendroglial tumors are characterized by 1p/19q deletion. Most oligoastrocytomas have either of these alterations. Molecular changes associated with progression to anaplastic glioma include 9p losses and inactivation of the *CDKN2A*, *P14*^{ARF} and *CDKN2B* genes on 9p21 as well as other changes, while progression to secondary glioblastoma is associated with frequent loss of 10q and *DCC* loss of expression amony others. The majority of pilocytic astrocytomas are characterized by duplication/fusion or point mutation of the *BRAF* gene on 7q34, while other genomic aberrations are rare. From Riemenschneider et al. [16], with kind permission of Springer Science+Business Media

was able to confirm the upregulation of miR21 and to identify the downregulation of miR-124, miR-128a, and miR-137 [52].

The deregulation of miRNAs, much like the ones mentioned above in gliomas, has been implicated in oncogenic pathways of other tumor types. For example, it has been suggested that, in cases of colorectal adenocarcinoma, miR-143 directly binds to KRAS transcripts at the 3′-UTR, thus potentially inhibiting KRAS protein expression [53]. In regard to gliomas, it has been shown that the dual activation of RAS and AKT in progenitor cells can induce the formation of glioblastomas [54].

Given this result and the knowledge that malignant gliomas commonly contain deletions or mutations of the tumor suppressor genes PTEN and p53, which are alterations that may lead to the activation of RAS-MAPK and PI3-AKT pathways, it raises the question to a likely correlation between miRNA deregulation and these oncogenic pathways in the development of central nervous system tumors [55].

Identification of dysregulated miRNAs in tumor tissue may provide a new therapeutic target as it may be possible to modulate miRNA through pre-miRNA molecules or anti-sense oligonucleotides.

1.4.1 miR-21

miR-21 was first noted to be upregulated in glioblastomas and its overexpression has since been documented in numerous solid tumors [48, 51]. Low-grade gliomas have also been found to overly express miR-21. Numerous studies have been conducted to elucidate the significance of increased miR-21 with findings to support the role of miR-21 in multiple oncogenic pathways.

In order to determine the significance of the overexpression, knockdown studies were performed in which glioblastoma cells without miR-21 expression resulted in apoptosis. This would suggest that miR-21 plays an anti-apoptotic role in gliomagenesis [51].

miR-21 indirectly affects the tumor-suppressing p53 pathway by targeting activators, homologs, and cofactors of the system [56]. Chemotherapeutic agents often induce DNA damage in tumor cells leading to apoptosis of the cell. In this way, miR-21 can compromise the p53-mediated apoptosis contributing to drug resistance of glioblastomas. miR-21 also plays a role in the inactivation of TGF-beta and mitochondrial apoptotic pathways [56].

The invasiveness of gliomas may also be attributed to miR-21. RECK and TIMP3, which act as inhibitors to matrix metalloproteinases (MMPs), are direct targets of miR-21. MMPs are peptidases involved in the breakdown of the extracellular matrix, allowing for a tumor to infiltrate with less resistance. MMPs are significantly elevated in gliomas [48, 57].

Expression of miR-21 is not at this time used for diagnosis or prognostication; however, future endeavors into targeted downregulation of miR-21 in gliomas and other tumors may prove to show a great therapeutic promise [48].

1.4.2 miR-221/222

The overexpression of miR-221 has been found in gliomas, exclusive to the high-grade types. miR-221 is often studied and reported in correlation with miR-222 due to their apparent conjunctive function in the cell. miR-221/222 negatively regulates $p27^{Kip1}$, a protein that acts to trigger cell cycle arrest by inhibition of cyclin-dependent kinases [48, 58]. By this means, overexpression of miR-221/222 leads to low levels of $p27^{Kip1}$ and hence continuous glioblastoma cellular proliferation.

1.4.3 miR-181a and miR-181b

The downregulation of the miR-181 family has been demonstrated in gliomas [50, 59]. Both miR-181a and miR-181b are under expressed; however, it is only miR-181a that showed a gradient decrease of expression negatively correlating with the tumor grade [59]. Although the exact targets of miR-181 in brain tumors has not been established, studies have shown that normal or overexpression of miR-181a and miR-181b lead to growth inhibition, apoptosis, and decreased invasion. Thus, decreased expression of these miRNAs plays a multifactorial role in tumorogenesis and may very well have more than one target.

1.4.4 miR-128

miR-128 is a neuron-specific miRNA that has been found to be downregulated in gliomas, with the most significant decrease of expression in glioblastomas [50]. Two targets of miR-128 have been identified thus far: E2F3a and Bmi-1. E2F3a is a transcription factor responsible for activating genes involved in cellular proliferation. miR-128 negatively regulates this transcription factor; thus decreased expression of miR-128 leads to an unchecked proliferation pathway [48, 60]. Bmi-1 is a factor necessary for the self-renewal of stem cells. Without the inhibition of Bmi-1 by miR-128, glioma cell self-renewal is possible [48, 61]. At the present time, loss of miR-128 could be used as a biomarker for tumorogenesis without significance of therapy or prognosis.

Although it is apparent that miRNAs are differentially expressed in gliomas of varying grades and in comparison to unaffected brain tissue, further investigation into the utilization of miRNA expression signatures as biomarkers for glioma diagnosis, prognosis, and therapeutic targets must still be performed.

1.5 Conclusions

The biology of gliomas is still far from understood and tremendous efforts are still required for further understanding of these complex tumors. The advances in molecular biology of gliomas have resulted in significant progress in the clinical management of patients. For example, a test for co-deletion of chromosomes 1p and 19q in oligodendrogliomas has brought not only a diagnostic tool for pathologists, but treatment options for oncologists, as well as prognostic information for patients. As long as the technique is available, there is potential for genomic tests in the future. From a clinical standpoint, a comprehensive approach using currently available biomarkers may be the best way to decide our patients' diagnosis, therapeutic choice, and prognosis.

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Chapter 2
Molecular Pathology and Diagnostics of Thyroid and Parathyroid Malignancies

Rodney E. Shackelford and Jason Savell

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R.E. Shackelford
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, LA 70112-2699, USA

J. Savell, M.D. (✉)
H. Lee Moffitt Cancer Center and Research Institute and University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: Jason.savell@moffitt.org

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Abstract Malignancies of the thyroid and parathyroid glands are rare oncologic entities that range in clinical behavior from relatively indolent to extremely aggressive malignancies. Presently, establishing a diagnosis and prognosis for thyroid and parathyroid malignancies largely depends on histology supplemented with immunohistochemical analysis. Over the past 20 years, different histologic subtypes of thyroid cancer have been shown to carry specific genetic alterations, which are often preferentially associated with, or unique to, each subtype. In many cases, these genetic alterations have been analyzed via molecular-genetic testing techniques to help establish a diagnosis in cases where histology and immunohistochemistry alone cannot. In addition, such testing has occasionally been used to determine prognosis. Presently, clinical molecular diagnostic testing is not performed on parathyroid tumors. However, differences between parathyroid hyperplasia, adenomas, and carcinomas have been detected via molecular testing. With additional research, these differences may become more fully understood and applied to molecular diagnostics. Thus, although presently not extensively employed, molecular diagnostics of the thyroid and parathyroid are likely to become increasingly important in determining the diagnosis and prognosis of these malignancies, especially for histologically difficult cases. Furthermore, pharmacologic inhibitors of many of the oncogenes mutated in these malignancies are being developed. With time, molecular diagnostic testing for these mutations is likely to be implemented to aid in choosing optimal chemotherapeutic treatment regimens.

Keywords Thyroid • Parathyroid • Malignancies • Tumor papillary • Follicular • Medullary • *BRAF* • *RAS* • *RET* • Molecular diagnostics • *NTRK1* • *PAX8-PPAR* gamma

Abbreviations

ACT	Anaplastic carcinoma of the thyroid
ASA	Allele-specific amplification
ATC	Anaplastic thyroid cancer
FA	Follicular adenomas
FC	Follicular carcinoma
FCT	Follicular carcinoma of the thyroid
FMTC	Familial medullary thyroid cancer
FNA	Fine needle aspirations
HRM	High-resolution melting
HT	Hashimoto's thyroiditis
IR	Ionizing radiation
MEN	Multiple endocrine neoplasia
MTC	Medullary thyroid cancer
PA	Parathyroid adenoma
PC	Parathyroid carcinoma
PCR	Polymerase chair chain reaction
PTC	Papillary thyroid carcinoma
SSCP	Single-strand conformational polymorphism
STAT	Shifted termination assay technology
TC	Thyroid cancers
TSH	Thyroid-stimulating hormone

2.1 Introduction

Cancers of the thyroid and parathyroid are relatively rare oncologic entities for which a straightforward diagnosis, based on histologic grounds and supplemented with immunohistochemistry, is possible in the majority of cases. However, in some cases, distinguishing between benign and malignant neoplasms can be difficult. For example, the follicular variant of papillary thyroid carcinoma (PTC) is easily distinguished from follicular adenomas (FA) or follicular carcinoma (FC) when the typical nuclear features of PTC are distributed diffusely throughout the tumor. However, some PTC cases have only focal and/or poorly developed PTC nuclear features, making the diagnosis of PTC vs. FA and FC quite difficult. Even very experienced thyroid pathologist have had high inter- and intraobserver variability in

the diagnosis of difficult cases [1]. Similarly, the use of histology alone to differentiate between parathyroid carcinoma (PC), parathyroid adenoma (PA), and atypical PAs can be difficult or even impossible unless obvious tumor invasion or metastasis is identified [2–4].

Since both thyroid and parathyroid malignancies can cause considerable morbidity and mortality, making the most accurate diagnosis is vital. Recently, molecular diagnostics has begun to play a role in the diagnosis and management of thyroid cancers (TC), especially for cytologically or histologically ambiguous cases. In addition, molecular testing can be valuable in predicting the probable clinical course, allowing risk stratification and possible changes in treatment plans. Lastly, molecular testing as a means of determining the specific chemotherapeutic agents employed in TC treatment is likely to become part of the management of these malignancies. Thus molecular-genetic testing is playing an increasingly important role in the diagnosis and management of thyroid neoplasia and is likely to eventually play a role in the diagnosis and clinical management of PC as well. Here, we will review current molecular-genetic testing for TC and PC with emphasis on the clinical utility of each test.

2.2 Thyroid Cancer: Epidemiology and Risks Factors

TC accounts for ~1 % of human malignancies and is the most common of the endocrine neoplasms. Interestingly, an increase in differentiated TC has been found in the United States and other developed countries that cannot be attributed solely to increased medical surveillance and increasingly sensitive diagnostic procedures [5–11]. TC is roughly three times more common in women, is very rare in individuals under age 10 years, and increases with age, having an average age of diagnosis of 40–45 years [12]. Some geographic and ethnic variations are seen with TC, with a low incidence seen in countries like Denmark, Holland, and Slovakia, and higher incidences seen in Iceland and the Hawaiian Islands [12, 13]. Population studies of different ethnic groups moving from low-risk to high-risk areas for TC suggest that both environmental and genetic factors contribute to the development of TC [12, 13]. Risk factors for TC cancer are described below.

2.2.1 Thyroid Cancer Risk Factor: Ionizing Radiation Exposure

Ionizing radiation (IR) exposure has long been known to be a risk factor for TC. Studies of the nuclear bombings of Hiroshima and Nagasaki, the aftermath of the Chernobyl nuclear accident, and effects of external radiotherapy on individuals treated for various head and neck malignancies have all demonstrated that IR to the thyroid significantly increases the TC risk. As expected, the risk for TC increases with higher

IR doses, with exposed children and pediatric patients receiving external IR showing the greatest TC increases. Interestingly, children exposed to the Chernobyl fallout most often presented with the solid and follicular variant of PTC that followed an unusually aggressive clinical course [14–21]. The latency period for TC following IR exposure ranges from a minimum of 4–5 years to a maximum at of 20 years, thereafter declining [21, 22]. Experimental evidence suggests that IR-induced *RET/PTC1* gene rearrangements play an early and important role in IR-induced TC development [14].

2.2.2 Thyroid Cancer Risk Factor: Iodine Deficiency

Areas with chronic dietary iodine deficiency show an increased incidence of benign thyroid nodules and TC, with FC and anaplastic TC (ATC) significantly increased. The underlying molecular mechanism of TC carcinogenesis results, in part, from iodine-deficiency causing increased thyroid-stimulating hormone activity, increased thyroid cell thyroid-stimulating hormone sensitivity with concomitant increased epidermal growth factor-induced cell proliferation, and increased angiogenesis, all of which promote carcinogenesis [23, 24]. Interestingly, some molecular and epidemiologic data have suggested that a high-iodine diet promotes PTC cancer by increasing the incidence of the *BRAF T1799A (V600E)* mutation [25].

2.2.3 Thyroid Cancer Risk Factor: Hormonal Factors

TC shows a strong female predominance that is greater in women in the childbearing years than in post-menopausal women. Additionally, pregnancy causes an increase in thyroid volume and an increased incidence of thyroid nodules and goiters. The reasons for the TC gender differences are poorly understood, although estrogen levels can influence cancer cell growth. In TC, estrogen receptor-alpha agonists promote cell proliferation, whereas estrogen receptor-beta agonists or beta-receptor overexpression suppresses cell proliferation. Several studies have demonstrated that alpha-receptor expression is increased in TC, while the beta-receptor expression is either decreased or absent [26, 27].

2.2.4 Thyroid Cancer Risk Factor: Other Factors

Several other factors influence the incidence of TC. Oddly, current smoking reduces TC risk, whereas alcohol and caffeinated beverages have no effect on TC risk. A diet high in vegetables reduces TC risk, and a high body mass index increases risk [28–30]. Lastly, TC is increased in individuals with benign thyroid nodules and goiters and possibly in individuals with HLA-DR7 [31–33].

2.3 Molecular-Genetic Testing for TC

Molecular testing on TC is not commonly performed, although presently two laboratories offer molecular TC testing within the United States. Testing can usually be performed on fine needle aspirates, paraffin-embedded formalin-fixed tissue, and fresh or frozen tissue. TC has multiple histological variants that range in clinical behavior from the usually indolent and often curable PTC, to anaplastic carcinoma (undifferentiated carcinoma), which almost invariably follows a rapid and lethal course, having a median survival rate of less than 6 months [34–37]. Over the past 20 years, molecular analyses of the different TCs have revealed distinctive gene mutations and expression patterns that correlate with the histological TC type. Additionally, within one histological TC type, specific mutations and gene expression patterns often correlate with a more aggressive clinical course. For example, PTCs with the *BRAF V600E* mutation are more likely to recur and exhibit increased aggressiveness by the recurrent tumor [38]. The molecular alterations seen in TC involve a relatively low number of genes, including *BRAF, RAS, p53, PTEN, NTRK1, RET, CTNNB1,* and the *PAX8/PPAR-gamma* translocation. In the sections, we will review the genetic alterations that occur in the major histological variants of TC and the molecular-genetic tests employed in TC diagnosis and clinical management.

2.4 Papillary Thyroid Carcinoma: Mutations and Molecular Testing

PTC is the most common TC, accounting for ~80 % of TCs in developed countries and up to 90 % of IR-induced TCs. PTC is most commonly composed of cuboidal columnar thyroidal epithelium, usually in papillary projections surrounding fibrovascular cores and often with associated follicles and dense fibrosis. The nuclei are overlapping, vesicular, hypochromatic, and often described as having an "Orphan Annie Eye" appearance, and 50 % of PTC will have psammoma bodies. The usual clinical course is indolent, at least in younger patients, with 98 % of individuals surviving 10 years, a survival rate similar to the general population. Interestingly, cervical lymph node involvement does not alter the prognosis, although etrathyroidal and/or extranodal tumor extension and loss of radioiodine avidity predict a poorer survival. Additionally, local lymph node metastasis is common, whereas hematogenous dissemination is rare. PTC has many variants, including conventional, solid, tall cell, Warthin-like, oncocytic, nodular fasciitis, microcarcinoma, macrofollicular, follicular, columnar cell, encapsulated follicular, diffuse sclerosing encapsulated, diffuse follicular, and cribiform-morular [39–42].

PTC is characterized by a number of different gene mutations, most of which are almost always mutually exclusive of each other and which often correlate with different histological subtypes and clinical courses. At present, there have

been no defined mutations found in a subset of PTC (~25 %), indicating the likely presence of unknown genetic alterations or the use of inadequate molecular testing methods [42].

2.4.1 BRAF Mutation Testing

BRAF somatic mutations are found in ~45 % of PTCs [43–45]. *BRAF* is found at 7q34 and consists of 19 exons. It is also known as the V-raf murine sarcoma viral oncogene homolog B1 (B-raf-1) and functions as a serine/threonine kinase. Under normal conditions, BRAF is phosphorylated and activated by KRAS, leading to an increase in cyclins D1-3, mdm2, and c-myc levels, promoting tumorigenesis via promotion of cell cycle entrance, increased growth potential, and apoptosis inhibition. BRAF is commonly constitutively activated in multiple human malignancies, including colorectal carcinomas, low-grade serous ovarian adenocarcinomas, and PTC [45–48]. More than 40 different *BRAF* mutations have been identified in PTC, with ~90 % of mutations involving a T→A transversion at position 1,779 in exon 15, causing a valine→glutamate substitution at residue 600 (V600E). The resulting mutant BRAF kinase is ~500-fold more active than the wild-type kinase and has ablated GTPase activity, allowing the kinase to be constitutively active [49]. Studies on thyroid cell lines and animal models have suggested that the *BRAF V600E* mutation promotes genomic instability, dedifferentiation, metastasis, proliferation, and transformation [50–53].

TCs with *BRAF V600E* often show a worse clinical course than TCs without the mutation and are associated with extrathyroidal extension, advanced disease stages III and IV, recurrence, loss of radioiodine avidity, and distant metastases. Interestingly, *BRAF V600E* mutations are more common in the tall cell, conventional, and Follicular variants of PTC, the three variants that exhibit the most aggressive clinical courses [54]. The *BRAF V600E* mutation is rare in FC [43].

Clinical *BRAF* mutation testing in TC has been performed via many different molecular techniques. Because the V600E mutation makes up the majority of mutations, many testing methods specifically target the 1,779 position T→A transversion. For example, allele-specific amplification polymerase chain reaction (ASA-PCR) and Shifted Termination Assay Technology (STAT) both use the one base-pair difference to detect *BRAF V600E* mutations.

2.4.2 Allele-Specific Amplification-PCR

Also known as amplification refractory mutation system-PCR and allele-specific oligonucleotide-PCR, this method is useful for detecting one to several base-pair changes (transversion or transitions) by taking advantage of the different initiation rates for the *Taq* polymerase from matched vs. mismatched template-primer

3′-termini, with the latter 10^{-3} to 10^{-6} less efficient depending on the mismatch type. ASA-PCR employs multiple primers with 3′ ends, often three base pairs from the 3′-terminus that match either the wild-type or the mutant sequence [55]. Mutation detection is based on the detection of an amplified wild-type or mutant sequence. Amplicon detection methods include the degradation of a probe labeled with a quencher held adjacent to a fluorescent dye. Upon probe degradation by the 5′–3′ exonuclease activity of the *Taq* polymerase, the quencher and fluorescent dye are no longer held in close proximity, allowing fluorescent dye emission detection and hence amplicon detection. Samples can be run multiplexed with different probes carrying different fluorophores or can be run separately with one primer pair, depending on the requirements of the test. Alternatively, other systems can be employed, such as Scorpion technology for amplicon detection. Due to the extremely specific base pairing requirements of the *Taq* polymerase, ASA-PCR can detect as little as one mutant in a background of 10^{-4} wild-type alleles [56]. Due to the low amplification that occurs from mismatched primer-templates with the *Taq* polymerase, ASA-PCR can produce amplicons at very high Cp values. Therefore, to be counted as mutant positive, some testing protocols using ASA-PCR require Cp values (rounds of primer annealing, extension, and denaturation) of 35 or lower [57]. Additionally, ASA-PCR with only one nucleotide difference requires very exact annealing temperatures for both the wild-type and mutant primers to optimize specific primer annealing [58].

Sapio et al. [57] employed ASA-PCR to examine *BRAF* mutations in PTC. One reverse primer was employed, with two forward primers differing at the mutant base pair: 5′-GTGATTTTGGTCTAGCTACAG*T*-3′ (wild type) and 5′-GTGATTTTGGTCTAGCTACAG*A*-3′ (mutant). Analysis of 43 archival PTCs revealed that 44.2 % harbored *BRAF V600E* mutations. No *BRAF* mutations were identified in 35 benign lesions or FCs. Interestingly, when single-strand conformational polymorphism (SSCP) and DNA sequencing testing methods were compared to ASA-PCR, SSCP and DNA sequencing both required more than 40 % mutant DNA to be present for mutation detection, showing a relatively low sensitivity, whereas ASA-PCR demonstrated two to threefold greater sensitivity.

2.4.3 STAT

ASA-PCR has drawbacks, including requiring extremely precise annealing temperatures and giving false-positive results at high Cp values [57, 58]. One technique that partially overcomes these limitations is STAT. With STAT, a detection primer is designed that is complementary to the target DNA, with the 3′-terminal detection primer binding site ending just before the mutant target base. A labeled detection primer then binds to the target sequence. A primer extension reaction is then performed. If the first target base is present in the wild type DNA, the detection primer is not extended. If there is mutational change by any type of mutation, including

point mutations, deletions, insertions, or translocations, a primer extension reaction will continue through the target base position, incorporating multiple labeled nucleotides. Colorimetric, fluorometric, or chemiluminescent detection systems can be used to quantify the amount of the mutant transcript. STAT is very sensitive and can detect as little as 1 % mutant DNA [59].

STAT has been employed to analyze PTC *BRAF V600E* mutations with good results. For example, Cohen et al. [60] employed STAT to analyze *BRAF V600E* mutations in 95 excised PTCs and 42 corresponding fine needle aspirations (FNAs). In this study, 100 ng genomic tumor/aspirate DNA was isolated and the region surrounding exon 15 carrying the V600E mutations was amplified via 39 cycles of standard PCR. Both STAT and automated DNA sequencing were performed on 10 μm of the resulting amplicon. *BRAF* mutations were detected in 67 % of the resected PTC and only 12 % of the FC, with only two ATC showing *BRAF* mutations in both the differentiated and undifferentiated areas. Forty-nine preoperative FNAs, which corresponded to the resected PTCs, were analyzed for *BRAF V600E* mutations. A 94 % concordance was found for *BRAF* status. Of three discordant samples, two were sparsely cellular; thus STAT testing is useful for aspirate analysis. Further analysis of a large number of FNAs revealed that clearly benign aspirates had no *BRAF* mutations, 39 % of the clearly malignant aspirates had mutations, as did 16 % of the indeterminate group. Thus STAT *BRAF* testing can be used to identify PTC within the indeterminate group. When STAT testing and DNA sequencing were compared, a 100 % correlation was found between the two methods. Thus STAT testing is comparable to sequencing in accuracy and reliability and has greater sensitivity.

2.4.4 Single Strand Conformation Polymorphism Testing

SSCP testing has been used to detect TC *BRAF* mutations [61]. In SSCP, wild-type and DNA undergoing mutation testing are PCR amplified, along with a known wild-type control. The two DNA samples are heat denatured, rapidly renatured, and subjected to electrophoresis. A ssDNA segment carrying a mutation, even if a single base pair change, will assume different secondary and tertiary structures, which will cause a different migration pattern compared to the wild-type control(s). SSCP has worked well and has the advantage of detecting more than the single *V600E BRAF* mutation, although it does not identify which specific base pair(s) is altered. It is also less sensitive than the ASA-PCR or STAT testing and is often technically challenging to perform. It does have use as an initial mutation-screening tool, which if initially positive, can be followed up by more accurate testing methods. Additionally, SSCP can initially use either genomic DNA or RNA, which is converted into DNA via reverse transcriptase and then PCR amplified. Conformation strand gel electrophoresis thyroid *BRAF* mutation testing, similar to SSCP, has not often been employed in thyroid *BRAF* mutation testing.

Fugazzola et al. [61] employed DNA sequencing and SSCP to analyze *BRAF* mutations in a large multicentric study. *BRAF* RNA was isolated, and cDNA was made via reverse transcriptase with radon hexamer primers. The cDNA immediately around the *V600E BRAF* mutation was PCR amplified with the forward and reverse primers 5′-CATTGCACGAQCAGACTGCAC-3′ and 5′-TCTGACTGAAAGCTGTATGG-3′ and subjected to both SSCP and direct DNA sequencing using ABI PRISM big dye terminators and run on an ABI PRISM 310 genetic analyzer. *BRAF V660E* mutations were found in 38 % of PTCs in an Italian population, with a tendency for the mutation frequency to increase with increasing patient age.

2.4.5 DNA Sequencing

DNA sequencing has often been employed in *BRAF* mutation analyses [58, 61]. Although considered the gold standard in molecular testing, especially Sanger sequencing, direct sequencing has several disadvantages: (1) it can be relatively expensive, especially for longer sequences; (2) other techniques such as ASA-PCR and STAT have higher sensitivity; and (3) it can give false results when applied to formalin-fixed tissue [62, 63]. Direct sequencing has the advantage of interrogating all possible nucleic acid sequences analyzed, thus it identifies rare mutations missed by ASA-PCR and STAT.

2.4.6 High-Resolution Melting Analysis in BRAF Mutation Analysis

High-resolution melting (HRM) analysis is performed on dsDNA. Most often a specific nucleic acid sequence containing the possible mutation of interest is PCR amplified. It is then mixed with a fluorescent dye, such as SYBR green, which emits strongly when bound to dsDNA and poorly in the presence of ssDNA. The amplified dsDNA is then heated, and the transition from dsDNA to ssDNA is followed via the loss of florescence at the DNA melting point. Typically, the data are presented as the negative first derivative of the melting curve, as this makes it easier to identify the exact temperature of dissociation.

Rowe et al. [64] analyzed 42 archival formalin-fixed, paraffin-embedded thyroidal samples, 37 PTC, and 5 benign lesions. The region around the *BRAF V600E* mutation was PCR amplified with 5′-CTC TTCATAATGCTTGCTCTGATAGG-3′ and 5′-TAGTAA CTCAGCAGCATCTCAGG-3′ forward and reverse primer for 45 cycles. The resulting amplicon was cooled and then heated at 0.1 °C/s from 45 °C to 95 °C, with fluorescent peaks determined by plotting the negative derivative of fluorescence with respect to temperature. Of the PTC samples, 62.2 % had the *BRAF V600E* mutation, whereas 0 % of the benign thyroid samples had mutations.

In this study, when HRM was compared to DNA sequencing, a 100 % concordance was revealed. Although useful in detecting *BRAF* mutations, the authors did note that HRN had poor sensitivity and required at least 50 % mutant DNA present to be accurate. Thus, HRM as a testing method is useful, but requires very pure tumor DNA to ensure testing accuracy.

There are several advantages of HRM: (1) it is often cost effective, thus allowing a large number of samples to be interrogated simultaneously; (2) it has a relatively short turn-around time and high accuracy; and (3) once "up and running," the assay is relatively simple to run and analyze. Disadvantages of HRM are that it does not identify the specific base pair(s) mutated and requires pure concentrated samples.

2.4.7 BRAF2

BRAF mutation testing is complicated by the closely related *BRAF2* pseudogene located at Xq13.3. The two genes are similar, and careful primer design is required to avoid pseudogene amplification during *BRAF* testing [65, 66]. *BRAF2* is often expressed in *BRAF* mutation-negative PTCs. Additionally, it is expressed in tumorigenic nude mice, it activates MAP kinases, and it can transform NIH3T3 cells [67]. Presently, molecular genetic testing is not performed for *BRAF2,* although this could change, as one study showed nearly 50 % of classic PTC had *BRAF2* expression [67].

2.4.8 RET/PTC Mutation Testing

The *RET* oncogene, also known as *ret-proto-oncogene*, is located at 10q11.2, has 21 exons, and has three main isoforms of 1,114, 1,106, and 1,072 amino acids, encoding a protein of ~124 kDa. The RET protein has an extracellular domain that binds extracellular ligands, a single transmembrane domain, and an intracellular domain that contains a tyrosine kinase. The physiologic RET ligands all belong to the glial-derived neurotrophic factor family: peresphin, artemin, neurturin, and glial-derived neurotrophic factor. *RET* has multiple functions; for example, it is required for the development of the sympathetic, parasympathetic, and enteric neurons, the kidneys, and male germ cells. Not surprisingly germline loss of *RET* function can cause colonic congenital aganglionosis (Hirschsprung's disease), whereas germline *RET* activation can lead to medullary thyroid cancer (MTC) or multiple endocrine neoplasia type two (MEN2A/B) [68].

For PTC, 20–30 % carry *RET* gene mutations. Unlike the point mutations that activate BRAF and KRAS or the activating *RET* point mutations seen in hereditary MTC or MEN2A/B, *RET* mutations in PTC are typically activating intra- and interchromosomal rearrangements and are unique to PTC. Most rearrangements

are in-frame fusions of the RET C-terminal/tyrosine kinase domain with the 5′ end of many heterologous donor genes, of which ~90 % involve the *RET/PTC1* and *RET/PTC3* genes, which result from fusion with the H4 (*D10S170*) gene and the NCOA4 (*ELE1, RFG*, or *ARA70*) genes, respectively [69, 70]. The molecular mechanisms by which these RET rearrangements cause tumorigenesis are incompletely understood, although the fusions lead to constitutive activation of the RET tyrosine kinase. Additionally, the rearrangements induce the nuclear changes typical of PTC, which are found in occult PTCs (making them an early event in PTC tumorigenesis) and induce TC resembling human PTC when expressed in the thyroid tissue of transgenic mice [71–73].

2.4.8.1 Real-Time PCR (RT-PCR) for RET/PTC Rearrangements

RT-PCR is useful for detecting gene rearrangements, as primers can be designed to amplify gene fusion regions (nucleic acid sequences likely unique to the particular tumor type and not found in normal cells). As in the case of the *BCR-abl* gene fusion characterizing chronic myelogenous leukemia, the initial analytic step is purifying RNA, priming with radon heximers, and employing reverse transcriptase to produce a cDNA library. The specific fusion can be PCR amplified and quantified through the use of fusion region flanking primers and a labeled probe.

Zhu et al. [74] used RT-PCR to analyze *RET/PCT1* and *RET/PCT3* rearrangements in 65 snap-frozen PTC samples. Total RNA was isolated with Trizol reagent, and a cDNA library was made via Superscript reverse transcription of 3 μg of RNA primed with radon heximers. The *RET/PTC1* and *RET/PTC3* gene fusions were detected by RT-PCR employing a common reverse primer of 5′-GCAGGTCTCAAGCTCACTC-3′ and RET/PTC1 and RET/PTC3-specific forward primers of 5′-CAAGAGAACAAGGTGCTGAAG-3′ and 5′-CGGTATTGTAGCTGTCCCTTTC-3′, respectively. Appropriately labeled probes were employed for amplicon detection. In PTCs, 22 % showed RET/PTC rearrangements.

Interestingly, the authors compared the ability of standard-sensitivity RT-PCR, high-sensitivity RT-PCR, real-time LightCycler RT-PCR, Southern blotting, and fluorescence *in situ* hybridization FISH to detect *RET/PCT1* and *RET/PCT3* rearrangements [74]. High-sensitivity RT-PCR detected the 14 *RET/PCT* rearrangements detected by standard PCR and 12 additional cases, for a 40 % detection rate. LightCycler RT-PCR detected 12 rearrangements, Southern blot 11, and FISH 14. Overall, 14 % of PTC had rearrangements detected by all five methods. Twelve (18 %) of PTCs had nonclonal rearrangements detected only by high-sensitivity RT-PCR. The authors concluded that the prevalence of different *RET/PTC* rearrangement seen in the literature is in part due to different molecular detection methods and tumor heterogeneities. Other techniques, such as DNA sequencing, have also been used to analyze these rearrangements [75]. However, different RT-PCR protocols are most commonly employed.

2.4.9 RAS Family Proteins and PTC

HRAS, NRAS, and *KRAS* are members of a superfamily of GTPases which has more than 150 members and is divided into five different groups: Ras, Rho, Ran, Rab, and Arf. Members of this group share a conserved G box motif, which transiently binds GTP, resulting in kinase activation and concomitant downstream signaling events that promote cell survival and proliferation. Under normal conditions, RAS proteins rapidly hydrolyze GTP to GDP, rendering the kinase inactive [76, 77]. A number of specific point mutations in codons 12, 13, and 61 ablate the GTPase activity, rendering the kinases constitutively active and oncogenic [76–80]. RAS point mutations are found in 10–20 % of PTC [81–83].

Molecular testing for RAS mutations in PTC has been done by multiple methods. For example, Vasko et al. [82] examined *NRAS* point mutations in 10 FA, 9 atypical follicular tumors, 10 FC, and 10 follicular variants of PTC. The tumors on slides were microdissected, genomic DNA was extracted and purified, and 500 ng was PCR amplified with primers specific for NRAS exon 2. The resulting amplicon was sequenced with an Applied Biosystem 373XL sequencer. No *NRAS* mutations were detected in any FA. Exon 2 *NRAS* mutations were identified in 33 % of the atypical follicular tumors, 20 % of the FC, and 20 % of the follicular variant PTC. Ezzat et al. [83] used SSCP testing and DNA sequencing to examine point mutations in exons 12, 13, and 61 of *HRAS* and *NRAS*. These exons were PCR amplified from 100 ng genomic DNA, and the DNA was heat denatured and analyzed by PAGE. Of 45 histologically characterized TC, adenomas, and hyperplastic nodules, one *HRAS* at codon 13 mutation was identified in one PTC and two PTC and one FA carried the *NRAS* codon 61 mutation. No tumors carried *KRAS* mutations.

2.4.10 NTRK1 Mutation in PTC

NTRK1, also known as *TrkA*, is found at 1q21-22 and has at least 17 exons that encode a 796-amino acid glycosylated protein of ~140 kDa. NTRK1 is widely expressed in neuronal and non-neuronal tissues and functions as a nerve growth factor receptor tyrosine kinase. Roughly 5–25 % of PTCs carry *NTRK1* mutations, depending on the population examined [84–87]. Similar to *RET, NTRK1* is constitutively activated by rearrangements involving at least three other genes, with the 3′ terminus of *NTRK1* fusing with the 5′ of genes, such as TPM3, TPR, and TFG. All rearrangements lead to constitutive NTRK1 tyrosine kinase activation [85, 88–92]. Once activated, NTRK1 activates RAS, RAC, PI3K, PLC-gamma, and MAPK, leading to cell proliferation [92].

NTRK1 has been analyzed by multiple methods, with PCR amplification of tumor-derived reverse transcribed RNA, followed by amplicon sequencing being a commonly used technique. Beimfohr et al. [93] used this technique with multiplex PCR and Sanger sequencing to examine 81 pediatric PTCs from children who had

been exposed to the Chernobyl fallout. All tumors were previously determined to be devoid of *RET* rearrangements of types PTC1 to PTC5. *TPM3/NTRK1* fusions were identified in five tumors. Reciprocal *NTRK1/TPM3* transcripts were found in four of five of these tumors. Tumors with the *TPM3/NTRK1* fusions were histologically no different than other post-Chernobyl childhood PTCs. The authors concluded that, compared to the high number of *RET* mutations seen in this population [14], NTRK1 rearrangements play a small role in Chernobyl fallout-induced pediatric PTCs. Presently, clinical testing for *NTRK1* mutations/rearrangements in TC is not routinely performed.

2.4.11 c-MET *Mutation in PTC*

c-MET is located at 7q31.2, consists of 21 exons, and encodes a hepatocyte growth factor/scatter factor receptor with tyrosine kinase activity. c-Met is synthesized as a 170 kDa single-chain precursor that is cleaved post-translationally to yield an internal 145 kDa beta-subunit and a 45 kDa extracellular alpha-subunit domain [94]. c-MET is overexpressed in ovarian, colorectal, pancreatic, renal cell, non-small lung, and thyroid malignancies [95–101]. Inappropriate ligation of c-MET by the hepatocyte growth factor increases epithelial cell growth and division and causes epithelial cells to acquire increased motility and invasive capacities [94, 95, 102]. Hence, c-MET expression is likely to play an important role in PTC acquiring invasive/metastatic potential [97].

c-MET is barely detected in normal thyroid or thyroid tissue affected by non-neoplastic disease. c-MET expression is increased roughly 100-fold in thyroid carcinomas, and its overexpression strongly correlates with locally advanced disease and/or distant metastases [103]. Immunohistochemical studies of benign thyroid tissue revealed that c-MET is usually located along the basal membrane, occasionally found with the cytoplasm, and not found at the apical cell membrane. In PTC, these analyses identified increased c-MET immunoreactivity in the cytoplasm and apical membrane, with loss of reactivity seen along the basal membrane. In PTC, c-MET was co-localized with tight junction protein zona occludens-1 in the cytoplasmic/apical membrane locations. These findings indicate that a change in cell polarity occurs in PTC expressing c-MET, which likely relates to the increased invasive/metastatic abilities of PTC overexpressing c-MET [102, 104]. c-MET overexpression did not increase PTC Ki-67 levels or increase tumor cell proliferation, and is reduced or absent in poorly differentiated tumors. c-MET is overexpressed in roughly 77 % of PTCs and 70 % of Hürthle cell tumors [102]. Interestingly, c-Met expression is significantly higher in the most aggressive PTC subtype, Tall Cell PTC, where its increased expression correlates with extracapsular spread, skeletal muscle invasion, and lymphatic invasion [105].

Currently clinical molecular diagnostic testing in not routinely preformed for c-MET overexpression. c-MET expression has been detected in PTC via immunohistochemistry, Western blotting, tissue microarray, cDNA expression arrays, and

quantitative real-time PCR, typically as basic research tools [106–108]. Most likely c-MET detection methods will be developed that will be applicable to molecular diagnostics as c-MET inhibitors, including monoclonal antibodies, and small-molecule tyrosine kinase inhibitors have shown encouraging results in *in vitro* studies, animal models, and in human phase I and II clinical trials [109, 110].

2.4.12 p53 *and* CTNNB1 *Mutations in PTC*

PTC rarely carries mutations of the *p53* and *CTNNB1* genes [111]. Presently, no clinical molecular testing is performed for mutations or changes in these genes in PTC.

2.4.13 *Hashimoto's Thyroiditis Versus PTC*

Hashimoto's thyroiditis (HT) can present with different histological patterns, such as the classic features of chronic autoimmune thyroiditis, or HT associated with hyperplastic/adenomatous lesions, or even HT associated with concurrent TC. HT can be nodular, sometimes with one or more large, so-called "dominant nodules," which histologically can have some features of PTC, including nuclear membrane irregularities, nuclear clearing, and increased size. In some cases, differentiating between reactive and malignant morphologic changes can be difficult. Sadow et al. [112] examined 28 cases of HT with a dominant nodule and looked for *BRAF, RET, KRAS, NRAS,* and *HRAS* mutations and *RET/PTC1* and *RET/PTC3* rearrangements within the dominant nodule. No *BRAF* or *RAS* mutations or *RET/PTC* rearrangements were detected in the dominant HT nodules, even in those with atypical, worrisome histological features. BRAF V600E mutations were only identified in HT with concomitant PTC. Thus dominant nodules in HT are neither malignant, nor precursor lesions, and molecular testing can separate even very atypical HT from PTC.

2.4.14 *Conclusions on Molecular Testing for PTC*

Molecular testing for PTC can be performed on resected thyroidal lesions and FNAs. Although not presently widely used in the clinical setting, molecular testing of the thyroid can be useful for histologically ambiguous cases and FNAs deemed indeterminate or suspicious for malignancy. Testing such as *BRAF* mutational analysis also has pronostic value. In particular, simultaneous testing for *BRAF V600E* and *RET/PTC* mutations, which together cover ~70–80 % of PTCs, is a useful aid in the diagnosis of PTC, especially for cytologically/histologically difficult cases, as these two mutations are almost exclusively found in PTC [1, 14].

2.5 Anaplastic Carcinoma of the Thyroid

Anaplastic carcinoma of the thyroid (ACT) accounts for roughly <2 % of TCs. ACT is one of the most aggressive human malignancies and carries a dismal prognosis, with a median survival of 6 months and a 5-year survival rate ranging from 1 % to 7.1 %. These tumors are so aggressive that the original diagnosis is often questioned in long-term survivors; in addition, despite its low incidence, it accounts for 50 % of deaths due to TC. ACT usually presents with symptoms related to a rapidly growing thyroid mass that shows extensive local invasion and early metastases to the bones, liver, lungs, and brain. Although it is rarely seen in younger individuals, it is usually a malignancy of those in their sixth and seventh decades. Histologically ACT shows extreme cellular pleomorphism and a high mitotic index, often with spindle cells resembling a sarcoma, undifferentiated squamoid cells, and pleomorphic giant cells resembling osteoclasts. The cell types may predominate in any given tumor or may occur in any combination within a tumor [113–118].

ACT carries multiple mutations that occur with different frequencies, including *RAS* (22 %), *BRAF* (26 %), *p53* (55 %), *beta-catanin* (38 %), *Axin* (82 %), *PTEN* (12 %), and *PIK3CA* (17 %). Although several of these mutations occur in well-differentiated TCs, like PTC, mutations of *p53, beta-catanin,* and *PIK3CA* are usually found only in ATC and likely represent late mutational events that contribute to the aggressive course of ATC [119]. The common finding of more differentiated areas with ACTs resembling PTC or follicular carcinoma of the thyroid (FCT), combined with many ACTs carrying the same mutations as PTC and FCT, indicates that many ACTs probably arise from well-differentiated TCs. Clinical molecular diagnostic testing is not commonly performed on ACT; however, because of its lethal clinical course, intensive research into the molecular pathology of ACT is being done in the hope of finding more effective treatments, especially as related to targeted molecular therapies [119].

2.6 Follicular Carcinoma of the Thyroid: Mutations and Molecular Testing

FCT accounts for 5–15 % of TCs, making it the second most common TC. FCT and PTC together make up 90 % of TCs, making up the “differentiated TC” category [111]. The incidence of FCT peaks in the fifth decade, usually presenting as slowly enlarging non-tender nodules and typically following a more aggressive clinical course than PTC. Patients with FCT often show hematogenous metastases to the brain, lung, and bone. Individuals with tumors that do not show capsule invasion have an 86 % 10-year survival rate, whereas those with invasion have a 44 % 10-year survival rate. Histologically, FTCs show follicular epithelial cells with vesicular nuclei and follicles that are often devoid of

collagen. Diagnosis of FCT vs. FA rests on the identification of capsular or vascular invasion or the identification of metastases [120, 121].

FCT often have *RAS* mutations (40–50 %), *PAX8-PPAR-gamma* gene rearrangements (~35 %), and less common *PTEN* and *PI3KCA* mutations (7 %). *BRAF* mutations are rare, and *RET* gene alterations have not been identified in FTC (115). Currently, clinical molecular testing is uncommon for FTC; when it is done, testing focuses on *PAX8-PPAR-gamma* gene rearrangements and *RAS* mutations.

2.6.1 RAS Mutations

Molecular testing for *NRAS, KRAS,* and *HRAS* were addressed above (Sect. 2.4 on PTC). However, many testing methods combine two or more standard protocols. For example, Nikiforova et al. [122] analyzed *NRAS, KRAS,* and *HRAS* in a series of 88 TCs consisting of 33 conventional FCTs of the thyroid, 23 conventional FAs, 19 Hürthle cell carcinomas, and 13 Hürthle cell adenomas. *RAS* mutation testing was performed using a combination of RT-PCR and HRM to analyze *NRAS, KRAS,* and *HRAS* activating point mutations at codons 12, 13, and 61. RNA was isolated from paraffin-embedded or frozen tissue, and 3 μg of RNA were converted in a cDNA library by reverse transcriptase with random heximer primers. The resulting cDNA was PCR amplified with two primers specific for each point of mutation being interrogated. Within each PCR reaction were two fluorescently labeled oligonucleotide hybridization probes. The two probes would hybridize internal to the primers such that the two fluorophores would be adjacent at the annealing step. One fluorophore functions as a donor and the other as an acceptor and emitter of a specific light wavelength. After 40 rounds of conventional PCR, the amplicons were cooled to 45 °C and heated at 0.2 °C/s to 95 °C. For detection purposes, one probe spans the mutation site. The wild-type and mutant amplicons are distinguished by different denaturation temperatures, reflecting the thermodynamic stability of complementary vs. point-mismatched probe-target duplexes. Using this technique, the group found 49 % of FCT had *RAS* mutations, whereas 48 %, 11 %, and 8 % of FAs, Hürthle cell carcinomas, and Hürthle cell adenomas had mutations, respectively. Comparison with DNA sequencing demonstrated that the method was both sensitive and accurate.

2.6.2 PAX8-PPAR-Gamma

PAX8-PPAR-gamma rearrangements are found in ~35 % of FTCs and ~55 % of FAs, but not in PTC or ATC [111, 122]. *PAX8-PPAR-gamma* rearrangements result from a t(2;3)(q13;p25) translocation, which encodes a protein that promotes the development of differentiated FTC, but is not sufficient for carcinogenesis [122–124]. PAX8 is a 48-kDa nuclear thyroid-specific paired domain transcription factor

found at 2q13 that regulates thyroid follicular cell development and the expression of thyroid-specific genes [125]. *PPAR-gamma* or the peroxisome proliferator-activated receptor-gamma is found at 3p25.2. *PPAR-gamma* is a ligand-activated transcription factor that dimerizes with the retinoic X receptor and is expressed primarily in adipose and lymphoid tissues, colon, liver, and heart. PPAR-gamma activity plays a role in many diseases, including diabetes, atherosclerosis, cancer, and metabolic syndrome. Additionally, PPAR-gamma binds antihyperglycemic/antidiabetic agents such as the thiazolidinediones [121].

PAX8-PPAR-gamma rearrangements have been detected with many different methods, including interphase FISH, immunohistochemistry, Northern blotting, sequencing, and reverse-transcriptase-PCR [123, 124]. Most commonly reverse-transcriptase PCR is employed, as this technique is highly specific and sensitive for the *PAX8-PPAR-gamma* rearrangement. Cheung et al. [123] analyzed *PAX8-PPAR-gamma* rearrangements by isolating total tumor RNA and producing a mixture of cDNAs with Superscript II reverse transcriptase. The *PAX8-PPAR-gamma* rearrangement was then PCR amplified with primers specific for *PAX8* exon 6, 5′-CGCGGATCCGCATTGACTCACAGAGCA-3′, and exon 1 of *PPAR-gamma,* 5′-CCGGAATTCGAAGTCAACAGTAGTGAA-3′. The integrity of the RNA was tested by simultaneous amplification of the beta$_2$-microglobulin RNA. Additionally, other primers were employed to amplify other *PAX8* exons sometimes involved in variants of the *PAX8-PPAR-gamma* translocation. Amplicons were measured and resolved by 5 % acrylamide gel electrophoresis. The identities of the amplicons were then checked via sequencing. *PAX8-PPAR-gamma* rearrangements and *RAS* mutations are found together in FTC at a low rate of ~3 % [126]. Interestingly, some data exist indicating that *PAX8-PPAR-gamma* rearrangements may be specific to some ethnic groups and not to others [127].

2.6.3 p53 *Mutations in FTC*

p53 mutations are a late event in thyroid carcinogenesis and are associated with the progression of differentiated TC to ACT [111, 128, 129]. *p53* mutations are only rarely found in FTC, and analysis of its mutation has little value in the molecular diagnostics of FTC.

2.6.4 PTEN and CTNNB1 Mutations in FTC

PTEN, also known as phosphatase and tensin homolog, is located at 10q23.3 and functions as a tumor suppressor via dephosphorylation of protein tyrosine, serine, and threonine phosphates, as well as by catalyzing the removal of the D3 phosphate of phosphatidylinositol 3,4,5-trisphosphate. These activities antagonize the PI3K-AKT/PBK signaling pathway, thereby attenuating signaling that promotes cell cycle

progression and cell survival [130]. *PTEN* is mutated in about 7 % of FTC, but not FAs [131, 132]. *CTNNB1* mutations are rare in FTC [111]. Interestingly, down-regulation of unmutated CTNNB1 membrane expression in FTC is associated with progressive loss of tumor differentiation, while *CTNNB1* mutation is associated with poorly or undifferentiated carcinomas [111, 133]. Molecular diagnostic testing is not routinely performed for *PTEN* or *CTNNB1* mutations in FTC.

2.7 Hürthle Cell Tumors

Hürthle or oxyphillic cell carcinoma accounts for ~5 % of differentiated TC. Hürthle cell tumors arise from follicular cell cells and are defined as encapsulated thyroid lesions consisting of at least 75 % Hürthle cells, which typically have very few or no lymphocytes, scant or no colloid, and large polygonal cell cells with indistinct cell borders, pleomorphic hyperchromatic nuclei with a prominent nucleus, and pink finely granular cytoplasm on hematoxylin-eosin staining due to abundant cytoplasmic mitochondria [120, 134]. Roughly 30 % of these tumors are malignant and hence show an invasive growth pattern and/or distant metastases. Interestingly, features such as pleomorphism, anaplasia, and atypia are seen in benign as well as malignant Hürthle cell tumors and are of no value in determining malignancy. The aggressiveness of these tumors is poorly defined with some studies showing them to be more aggressive than FTC and other equally of less aggressive [134]. Hürthle cell tumors show somatic mitochondrial, GRIM-19, RET/PTC gene mutations, and chromosomal gains at 5p and losses at 2q and 9q [120, 134, 135]. Presently, molecular resting is not performed on Hürthle cell tumors.

2.8 Medullary Thyroid Cancer: Mutations and Molecular Testing

Medullary thyroid cancer (MTC) accounts for ~4 % of TCs and arises from neural crest-derived parafollicular C-cells of the thyroid, giving them the clinical and histological features of neuroendocrine tumors such as carcinoid and islet-cell tumors; 25–30 % of MTCs occur in inherited autosomal dominant disorders that are usually preceded by bilateral, multicentric C-cell hyperplasia/calitonin-producing lesions. Hereditary MTCs occur in younger individuals and are often associated with other endocrine tumors, usually in well-defined genetic syndromes such as MEN2 and MENB (see below). The remaining MTCs are sporadic, occurring at an older age than hereditary MTCs and having a poorer prognosis. Histologically, MTCs consist of small polygonal to spindled cells in a solid or trabecular patterns, often with associated amyloids. MTC nearly always occurs in the lateral thyroidal lobes, with other thyroidal locations being far less common [136, 137]. Interestingly, residual C-cell hyperplasia near an MTC often indicates that a particular MTC is hereditary,

although C-cell hyerplasia can rarely occur next to sporadic MTC or other benign and malignant thyroid lesions [138]. MTC has a worse prognosis than other TCs such as PTC, due to early metastases, with about half of affected individuals having local lymph node involvement at diagnosis. Distant metastases are common, often to the adrenal glands, lungs, liver, and bones. The 5-year survival rate is 60–70 %, and the 10-year survival rate is 40–50 %. Presently, establishing a diagnosis and prognosis of MTC depends largely on histologic analysis combined with immunostaining for calcitonin, and in some cases analysis of serum calcitonin and carcinoembryonic antigen levels, both of which are secreted by MTC [136–139]. Molecular diagnostic testing has an import role in patient care for these tumors.

Both sporadic and hereditary MTC are characterized by activating or "gain of function" *RET* mutations and less by *RET* gene rearrangements, as are found in PTC [69, 70]. Molecular diagnostic testing is mainly performed for possible hereditary MTC, as testing results are an important part of establishing a diagnosis (in conjunction with clinical data) and determining whether kindred testing is required. About 98 % of individuals with hereditary MTC syndromes carry germline *RET* mutations, whereas 40–50 % of sporadic cases carry *RET* mutations [140–146]. Last, about 4–11 % of individuals with apparently sporadic MTC actually carry germline *RET* gene mutations. Thus screening sporadic MTC cases for germline *RET* mutations can identify kindreds who can benefit from early medical intervention, such as prophylactic thyroidectomy [145–148].

Since the *RET* mutations in MTC are activating, they typically involve single or a few nucleotide changes that cause constitutive tyrosine kinase activation via loss of normal kinase inhibitory mechanisms, as are also found in other proto-oncogenes such as *KRAS* and *BRAF*. For example, 23–85 % of sporadic MTCs have an exon 16, codon 918 (ATGACG; MetThr) activating mutation, and this mutation along with exon 15, codon 833 (GCTTTT; ArgCys) accounts for more than 98 % of MEN2B cases [140–148]. *RET* mutations have been detected by many different molecular methods, including SSCP, microarrays, sequencing, temperature gradient capillary electrophoresis, denaturing gradient gel electrophoresis, and HRM of PCR products [149–154].

2.8.1 RET DNA Sequencing

Since one or a few *RET* mutations often characterize a specific MEN syndrome, mutation-specific *RET* testing is often performed. However, mutation-specific testing will not locate unknown or novel mutations. To find such mutations, DNA sequencing works well. Ahmed et al. [153] employed PCR and sequencing to identify nine infrequent novel *RET* gene variants; 6,700 cases of suspected MEN2A or familial MTC (FMTC) or a family history of MEN2A of or FMTC were analyzed. Genomic DNA was extracted from peripheral blood leukocytes and amplified via PCR employing a sense primer; 5′-CAACATTTGCCCTCAGGACTG-3′, and an antisense primer; 5′-CTTGAAGGCATCCACGGAGA-3′. The resulting 1,785-base

pair amplicon was analyzed for variants by forward and reverse sequencing using a radiolabeled terminator cycle sequencing kit (Thermo Sequenase no. US79770; Amersham Life Science, Piscataway, NJ). Although sequencing identifies all possible mutations/variants within an amplified DNA sequence, it is also relatively expensive compared to other methods and has lower sensitivity than techniques such as ASA-PCR.

2.8.2 HRN Analysis of RET Mutations with Asymmetric PCR

RET gene analysis lends itself to mutation-specific testing, allowing more sensitive, lower cost tests to be employed than sequencing. For example, Margraf et al. [154] used modified HRN and PCR to identify RET codon 883 and 918 mutations in individuals with suspected MEN2B. Normal and codon 883 and 918 heterozygous mutants DNA samples were obtained, the DNA purified, and PCR amplified with primer sets that cover the codons mutated in MEN2B. Unlabeled oligonucleotide probes were added to the mix that had incorporated 3′ amino modifiers that prevent DNA polymerase extension during PCR. The unlabeled oligonucleotide probes carried the wild type of mutant probe sequences, corresponding to the known MEN2B *RET* mutations. LCGreen dye was added to the reaction. The PCR reaction was carried out with an excess of the primer for the strand targeted for amplification over 55 PCR cycles. Asymmetric PCR results in the accumulation of ssDNA, facilitating later HRN analysis. At the end of the PCR reaction, the reaction tube was heated to 95 °C for 30 s and cooled to 50 °C to generate probe-ssDNA duplexes. HRN data were collected by heating the reaction chambers to 95 °C at 30 acquisitions/s while analyzing LCGreen emission with a 450 nm excitation and 500-nm emission filter combination. All samples were later sequenced for assay validification. This technique allowed rapid and accurate identification of MEN2B mutations in a closed-tube system that is less time-consuming and expensive than sequencing.

2.8.3 Reverse Transcriptase-PCR in the Diagnosis of MTC

Thyroid tumors are often diagnosed by cytologic examination of FNAs. In some cases a diagnosis is not possible due to inadequate FNA sampling, poor fixation, or a lack of epithelial cells. To facilitate diagnosis in such cases Takano et al. [155] developed a new molecular technique termed aspiration biopsy-reverse transcriptase-PCR. In this technique, cells left over in the FNA needle are used for reverse transcriptase-PCR of genes specifically expressed in one TC type. *RET* proto-oncogene, calcitonin, and carcinoembryonic antigen expression is restricted to MTC [156–158]. Takano et al. [155] examined 35 thyroid tissue samples consisting of 6 normal thyroids, 3 adenomatous goiters, 6 FAs, 7 PTCs, 2 FTCs, and 11 MTCs. Total RNA was extracted and reverse transcribed with

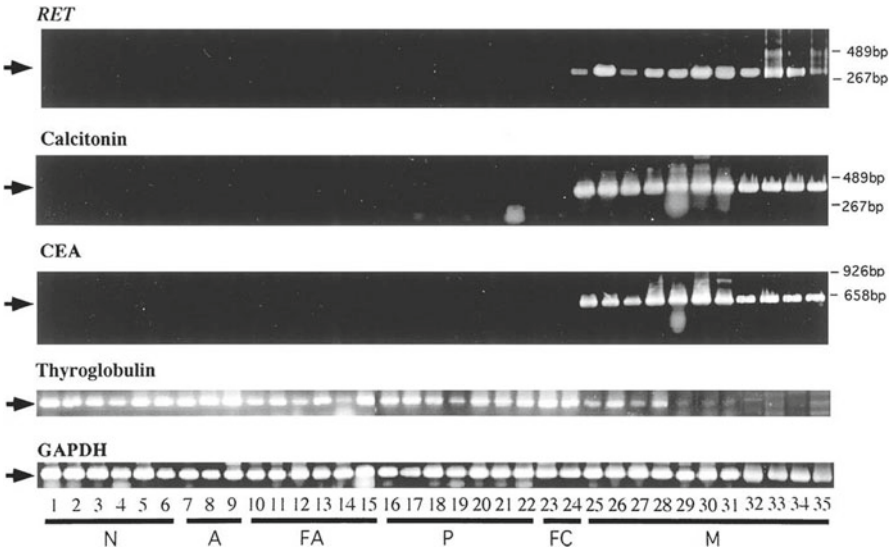

Fig. 2.1 Aspiration biopsy-reverse transcriptase-PCR analysis by Takano et al. [155] of the expression of RET, calcitonin, and CEA mRNAs. Six normal thyroid tissues samples (*N*), three adenomatous goiters (*A*), six follicular adenomas (*FA*), seven papillary carcinomas (*P*), two follicular carcinomas (*FC*), and 11 MTCs (*M*). *Arrows* indicate the expected positions of the PCR products

M-MLV reverse transcriptase. One microliter of the resulting cDNAs were PCR amplified with the following primers over 30 cycles and analyzed by gel electrophoresis:

- *RET:* 5′ (5′-GCAGCATTGTTGGGGGACA) (codon 588–594)
- *RET:* 3′ (5′-CACCGGAAGAGGAGTAGCTG) (codon 685–692)
- Calcitonin: 5′ (59-CCTTCCTGGCTCTCAGCATC) (base 57–76)
- Calcitonin: 3′ (5′-GAGTTTAGTTGGCATTCTGG) (base 445–464)
- Carcinoembryonic antigen: 5′ (5′-ACAAGGATGCTGTGGCCTTC) (base 1,658–1,677)
- Carcinoembryonic antigen: 3′ (5′-AGGGCTGCTATATCAGAGCA) (base 2,211–2,)
- Thyroglobulin: 5′ (5′-GTTGGCAACCTCATCGT) (base 7,011–7,027)
- Thyroglobulin: 3′ (5′-AATTCTGCAGTGCCTGGT) (base 7,657–7,674)
- GAPDH: 5′ (5′-CCATGGAGAAGGCTGGGG) (base 371–388)
- GAPDH: 3′ (5′-CAAAGTTGTCATGGATGACC) (base 546–565)

GAPDH was detected in all samples. Thyroglobulin was detected in all normal thyroid tissue, the adenomatous goiters, FAs, and PTCs and weakly in 4 of the 11 MTCs. RET, calcitonin, and carcinoembryonic antigen expression was detected in all 11 MTCs and in none of the non-MTC samples (Fig. 2.1). This technique was

confirmed to work well for left over cells in FNA needles. Thus aspiration biopsy-reverse transcriptase-PCR can be a useful molecular diagnostic test where standard cytologic sampling is inadequate.

2.9 Hereditary Thyroid Cancer Syndromes

Several hereditary TC-prone syndromes have been identified, including MEN2A, MEN2B, FMTC, and hereditary PTC. Molecular diagnostic testing plays a role in the first three syndromes, where once identified in a proband, kindred analysis and subsequent early medical intervention can significantly reduce mortality and morbidity in affected individuals. Here, we will briefly discuss each syndrome.

Multiple endocrine neoplasia type 2A and 2B and familial MTC (MEN2A, MEN2B, and FMTC, respectively) syndromes are all characterized by an autosomal dominant inheritance pattern, activating *RET* gene mutations, and MTC. Virtually all individuals with these syndromes will develop C-cell hyperplasia followed by MTC. In fact, MTC is typically the first clinical manifestation of these syndromes, and, without medical intervention, the most common cause of death in individuals with these syndromes. Once germline *RET* gene mutations are identified in individuals with one of these syndromes, prophylactic thyroidectomy is recommended to prevent MTC development [159].

2.9.1 MEN2A

MEN2A or Sipple's syndrome is an autosomal dominant disorder characterized by MTC and pheochromocytomas and hyperparathyroidism in about 50 % and 30 % of affected individuals, respectively. Other less common manifestations of MEN2A include cutaneous lichen amyloidosis and Hirschsprung's disease [159]; 97 % of MEN2A is caused by activating missense germline *RET* mutations in exon 10, codons 609, 611, 618, and 620, and in exon 11, codons 630 and 634, all of which enhance receptor dimerization, with increased receptor autophosphorylation and hence increased kinase activity [139–148, 160]. Molecular diagnostic testing for MEN2A is usually directed at these specific mutations with methods described above.

2.9.2 MEN2B

MEN2B or Wermer's syndrome is characterized by MTC, pheochromocytoma, mucosal neuromas (of the tongue, lips, conjuctivae), ganglioneuromas of the intestines, characterized by special facial appearance, and marfanoid habitus.

Interestingly, the MTC of MEN2B is more aggressive than those seen in MEN2A or FMTC; 98 % of MEN2B carry *RET* mutations in either exon 16, codon 918, or exon 15, codon 833 [140–148, 160]. Directed testing for these mutations is the most sensitive, rapid, and accurate method for identifying individuals with MEN2B [154].

2.9.3 FMTC

MTC is the only manifestation of FMTC and typically follows a very indolent course. A kindred should have 10 or more *RET* mutation carriers with MTC and sufficient history to rule out pheochromocytoma or hyperparathyroidism [159]. FMTC is characterized by the same mutations as are found in MEN2A, with a few individuals having germline mutations in codons 768, 790, 791, 804, and 891 in *RET* exons 13, 14, and 15 [139–148, 160]. Molecular diagnostic testing is usually directed at the more frequently occurring of these mutations.

2.9.4 TC and MicroRNA Expression Profiling

Although not presently employed in TC molecular diagnostic testing, differences have been detected in the levels of specific microRNAs in PTC, ATC, MTC, oncocytic tumors, and FTC, with each histopathological TC type showing a unique microRNA expression pattern [161–164]. Additionally, the microRNA expression pattern changed with concomitant *BRAF, RET/PTC,* and *PAX8-PPAR-gamma*, but not with *RAS* mutations, showing possible microRNA expression pattern alterations with mutational status [164]. Although not presently employed in the molecular diagnostics of TC, different microRNA patterns are well established in TC and are likely to be incorporated into TC molecular testing in the future. Interestingly, microRNA studies have proven valuable in increasing our understanding of some thyroid lesions. For example, the hyalinizing trabecular adenoma is a distinctive thyroid neoplasm that shows some morphological features of PTC, including similar nuclear characteristics, psammoma bodies, and *RET/PTC* rearrangements [165–167]. Sheu et al. [168] analyzed microRNA expression and *BRAF V600E* mutation and *RET/PTC-1* and *-3* rearrangements in 18 hyalinizing trabecular adenomas. They found that microRNAs -146b, -181b, -21, -221, and -222, which are all significantly up-regulated in PTC, were suppressed in hyalinizing trabecular adenomas. Additionally, no *BRAF V600E* mutation and *RET/PTC-1* and *-3* rearrangements were detected. Based on this, the authors concluded that the hyalinizing trabecular adenoma and PTC are separate entities. The reasons for the discrepant findings of this study vs. previous studies concerning *RET/PTC-1* and *-3* rearrangements were not explained [167, 168].

2.10 Parathyroid Cancer: Mutations and Molecular Testing

Parathyroid cancer (PC) is a rare malignancy with an incidence of 0.4–4 % in all patients surgically treated for primary hyperparathyroidism [169, 170]. The majority of patients present with hypercalcemia, often found during a routine health screening, leading to a diagnosis of primary hyperparathyroidism. Typically, the clinical features will include high serum calcium levels (>14 mg/mL), parathyroid hormone levels at least five times above normal, a palpable lesion, bone symptoms, and operative findings suggestive of invasive growth. Histologic features of PC include broad intersecting fibrous bands within the tumor, increased mitotic index, stromal invasion, and angiolymphatic/perineural invasion. A definitive histological diagnosis of PC requires obvious tissue invasion or metastasis [169–171]. Interestingly, up to 86 % of PCs are not recognized as such intraoperatively and do not receive adequate surgical resection. In addition, in one series, half of all metastatic and recurrent PCs were initially diagnosed as benign [172, 173]. Currently there are no molecular diagnostic tests that can differentiate parathyroid adenoma (PA) from PC. Since clinically diagnosing a tumor as malignant based on metastasis is unacceptable, there is a strong need for such tests. Several areas of research appear promising.

2.10.1 Parafibromin Immunoreactivity

HRPT2 is a recently identified PC tumor suppressor found at 1q25 and has 17 exons that encode a 61 kDa nuclear protein parafibromin [174]. Germline mutations of the gene have been identified in kindreds with hyperparathyroidism-jaw tumor syndrome, which is characterized by childhood parathyroid hyperplasia, fibro-osseous jaw tumors, renal lesions, with a ~15 % lifetime chance of developing PC [175, 176]. Howell et al. [177] analyzed 60 parathyroid tumors , including five from individuals with hyperparathyroidism-jaw tumor syndrome, three with familial isolated hyperparathyroidism, three with MEN1, one with MEN2A, 25 sporadic PAs, 17 hyperplastic parathyroid glands, two lithium-associated tumors, and four sporadic PCs. All 17 exons and intron-exon boundaries were PCR amplified and analyzed by denaturing high-performance liquid chromotography. *HRPT2* mutations were found in all four sporadic PCs, all five tumors from individuals with hyperparathyroidism-jaw tumor syndrome, and in one of the two tumors from individuals with familial isolated hyperparathyroidism. The authors hypothesized that *HRPT2* mutation is an early event in PC carcinogenesis and may be a potential marker for both familial and sporadic PCs. Interestingly, parafibromin immunostaining is reduced in PCs compared to PA, with positive parafibromin nuclear staining largely excluding carcinoma and reduced staining likely indicating carcinoma or rare PAs with low staining [178, 179].

2.10.2 Cyclin D1

Cyclin D1 or *PRAD1* is located at 11q13 and encodes a cyclin-dependent kinase that plays a central role in regulating and promoting G_1S phase cell cycle progression [180]. Chromosomal rearrangements of the *cyclin D1* regulatory region have been detected in 8 % of PAs and 20–40 % of PCS, and 31 % of hyperplastic parathyroid glands overexpress cyclin D1 [181, 182]. The overexpression appears to result from the effects of growth factor stimulation and calcium-sensing receptor activation [183].

2.10.3 Cellular Proliferation Markers

Ki-67 and PCNA immunohistochemical staining may have some value in separating PC from benign hyperplasia and adenomas, although the literature is contradictory and due to the rarity of PC most studies include relatively few cases [184, 185]. Last, the proapototic gene product FHIT appears to be preferentially mutated in PC compared to hyperplastic or adenomatous parathyroid lesions [185].

2.10.4 Chromosomal Changes

PC and PAs show gains and losses in specific chromosomal locations, often where tumor suppressor genes (at 1p, 3q, 4q, 13q, and 21q) and oncogenes are located (at 1q, 5q, 9q, 16p, 19p, and Xq). Interestingly, these areas tend to be different between PCs and PAs, likely indicating a different molecular pathogenesis of these neoplasms [186]. Presently, none of the above molecular alterations identified in PC, PA, or hyperplasia are used in molecular diagnostics, and many of these alterations appear to be quite similar between PC and PAs. Further research is required before molecular diagnostic tests for parathyroid neoplastic lesions can be implemented.

2.11 Conclusion

Over the past 20 years, different histopathologically defined thyroid malignancies have been shown to have mutation and gene expression patterns that allow them to be clearly distinguished via molecular analysis. In many cases, such analyses allow a clear diagnosis where the histology is ambiguous [1, 14]. Additionally, in many cases, as with PTC *BRAF* and *c-MET*, these mutations give useful prognostic information. Last, inhibitors for many of the constitutively active mutated kinases found in the TCs are being developed, which will eventually necessitate molecular diagnostic testing, as is currently done for *KRAS* mutations in colorectal carcinoma

(see Chap. 5, Molecular Pathology and Diagnostics of Colorectal Cancer). Thus molecular diagnostics applied to these TCs are likely to increase. Currently, there are few molecular diagnostic procedures applied to parathyroid malignancies. However, there is a strong need for diagnostic tests that can differentiate PAs from PCs. Testing should become available as our knowledge of the molecular pathology underlying this malignancy increases.

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Chapter 3
Molecular Pathology and Diagnostics of Breast Cancer

William Kong, Ted Richards, Jin Q. Cheng, and Domenico Coppola

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Abstract Proper treatment of breast cancer often depends on timely and accurate diagnosis. Due to the heterogeneic nature of breast cancer, it is important to continually develop diagnostic methods and tools that strive to provide consistent and reliable results for optimal patient specific care. Early microscopic observations of tumor sections have revealed that specific morphological structures of the mammary gland, such as luminal or basal layers, are often mimicked by cancer cells. This has resulted in a hypothesis that hyperplastic cells originate from their respective anatomical sites. Newer findings now suggest breast cancer arises from mammary stem

W. Kong
Stanford University School of Medicine, Stanford, CA 94305-5101, USA

T. Richards • J.Q. Cheng • D. Coppola, M.D. (✉)
Department of Anatomic Pathology and Oncological Sciences,
H. Lee Moffitt Cancer Center & Research Institute and University
of South Florida, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: Domenico.coppola@moffitt.org

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or progenitor cells, which then differentiate into various lineages by molecular mechanisms not yet fully understood. Subtypes of breast cancers are reliably labeled aggressive, including triple negative and hereditary breast cancers. Molecular diagnosis is currently available through various platforms, including: MammaPrint®, Veridex®, Theros®, and Oncotype DX®, which have improved the resolution of diagnosis. Significant advancements in a variety of scientific disciplines in basic research, integration of next-generation sequencing technologies, and innovative computational and mathematical methods in integration of diverse data types on a large scale will likely translate into clinical applications such as breast cancer diagnosis.

Keywords Molecular diagnosis • Breast cancer • Mammary development • miR-155 • BRCA

Abbreviations

ER	Estrogen receptor
HER2/NEU	Human epidermal growth factor receptor 2
miRNA	microRNA
NMuMG	Normal mouse mammary gland epithelial cells
PR	Progesterone receptor
RS	Recurrence score

3.1 Introduction

Breast cancer is a heterogeneous disease first appreciated by histopathologists when they noted a large diversity of phenotypes while observing specimens under the microscope [1–3]. It was also apparent that different phenotypes often associated with different biological and clinical behaviors; leading to early diagnostic schemes based on appearance. Under the microscope, specimens had features that resembled microanatomical structures of the normal mammary gland, misleading histopathologists to hypothesize that breast cancer likely arises from these structures; hence, the terms ductal and lobular carcinomas were coined [4]. The first part of this chapter will provide background information on mammary gland development from the embryonic stages to post-pregnancy involution. It is important to grasp this concept to fully appreciate the original classification schemes of breast cancer. Then, we will briefly discuss the importance of histological grading and estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu) status. This is the foundation for understanding use of microarray technology to diagnose breast cancer, since it was proposed that phenotype is reflected by its gene expression signature. Numerous platforms, such as MammaPrint®,

Oncotype DX®, Theros®, have been developed in recent years to serve this purpose. Rapid development in breast cancer research utilizing various approaches ranging from biology to genetics and covering specific areas of interest including normal and cancer stem cell identification, protein-protein interaction(s), non-coding regulatory RNAs, genetics and epigenetics may provide improvements to current diagnostic approaches in the future.

3.2 Mammary Gland Development

The murine model has been utilized dependably to study basic mechanisms of mammary development. Development can be classified into stages: embryonic, pre/post-puberty, pregnancy, and involution. Each pregnancy begins a cycle of alveolar differentiation, milk production, and then involution. During embryonic development, the first significant event is marked by formation of the bilateral milk lines on embryonic day 10. These lines fragment and coalesce into five pairs of mammary placodes, forming elevated buds by embryonic day 11. The mammary buds then invaginate below the epithelial surface into a bulb and induce transformation of their underlying stroma into the mammary mesenchyme. Next, sprouts appear from each bud and penetrate into the mammary mesenchyme, forming a primitive tree-like structure by embryonic day 18 [5]. The sprouts extend, forming hollow ducts comprising an inner layer luminal epithelial cells surrounded by outer layer of basal myoepithelial cells. These layers play important functional roles during pregnancy where luminal epithelial cells and basal epithelial cells provide a niche for progenitors of alveolar sacs, while myoepithelial cells give rise to contractile cells for milk secretion [6, 7]. Different types of breast cancer were originally believed to arise from these distinct microanatomical structures of the normal breast, hence giving origin to the terminology of ductal and lobular carcinomas [8–10].

Mammary development seizes until puberty, where production of ovarian steroids initiates a second phase of growth that drives expansion and further branching of the ductal system. Terminal end buds, a structure resembling Edison's incandescent glass bulb, are located at the ends of each duct. Stem cells are believed to be located within this structure and are housed in a niche environment that enables them to self-renew and remain undifferentiated and quiescent [5–7]. Some stem cells located within the terminal end buds are programmed to undergo progenitor commitment and further differentiation as the ducts elongate, branch, and invade into the outer borders of the mammary fat pad. Additionally, both stem and progenitor cells are seeded along the ducts as sources of alveolar differentiation for each pregnancy event. After weaning is completed, these alveolar sacs undergo involution, returning the mammary gland back into a mostly ductile organ while adipogenesis fills in the voids to replenish the mammary fat pad [4, 6, 11, 12] (Fig. 3.1). It is believed that most in situ precursors lesions and invasive breast cancers arise from stem/progenitor cells and are able to give rise to all histological types [4, 13, 14]; therefore, the luminal and basal resemblance that have long since been appreciated by histopathologists.

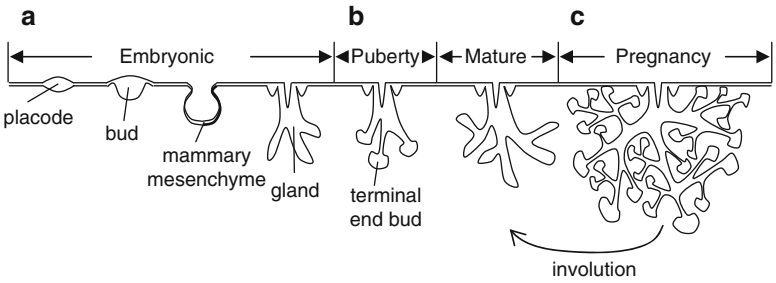

Fig. 3.1 Murine mammary gland development. (**a**) During embryonic development, bilateral milk lines are established by embryonic day 10 (E10). The milk line breaks up and coalesces into individual mammary placodes which transform into elevated buds by E11. Each bud continually develops and invaginates below the epithelial surface to form a bulb structure that condenses the underlying stroma and pushes into the mammary mesenchyme towards the fat pad. Sprouts branch from the bulb and penetrate into the mammary mesenchyme in formation of a primitive tree like structure by E18. (**b**) Formation of the mature mammary gland begins in the presence of circulating steroid and peptide hormones systemically produced at the onset of puberty. (**c**) Each pregnancy cycle involves the formation of milk producing alveolar sacs. The mammary gland undergoes post-lactational involution after weaning

3.3 ER, PR, HER2/neu, BRCA1/2, Grade, Stage, and Lymph Node Status

Current diagnosis emphasize standard clinical features such as the tumor size, extent of lymph node involvement, and distant metastasis and have all been integrated into the American Joint Committee on Cancer TNM classification standard and still play a central role in determining prognosis and treatment course by the physician [15–20]. Similarly, ER and PR status have been defined as important markers of breast cancer pathology decades ago, but now are gold standards in prescribing adjuvant therapy [21–28]. Another important marker for therapy response of breast cancer is the amplification of HER2/neu. Testing for this marker has become routine before prescribing Herceptin therapy. HER2/neu is a cell membrane surface-bound receptor tyrosine kinase belonging to the epidermal growth factor receptor family (ErbB) involved in signal transduction pathways promoting cell growth and differentiation [29–35]. Although its presence indicates aggressive tumor, trastuzumab, a specific antibody that targets the cell surface receptor, is supplemented, so that the receptor can be blocked from receiving growth signals [36–39].

A subtype of aggressive breast cancer, called "triple negative," lacks ER/PR/HER2 expression and grows without the need for external growth hormones [40–46]. It is more prevalent in younger women and characterized by early recurrence and visceral metastasis to the brain [40, 47, 48]. Fortunately, it is still sensitive to conventional chemotherapy and often prescribed concurrently with surgery [40, 41, 49–51]. Although the molecular biology of this breast cancer subtype is not yet fully understood, it has been recently associated with mutations in the two major breast cancer susceptibility genes, BRCA1 and BRCA2 [52–56].

BRCA1 and BRCA2 function as tumor suppressors involved in DNA damage response and DNA repair. Mutation in one copy of either BRCA1 or BRCA2 can increase the risk of hereditary breast and ovarian cancer with a lifetime risk of 50–80 % and 30–50 % for breast and ovarian cancer, respectively. Additionally, cancer development occurs earlier than non-hereditary breast cancer and is often aggressive [57].

Other points in early classification schemes are stage, grade, and lymph node status. Tumor size is used to assign stage, whereas the Nottingham histological grading system defines proliferative activity (mitotic index) and degree of differentiation (tubule formation and nuclear pleomorphism) of the tumor [58, 59]. Assessment of lymph node status is also part of tumor staging, and it can provide prognostic value. However, the combination of all three is often used to predict tumor prognosis. It has been reported that late stage and high grade can override the positive effects of negative node involvement [15, 17, 60–64].

Although determining ER, PR, and HER2 status may seem trivial and often conflicts with classification schemes, it is significant since it allows for targeted therapy. Targeting the ER and widespread use of the selective ER modulator tamoxifen in breast cancer are well-established targeted therapeutic modalities. These are responsible for the major improvements in cure rates, quality of life, and disease prevention for breast cancer observed over the past two decades. Targeting both HER2/neu with trastuzumab and the vascular endothelial growth factor with bevacizumab in combination with chemotherapy has become a further milestone in molecularly targeted therapy of this disease [65–69].

3.4 Gene Expression Arrays

The Stanford group pioneered class discovery through the use of high-throughput technologies and re-emphasized that breast cancer is not a single entity and that it can be classified into at least five groups by their molecular signature: luminal subdivided into luminal A and luminal B; basal-like; HER2; and a least characterized normal breast-like. Luminal tumors, like normal luminal epithelial cells of the breast, express cytokeratins 8/18, and are ER positive. This group is further subdivided into luminal A (lower grade and excellent prognosis) and luminal B (higher grade and poorer prognosis exemplified by higher proliferation rates). The basal-like breast cancers express genes resembling normal basal/myoepithelial mammary cells. For instance, they express cytokeratins 5, 6, 14, and 17, P-cadherin, caveolins 1 and 2, and EGFR. They are often ER, PR, and HER2/neu negative and are of higher histological grade characterized by a high mitotic index. They exhibit necrotic regions with lymphocytic infiltrate and often metastasize. HER2 tumors are ER negative with high HER2/neu expression. This type of breast cancer is aggressive but, unlike the basal like tumors, responds to adjuvant therapy. The last group is the normal breast-like and is not well characterized, but their gene expression profile resembles fibroadenomas and normal breast samples [4, 70–78].

Although classification based on gene expression arrays may seem more objective and straightforward, there are always exceptions. For instance, not all breast tumors that are *HER2* amplified can be categorized into the HER2 group. Some *HER2*-amplified tumors express ER, and their gene expression signature resembles the luminal type. Furthermore, the basal type is not always triple negative (ER, PR, and HER2), and a small subset of triple negative breast cancers is not basal like. Therefore, the use of microarrays to classify breast cancer might not always align with previous histopathological classifications. Nevertheless, MammaPrint®, Oncotype DX®, and Theros® have been developed in recent years for molecular diagnosis of breast cancer gene expression array technologies, and we provide a brief overview below of each [4, 70–82].

3.4.1 MammaPrint®

The first breast cancer classification through utility of gene expression profiles was demonstrated by van't Veer et al. [83]. The original data sets were samples from patients younger than 55 years old, tumor size smaller than 5 cm, and lymph node negative. They analyzed the gene expression signature of samples taken from breast cancer patients who developed metastasis within 5 years after diagnosis and compared these against the gene expression signatures of samples taken from breast cancer patients who were metastasis free during the same time. The analysis identified a 70-gene expression signature that could provide a predictive value for 5 years metastasis. This resulted in administration of proper treatment to patients with aggressive breast cancer.

The results from this study were validated in a subsequent study by Vijver et al. that encompassed more patients. They determined this gene set was an independent predictor of outcome in a multivariate survival analysis. Beyond its ability to demonstrate likelihood of metastasis, this gene expression signature could stratify node-positive patients based on disease outcome prediction. This assay is currently undergoing phase III clinical trial organized by the European Organization for Research and Treatment of Cancer. The trial is setup to profile the 70-gene expression signature and to simultaneously determine the clinicopathological characteristics for 6,000 patients. If the 70-gene expression signature and clinicopathological data agree, patients will be treated accordingly. However, if the two diagnostic tools differ, patients will be randomized and treated according to the results of the 70-gene expression signature or clinicopathological parameters [84–89].

Finally, this 70-gene expression signature has been shown to outperform traditional clinical and histological parameters for providing a prognostic value [90–93]. A downside to this gene expression signature is that it fails to stratify ER-negative or HER2-positive breast cancer since it predicts 96–100 % and 78–95 %, of them, respectively, which will have poor prognosis. For the remaining 5–22 % HER2-positive breast cancers not predicted to be in the high-risk group, the controversial question is whether to provide trastuzumab treatment or

not [93–95]. Regardless, this assay received FDA approval for use in patients younger than 61 years old, at stages I/II, node negative, and tumor size smaller than 5 cm [84–89].

3.4.2 Veridex®

In the original study, Wang et al. [96] separated 115 lymph node-negative breast cancer samples, from systemic therapy naïve patients, into 35 ER-negative and 80 ER-positive for analyses. From the analyses, samples from patients who were ER-positive with distant metastasis expressed 16 genes differentially, whereas samples from patients who were ER-negative with distant metastasis expressed 60 genes differentially. This resulted in a 76-gene signature that can predict likelihood of metastasis. A subsequent study expanded to examine 171 lymph node-negative tumors from systemic therapy-naïve patients. This gene expression signature was shown to be an independent prognostic factor in multivariate analysis. It should be noted that this gene expression signature failed to provide a predictive value when applied to ER-negative patients [96–99].

3.4.3 Theros®

Ma et al. analyzed the gene expression signatures of 60 patients uniformly treated with adjuvant tamoxifen alone. They analyzed the whole tissue sections and laser-capture microdissected samples from patients who responded to tamoxifen treatment and identified three strongly predictive genes: homeobox gene HOXB13, interleukin 17B receptor (IL17BR) and EST AI240933. The study showed expression ratios between HOXB13 and IL17BR were strongly correlated with recurrence and outperformed other clinical pathological predictors. A validation study was carried out from RNA samples extracted from paraffin-embedded tissues from a cohort of 20 patients. Then the HOXB13:IL17BR expression ratio was re-evaluated in a cohort of 206 postmenopausal patients with ER-positive breast cancer who underwent tamoxifen-only treatment from the North Central Cancer Treatment Group (NCCTG) 89-30-52 trial. It was further expanded into a study with a cohort of 852 patients. In these studies, the HOXB13-to-IL17BR ratios were shown to identify ER-positive breast cancer patients that underwent tamoxifen treatment but at high risk of recurrence. It was also shown that the HOXB13-to-IL17BR ratios were a predictor of outcome for ER-positive patients who were either adjuvant naïve or who underwent tamoxifen treatment. Although the HOXB13:IL17BR ratios are practical in these aspects, it appears to have limited ability to predict outcome in lymph node-positive patients. These studies have led to the development of Theros®, a RT-qPCR-based test performed in a central laboratory and commercialized by Biotheranostics. This test is recommended for lymph node negative ER-positive

breast cancers patients treated with surgery alone, to define risk of recurrence and benefit from endocrine therapy. At the time of this review, the HOXB13:IL17BR index has not yet been shown to predict adjuvant hormone therapy or chemotherapy benefit and has not been included in the American Society of Clinical Oncology or National Comprehensive Cancer Network guidelines for breast cancer treatment [100–104].

3.4.4 Oncotype DX®

The OncotypeDX® platform (Genomic Health Inc., Redwood City, CA, USA) utilizes RT-qPCR to analyze the expression of 16 cancer-related genes: Ki67, STK15, survivin, CCNB1, MYBL2, GRB7, HER2, ER, PGR, BCL2, SCUBE2, MMP11, CTSL2, GSTM1, CD68, and BAG1, in addition to five reference genes (*ACTB*, *GAPDH*, *RPLPO*, *GUS*, and *TFRC*). The test utilizes RNA extracted from formalin-fixed paraffin-embedded ER-positive breast cancer samples. Selection of genes was originally derived from published literature, genomic databases, and microarray data.

OncotypeDX® predicts risk of recurrence in ER-positive, lymph node-negative breast cancer patients and provides treatment guidelines via a quantitative system of continuous Recurrence Score (RS) that ranges between 0 and 100 to predict the risk of recurrence within 10 years. This score has also been shown to be an independent predictor of outcome in multivariate survival analyses where samples are categorized, based on the RS into three risk group: low (RS < 18), intermediate (RS = 18–31), and high (RS ≥ 31).

Studies have validated OncotypeDX® and demonstrated that breast cancer patients who are ER-positive with a low RS tend to have a low risk of recurrence and will not benefit much from chemotherapy. On the other hand, breast cancer patients who are ER-positive with a high RS tend to have a high risk of recurrence and can benefit from chemotherapy. This justifies the use of endocrine therapy in addition to chemotherapy for ER-positive patients with a high RS score. This assay has been implemented into the American Society of Clinical Oncology guidelines for the use of tumor markers in breast cancer and into the National Comprehensive Cancer Network guidelines for breast cancer treatment [105–110].

3.5 MicroRNAs

The miRNA paradigm shifted from worm development to cancer when G.A. Calin et al. identified miR-15 and miR-16 as tumor suppressors at 13q14 in chronic lymphocytic leukemia in 2002 [111]. It has been shown that hemizygous or homozygous loss of 13q14 occurs in more than 50 % of B-cell chronic lymphocytic leukemia, mantle cell lymphoma, and prostate cancers and only slightly lower in multiple myeloma; however, detailed genetic analysis of traditional genes located in this

region yielded inconclusive evidence. The discovery of miRNA prompted Calin et al. to revisit information coded within this region. They discovered that the genetic code for two miRNAs, miR-15 and miR-16, are indeed located in this area and hypothesized that deletion of 13q14 results in loss of miR-15 and miR-16 expression. This was confirmed when analyses showed 68 % of RNA samples from B-cell chronic lymphocytic leukemia patients do not express these two miRNAs [111]. Shortly after, Michael et al. showed a similarly reduced expression of mature miR-143 and miR-145 in colorectal cancer [112]; Metzler et al. showed an accumulation of miR-155 in Burkitt lymphoma [113], and a similar trend was reported for other types of B-cell lymphomas, including diffuse large B-cell lymphoma [114, 115]. In 2004, Takamizawa et al. showed that let-7 was often lost in lung cancer and had a direct correlation to postoperative survival. They also found that overexpression of exogenous let-7 in lung cancer cell lines leads to growth inhibition [116]. Later, to complement Takamizawa's findings, Johnson et al. published data demonstrating that let-7 is indeed a probable tumor suppressor by targeting oncogenic RAS [117]. These landmark studies established the importance of miRNAs in cancer, leading to an exponential growth in miRNA cancer research. Numerous studies reporting miRNA profiling of cancer specimens were published with the intent to determine the functional significance of individual miRNAs in addition to the speculation that miRNA profiles could have clinical practicality for tumor classification.

3.5.1 Breast Cancer Subtype miRNA Profiles

Early and recent miRNA profiles showed a subset of miRNAs differentially expressed in normal tissues compared to solid tumors including breast cancers. Furthermore, some of these miRNAs were associated with biopathologic features of breast cancer such as ER+/−, PR+/−, cancer stage, nodal status, vascular invasion, proliferation index, and p53 status [118–122]. miRNA profiles were also performed on the different molecular subtypes of breast cancer as defined by gene expression signatures. Furthermore, the use of miRNA profiling was applied to determining a miRNA signature of different mammary cell types [123–128].

Blenkiron et al. showed that 133 miRNAs are differentially expressed in the human breast and in the different molecular subtypes of breast cancer. They first classified 93 cases of primary human breast tumors by gene expression profiling into luminal A, luminal B, basal-like, HER2+, normal-like, and ER+/−. Using a bead-based flow cytometric miRNA expression profiling method, they identified differentially expressed miRNAs in the various molecular subtypes. These miRNAs include pathologically important miRNAs: members within the let-7, miR-200, and miR-10 family; the miR-17–92 and miR-214 clusters; and individual miRNAs such as miR-155, miR-21. Furthermore, they showed miR-100, miR-99a, miR-130a, miR-126*, miR-136, miR-146b, miR-15b, miR-107, and miR-103 can differentiate between the luminal A and luminal B subtypes, in addition to providing a profile for differentiating ER+/− and tumor grade [128].

Sempere et al. used locked nucleic acid array to profile breast cancer cell lines in addition to normal and tumor breast tissue specimens. Then, they implemented an in situ hybridization method to determine the localization of miRNAs within specific cell types of formalin-fixed, paraffin-embedded tissue specimens. The rational was that the miRNAs of interest should be aberrantly expressed within malignant cells rather than the involved stroma, infiltrating lymphocytes, and/or vasculature. They found miR-145 and miR-205 expression in myoepithelial cells of normal mammary epithelial structures, whereas their expression was significantly reduced or eliminated in matching tumor specimens. Let-7a, miR-21, miR-141, and miR-214 were detectable within luminal epithelial cells in normal tissue where let-7a was often decreased while miR-21 was increased in malignant cells. Interestingly, although the authors did not extrapolate on this finding, their heat map indicated elevated expression levels of miR-221, miR-222, miR-214, and miR-21 in ER- tumors [124].

3.5.2 MicroRNA-155

One miRNA rising to prominence in breast cancer is microRNA-155 (miR-155). It was originally identified to be differentially expressed in miRNA profiles [118, 119, 128]. Kong et al. discovered miR-155 may have implications in aggressive, invasive breast cancer using TGFβ induced epithelial to mesenchymal transition in normal mouse mammary gland epithelial cells (NMuMG), an *in vitro* cell line model often used to study mechanisms of breast cancer metastasis. NMuMG expressing miR-155 exhibits greater invasion and migration, whereas NMuMG without miR-155 had the opposite effect. miR-155 expression also induced cellular plasticity that could be rescued with RhoA reconstitution [129]. In another study, Kong et al. showed miR-155 induced breast cancer cell proliferation and chemoresistance by targeting FOXO3a [130]. Furthermore, Blenkiron et al. showed that miR-155 expression is highly elevated in basal-like and high-grade breast cancer [128].

Chang et al. recently published data showing that the tumor suppressor BRCA1 epigenetically controls miR-155 expression. They demonstrated that the R1699Q moderate risk variant, which exhibits a point mutation in the BRCT domain, loses its ability to repress miR-155, explaining how BRCA1-deficient cells express high levels of miR-155. Orthotopic transplantation of BRCA1+ MDA-MB-468 cells ectopically expressing miR-155 into athymic nude mice exhibited accelerated tumor growth relative to control. Analyses of BRCA1-mutant human breast tumors show inverse correlation between BRCA1 and miR-155 expression [131]. The complementation of *in vitro* experiments by Kong et al., with the clinical breast cancer profile work by Blenkiron et al., suggested that miR-155 has important clinical implications in aggressive breast cancer. The data of Chang et al. solidified the physiological importance of miR-155 in the critical BRCA1 mutant breast cancers. This suggests miR-155 could be a potential biomarker in addition to a therapeutic target for specific types of aggressive breast cancers [128–131].

3.6 Conclusions

Diagnosis of breast cancer has evolved from observing the physical appearance of the tumor under the microscope to quantifying expression of genes at the molecular level. Although the latter method may seem less subjective and more quantitative, it is still subject to variations including but not limited to sample handling and reagent lot variations among other factors. Implementation should probably not replace the former method supported by the expertise of a trained pathologist. Molecular diagnosis should probably be used in a combinatorial manner, although the cost of doing so might not justify its implementation. Furthermore, basic research in science now covers a variety of subjects such as epigenetics, regulation by non-coding RNAs, identification of cancer stem cells and understanding their functions from genetics to biology, and a vast variety of other disciplines in between. Furthermore, next-generation sequencing technologies and the technological means to integrate and analyze diverse data types on a large scale, which allows investigation of biological networks in diseased state, will likely drive further changes in molecular diagnosis of not only breast cancers but other diseases.

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Chapter 4
Molecular Pathology and Diagnostics of Non-small Cell Lung Carcinoma

Brian Quigley, Steve Ducker, and Farah Khalil

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B. Quigley, M.D. • S. Ducker, M.D. • F. Khalil, M.D. (✉)
Department of Pathology and Cell Biology, University of South Florida, Tampa, FL, USA

Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive,
Tampa, FL 33612, USA
e-mail: farah.khalil@moffitt.org

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Abstract Of all the molecular alterations that may have predictive value in non-small cell lung cancer (NSCLC), testing for *EGFR* mutations is usually the first step in determining course of adjuvant therapy. Activation of *EGFR* mutations, and perhaps amplification, predicts the response of NSCLC to tyrosine kinase inhibitors (TKIs). The presence of activating *KRAS* mutations predicts resistance to TKI therapy, but the value of this test is questionable in NSCLC given that the coexistence of both *EGFR* and *KRAS* mutations in the same patient is extremely rare and the presence of a *KRAS* mutation may not have much different significance than the absence of an *EGFR* mutation. In patients who are considered for gemcitabine therapy, measurement of ribonucleotide reductase subunit 1 (RRM1) expression levels may help predict which patients are less likely to respond, as higher levels of RRM1 have

been shown to overcome the anti-metabolite of this drug. Similarly, in patients being considered for platinum-based therapy, determination of excision-repair cross complementing-1 protein (ERCC1) expression level may help predict which patients are less likely to respond, given that ERCC1 repairs platinum-induced DNA damage. The use of these predictive factors ideally will help target therapy to individual tumors to achieve the best chance for long-term survival and to avoid side effects from medications that are unlikely to have any effect. Further studies will continue to refine testing and treatment algorithms.

Keywords Cancer • Lung • Carcinoma • Non-small cell • Molecular • Smoker • Squamous cell carcinoma • Adenocarcinoma • EGFR • KRAS • ALK • FISH • ERCC1 • RRM1

Abbreviations

ALK	Anaplastic lymphoma kinase
ARMS	Amplification refractory mutation system analysis
dNTP	Deoxyribonucleoside triphosphate
EGFR	Epidermal growth factor receptor
ELM4	Echinoderm microtubule-associated protein-like 4
ERCC1	Excision-repair cross complementing-1 protein
ERK	Extracellular signal-regulated kinase
FISH	Fluorescence in-situ hybridization
IHC	Immunohistochemistry
LOH 3p	Loss of heterozygosity of 3p
MASI	Mutant allele-specific imbalance
MEK	MAPK/ERK kinase
MRP	Multidrug resistance protein
NSCLC	Non-small cell lung cancer
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RRM1	Ribonucleotide reductase subunit 1
SNP	Single-nucleotide polymorphisms
TKI	Tyrosine kinase inhibitors

4.1 Introduction

In the United States, lung cancer is the number one cause of cancer deaths in both men and women [1–3]. Not surprisingly, given this fact, survival from lung cancer is poor. Although most cases of lung carcinoma can be attributed to cigarette smoking, a number of cases occur in people who have never smoked. As could be

expected from the different exposure to carcinogens between smoking and non-smoking patients, lung carcinomas in these populations tend to have different molecular abnormalities, supporting the argument for differing mechanisms of carcinogenesis. A consequence of unique molecular alterations is differing responses to targeted therapeutic agents. Predicting responsiveness of a tumor to particular drugs often involves determination of some specific molecular characteristic. Description of such predictive molecular markers with background on their role in tumorigenesis and evidence for their value as predictive markers will be discussed in this chapter.

4.2 Lung Carcinoma Basics

4.2.1 Survival and Response Rates

Across all treatments and tumor stages, the overall survival rate for non-small cell lung cancer (NSCLC) is only 16 % at 5 years [1]. Patients with chemotherapy-treated advanced NSCLC have an 8–10-month median survival compared with only 4 or 5 months when untreated [2]. Response rates for treated metastatic NSCLC are only 17–37 %, with a median survival of only 6.7–11.3 months, 1-year survival rate of only 31–46 %, and 2-year survival rate of 9–21 % [1–4]. Time to progression averages 4–6 months, and response to second-line therapy is far worse at 8 % [5].

4.2.2 Gender, Smoking Status, and Histology

Although 85–90 % of lung cancers in men are attributable to smoking, 25 % of cases in men and women combined are not caused by smoking [6, 7]. Furthermore, lung cancers in smokers and never-smokers have different molecular characteristics, some attributable to carcinogenic agents in cigarette smoke [6–9]. Not only are there often molecular differences between lung cancers in smokers versus never-smokers [6, 8, 10], but frequencies of histologic types vary as well. Depending on the patient population, adenocarcinomas (Image 4.1) account for 62 % of lung cancers in never-smokers but only 19 % of lung cancers in smokers. Squamous cell carcinoma, on the other hand, accounts for 28–52 % of lung cancers in smokers but only 5.9–18 % in never-smokers [8, 11, 12].

Gender differences are present as well. Squamous cell carcinoma accounts for 44 % of lung cancers in men compared to 25 % in women, while adenocarcinomas comprise 28 % of lung cancers in men compared to 42 % in women. Frequencies of small- and large-cell carcinomas are roughly equal between men and women [7]. Part of the gender differences can be explained by the fact that a higher percentage of never-smokers are women [11, 12].

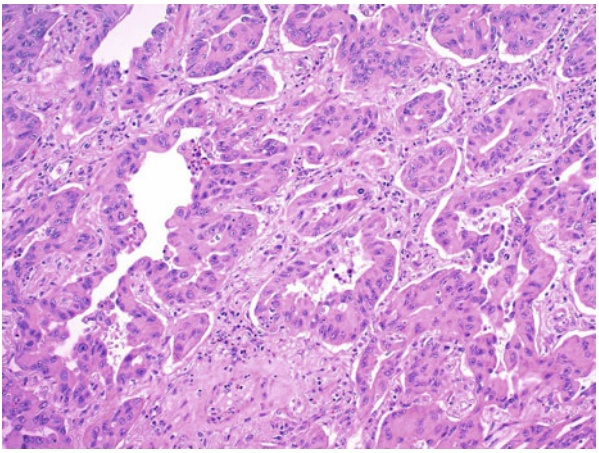

Image 4.1 Adenocarcinoma, 10× magnification

4.2.3 Treatment

The standard treatment for early NSCLC is surgery followed by adjuvant chemotherapy intended to prevent recurrences caused by micrometastases [2]. Advanced-stage or metastatic NSCLC is treated with a doublet chemotherapy regimen, for example, a doublet regimen, including cisplatin or carboplatin plus either docetaxel, paclitaxel, vinorelbine, gemcitabine, or irinotecan; other platinum combinations; or non-platinum combinations [1–4, 13–15]. Older patients or those with poor performance status may be treated with a single agent [14]. It has been shown that adjuvant chemotherapy in resectable NSCLC provides a 5–15 % absolute increase in overall survival [2, 16, 17].

4.2.4 Common Genetic Alterations in Lung Cancer

Various genetic alterations are often present in lung cancers, some of which may have specific prognostic or predictive value. The three most common genetic alterations found across all subtypes of lung cancer are loss of heterozygosity of 3p (LOH 3p), *TP53* mutations, and *PRb* mutations [7, 18–20]. Up to 80 % of cases demonstrate LOH 3p, which is the location of tumor suppressor genes such as *FHIT* [7, 9]. The *TP53* gene, which encodes the p53 tumor suppressor, is mutated in 50 % of NSCLC and 70 % of small cell carcinomas [7, 19]. Some alterations occur in specific histologic types. For example, squamous cell carcinomas frequently overexpress cytokeratins 5, 6, 13, 14, 16, 17, and 19 and infrequently harbor *KRAS* mutations [7]. In contrast, adenocarcinomas contain *KRAS* mutations in up to 30 %

(especially in smokers) [10], contain *TP53* mutations [10], and show overexpression of p27 [9, 21] and Cox-2 [7, 21].

Of the molecular alterations identified in lung carcinomas, some have been shown to have prognostic implications. Never-smoking status, high epidermal growth factor receptor (EGFR) protein expression, and increased *EGFR* gene copy number were all independent prognostic factors in the study by Hirsch et al. [22]. In squamous cell carcinomas, loss of PRb expression portends poor prognosis [7]. TP53 mutation has not been shown to have prognostic significance in squamous cell carcinomas but indicates poor prognosis for those with stage I and II adenocarcinomas [7, 19]. Poor prognosis may also accompany *KRAS* activating mutations and HER-2/neu overexpression [7]. Overexpression of p27 is correlated with well-differentiated tumors and indicates a good prognosis [7] (Fig. 4.1).

4.3 Theory Behind the Use of Molecular Predictive Markers

Despite the fact that, in the population as a whole, chemotherapy provides a survival benefit for NSCLC, not every patient will respond; worse yet, many may only suffer from the adverse effects. This includes death in 1–2 % of patients receiving platinum-based regimens [17]. To avoid treating patients who are unlikely to respond, it would be ideal to have predictive markers to determine whether a patient would be likely to respond to a given regimen [1]. Such predictive markers may be positive, such as the presence of an activating EGFR gene mutation predicting response to tyrosine kinase [22], or negative, such as a *KRAS* mutation predicting resistance to tyrosine kinase inhibitors (TKIs). Predictive markers are not to be confused with prognostic markers or factors that estimate a patient's likely outcome regardless of therapy [1].

Molecular alterations that serve as predictive markers are naturally going to fall into the category of a gene encoding drug metabolism, transmembrane transport, or target proteins [23]. Among the most studied predictive markers in lung cancer are EGFR, excision-repair cross complementing-1 protein (ERCC1), KRAS, and ribonucleotide reductase subunit 1 (RRM1). The following sections will discuss each of these markers, from the mechanisms by which they are thought to influence response to therapy and the clinical evidence supporting their value as molecular predictive markers.

4.4 Epidermal Growth Factor Receptor

4.4.1 Background

The most promising and widely reported molecular predictive factor in NSCLC is EGFR. The epidermal growth factor itself was first identified by Cohen in 1962 [24–26]. After epidermal growth factor binds to the EGFR, the receptor dimerizes,

autophosphorylates, and activates several pathways, including MAP kinase, Jak2/STAT3, STAT5, and phosphoinositide 3-kinase (PI3K/Akt), which stimulate proliferation, metastasis, and migration and prevent apoptosis [5, 27–31]. Levels of phosphorylated STAT3 are most significantly correlated with phosphorylated EGFR levels, indicating that most of the signals from activated EGFR are transmitted through the STAT3 pathway [5]. It has also been shown that EGFR that is constitutively active as a result of either an exon 19 deletion or L858R substitution preferentially stimulates STAT and AKT pathways, preventing apoptosis but not promoting proliferation [28, 32]. This is consistent with the observation that there is no association between *EGFR* mutation and stage or metastasis, indicating absence of a link to tumor progression [32].

Because the epidermal growth factor normally favors growth and proliferation, mutations of its receptor that render it constitutively active independent of ligand-binding lead to a malignant phenotype [33, 34]. Increased EGFR gene copy number or overexpression has also been investigated for each of their roles in oncogenesis. It has been suggested that, based on their mechanisms of action, EGFR monoclonal antibodies (such as cetuximab, matuzumab, and panitumab, which bind the extracellular domain of EGFR and prevent ligand binding) should be beneficial to patients with tumors overexpressing surface EGFR, whereas TKIs should benefit patients having tumor proliferation and metastases driven by EGFR autophosphorylation [24, 35, 36]. In the latter case, blocking the catalytic site of the receptor (responsible for activating downstream molecules) could conceivably prevent growth and proliferation and even favor apoptosis in cancer cells. Two promising drugs that target the catalytic domain of the EGFR by competing with ATP are the reversible TKIs erlotinib and gefitinib [5, 36, 37]. Tumors likely to respond to TKIs are the ones that are dependent on aberrant signaling through the EGFR pathway for survival [36, 38].

The TKI erlotinib has been shown in a randomized, placebo-controlled, phase III trial to provide significant survival benefit compared to placebo in unselected, previously treated advanced-stage NSCLC patients [25]. In this trial and in others published before the discovery that lung tumors harboring mutations in the *EGFR* gene are sensitive to TKIs, it was observed that patients with certain demographic characteristics responded better to these agents. Studies had demonstrated that higher response rates to gefitinib were observed in women, Japanese patients, and in those with adenocarcinomas [11, 29, 39, 40]. The higher response rate in Japanese patients and the better outcome in women were not attributable to differences in pharmacokinetics in these populations [40]. It was also known that never-smokers had higher response rates, longer times to progression, and longer median overall survivals when treated with TKIs than smokers [11, 41]. These clinical characteristics (female sex, Asian origin, never-smoking status, and adenocarcinoma histology) have been independently associated with response to erlotinib [25, 42] and gefitinib (never-smoking status and female sex in Japanese patients) [43], and it has been suggested that these clinical characteristics may be of value as surrogate predictors of response to TKIs when molecular profiles are not available [44, 45]. Three articles published in 2004 revealed that *EGFR* mutations are the common factor linking the aforementioned patient demographics with consistent response to TKIs [7, 46].

Although erlotinib was shown to provide survival benefit as compared to placebo in a phase III trial [25], it was disappointing that gefitinib was shown only to be non-inferior to docetaxel in a randomized, placebo-controlled phase III trial, with no benefit over docetaxel even in subgroups of women, patients with adenocarcinomas, never-smokers, or Asian patients [47]. Compared to placebo, however, gefitinib did provide significant survival benefit for never-smokers and Asian patients in the randomized, placebo-controlled ISEL trial [48]. Without this subgroup analysis, survival was not significantly longer with gefitinib even compared to placebo, although response rate was significantly higher with gefitinib in the whole group and most pronounced in subgroups of patients with adenocarcinoma, never-smokers, women, and Asian patients [48].

EGFR activity may be increased in NSCLC by several different mechanisms, which may be assessed separately in the lab. The gene, *EGFR* (located on chromosome 7p12), may be mutated and/or amplified and the EGFR protein may be overexpressed in the presence or absence of either of these abnormalities. Background information and clinical studies regarding the predictive value of each of these aberrations are reviewed in the following sections.

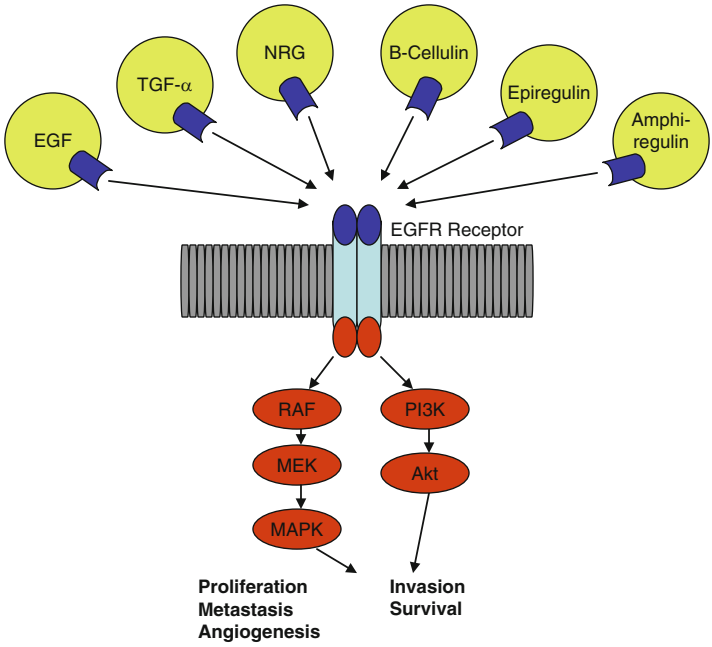

Fig. 4.1 EGFR Receptor involved in tumorigenesis of non-small cell lung cancer. *EGFR* epidermal growth factor receptor, *EGF* epidermal growth factor, *TGF* transforming growth factor, *NRG* neuregulin, *RAF* v-raf 1 murine leukemia viral oncogene homolog 1, *Akt* protein kinase B, *PI3K*, phosphatidylinositol-3-kinase, *MEK*, *MAPK* mitogen-activated protein kinase

4.4.2 EGFR *Mutations*

4.4.2.1 Types of Mutations

Many different types of mutations have been identified in the *EGFR* gene, approximately 90 % of which are either small deletions from exon 19 (codons 746–750) or substitution of arginine for leucine at position 858 in exon 21 (L858R). Another 3 % are substitutions of a variety of amino acids in place of glycine at codon 719 (G719X) and 3 % are in-frame insertions in exon 20 [7, 29, 37, 46, 49–51]. The overall incidence of *EGFR* mutations in NSCLC ranges from 12.1 % to 49 %, depending on the patient population [37, 46, 49–51]. Tyrosine kinase domain mutations of *EGFR* are rare in non-lung tumors [28].

The exon 19 deletions and L858R substitution are activating mutations that result in increased phosphorylation of EGFR independent of ligand stimulation [11, 28, 29, 52]. These mutant EGFRs have greater affinity for TKIs and are susceptible to inhibition by TKIs in vitro [34, 52]. Politi et al. and Ji et al. each showed that expression of either the exon 19 deletion or L858R substitution mutation in *EGFR* causes adenocarcinoma to develop in mice and that the tumors regressed when either expression of the mutant gene was blocked or erlotinib was administered [33–35, 53]. Greulich et al. obtained similar results in cell cultures and confirmed that these mutations result in constitutive ligand-independent receptor activation, which is further enhanced by the addition of ligand stimulation [54]. In summary, *EGFR* genes specifically with activating mutations produce EGFR that demonstrates ligand-independent stimulation of downstream growth and survival pathways that produce the malignant phenotype [5, 11, 33–35, 53, 55].

4.4.2.2 Clinical Characteristics of Patients with *EGFR* Mutations

Since the discovery that NSCLCs with *EGFR* mutations may be more responsive to TKIs, the characteristics of patients and tumors harboring these mutations have been validated again and again. A compilation of data from 2,880 patients generated in the review by Mitsudomi et al. in 2007 shows that *EGFR* mutations are found in 32 % of Asians compared to 7 % of non-Asians, 38 % of females compared to 10 % of males, 47 % of never-smokers compared to 7 % of smokers, and 30 % of adenocarcinomas compared to 2 % of non-adenocarcinomas [29]. Many other studies have also found *EGFR* mutations to be significantly associated with females, Asian origin, never-smoking status, and adenocarcinoma histology [22, 36–38, 41, 42, 45, 46, 51, 56–58], which overlap with characteristics of patients who respond to TKIs [25]. From a different angle, smoking has been shown not to be a risk factor for *EGFR*-mutant NSCLC, whereas female sex is a significant risk factor [59]. A significant association between presence of *EGFR* mutation and younger age was even demonstrated by Eberhard et al. in the phase III TRIBUTE study [49]. Compared to white patients, African Americans are significantly less likely to have

activating mutations of *EGFR* [60]. Among Korean patients with adenocarcinomas, the presence of *EGFR* mutations has not been shown to be associated with gender, smoking history, histological grade, age, bronchioloalveolar component, or stage [61]. Despite the statistical associations, it is important to remember that there will be exceptions to the rules. Park et al. in 2009 found that 3 of 20 male patients with squamous cell carcinoma of the lung had *EGFR* mutations, all of which responded to gefitinib, and, in addition, one of the *EGFR*-wild-type patients responded as well [38].

4.4.3 *Histology of* EGFR-*Mutant Tumors*

With respect to histology, it has been shown that, among adenocarcinomas that contain *EGFR* mutations, the majority are of terminal respiratory unit type [28, 62]. This subset of lung adenocarcinomas is characterized by cytologic resemblance to type II pneumocytes, Clara cells, or nonciliated bronchiolar cells [62]. Positive immunohistochemical staining for both thyroid transcription factor-1 and surfactant precursor protein B is a highly sensitive and specific method of identifying TRU-type lung adenocarcinomas [62]. *EGFR* mutations have also been identified in areas of atypical adenomatous hyperplasia, a preneoplastic lesion [62].

4.4.4 *Relationship of Smoking Status to* EGFR *Mutations*

The first molecular alteration that is actually found more often in non-smokers with NSCLC than in smokers is mutation of *EGFR* [28, 35]. Although some studies have shown a significant relationship between *EGFR* mutation presence and non-smoking status [6, 45, 56, 63, 64], and others have shown a trend toward lower *EGFR* mutation incidence with higher cumulative smoke exposure [29], not all studies have found a significant relationship between presence of *EGFR* mutation and smoking status [49, 61]. Even though a given never-smoker may be more likely to have an EGFR mutation than a smoker, *EGFR* mutations are still occasionally identified in smokers. Mitsudomi et al. in 2007 found *EGFR* mutations in 20 % of NSCLC from smokers, explaining that the reason *EGFR* mutations are found in a smaller percentage of tumors from smokers is that they are diluted by tumors arising from different smoking-induced mechanisms not usually present in never-smokers [30, 31, 35, 59]. To further investigate the relationship between smoke exposure and *EGFR* mutation status, Lee et al. examined the incidence of *EGFR* mutations in never-smokers with and without environmental tobacco smoke exposure, finding a significantly lower incidence of *EGFR* mutations in those with environmental tobacco smoke exposure than in those without. They also found a trend toward lower response to TKIs in patients with environmental tobacco smoke exposure compared to those without [64].

4.4.5 EGFR *Mutation as a Molecular Predictor of Response to TKIs*

The heart of determining the usefulness of *EGFR* mutation status as a predictive factor for response to TKIs lies in the clinical trials and retrospective studies investigating this relationship. In 2004, Lynch et al. was one of the first groups to demonstrate that *EGFR* mutations are associated with clinical response to gefitinib and to show in vitro that *EGFR* mutants were more sensitive to gefitinib. No mutations were present in patients who did not respond to gefitinib. In vitro, both the exon 19 deletion mutants and L858R mutants were more sensitive to gefitinib than tumors with wild-type *EGFR*. All eight mutants they identified were heterozygotes, indicating that these mutations act in a dominant manner (as do other oncogenes) [46]. Paez et al. promptly confirmed these results [41] and Pao et al. soon added that *EGFR* mutations were also identified in tumors sensitive to erlotinib [45]. Since then, many studies have confirmed association between *EGFR* mutation status and response to TKIs, and a review of the literature by Mitsudomi et al. in 2007 compiling data from 1,335 patients showed that EGFR TKI response was observed in 70 % of *EGFR* mutant tumors compared to only 10 % of patients with *EGFR* wild-type tumors [15, 29, 43, 58, 63, 65, 66]. In the phase II IDEAL trial, *EGFR* mutant tumors responded better to gefitinib than those with wild-type *EGFR* [58]. Kalikaki et al. demonstrated that patients with classic activating *EGFR* mutations treated with gefitinib had significantly longer median overall survival than those with wild-type EGFR. This relationship was not present in patients with other types of *EGFR* mutations [55]. Significantly higher objective tumor response to TKIs in *EGFR* mutants compared to wild-type has been demonstrated by several studies [37, 50, 65, 67], although others have found no relationship [42].

Differential response to treatment between the different types of *EGFR* mutations have been observed by some authors. Mitsudomi et al. reported that deletional mutations had higher response rates to gefitinib than other mutations, especially L858R [29]. Along those lines, Hirsch et al. found that patients who only had exon 19 deletions had significantly higher objective response than patients with exon 21 mutations [22]. On the contrary, Pao et al. found in vitro that wild-type *EGFR* and exon 19 deletion mutants responded similarly to gefitinib and erlotinib, whereas L858R mutants were about 10 times as sensitive [45]. As of yet, there has been no report of response to TKIs in a patient with an exon 20 insertion [29].

Not all patients with activating mutations of *EGFR* respond to TKIs [68]. Conversely, some patients without *EGFR* mutations still respond to TKIs [7, 67, 68]. In 2009, Yoshioka et al. published a phase II prospective study with *EGFR* mutation status determination followed by erlotinib treatment and determined that *EGFR* wild-type tumors have "modest" response to erlotinib without irreversible toxicity [69]. Some of the mechanisms by which patients harboring *EGFR* mutations become resistant to TKI therapy are discussed later in this chapter.

4.4.6 EGFR *Mutation as Molecular Predictor of Response to Chemotherapy*

TKIs are not FDA approved for use as first-line therapy for NSCLC in the United States [36]. Because first-line therapy is limited to conventional chemotherapy (a doublet regimen: for example, cisplatin or carboplatin plus either docetaxel, paclitaxel, vinorelbine, gemcitabine, or irinotecan; other platinum combinations; or non-platinum combinations) [2–4, 13, 14] (as mentioned previously), naturally the impact of *EGFR* mutation status on response to initial chemotherapy has been investigated. Results of such studies have been conflicting, with many studies reporting no association, while Hotta et al. showed that *EGFR* mutations were associated with longer overall and progression-free survival but not better response to front-line chemotherapy (consisting of various regimens, including both platinum- and non-platinum based) and other studies showed that only patients who carried deletions in exon 19 of *EGFR* had response to initial chemotherapy [15, 55]. Kalikaki et al. found a higher objective response rate to initial chemotherapy (particularly platinum-based regimens) in patients with classic activating EGFR mutations compared to wild-type *EGFR* [55]. First-line cytotoxic chemotherapy response was not correlated with *EGFR* mutation status in the study by Yoshida et al. (the exact chemotherapy regimens were not specified in this study) [69].

4.4.7 *TKIs Versus Chemotherapy in* EGFR *Mutant Tumors*

Although many studies have shown a benefit of erlotinib over placebo in *EGFR-mutation* positive patients, differential responses between TKIs and conventional chemotherapy have been more difficult to demonstrate. In the phase III TRIBUTE study, erlotinib did not provide any survival, objective response rate, time to progression, or duration of response advantage over carboplatin plus paclitaxel alone in *unselected* patients [44, 49]. Similarly, gefitinib did not provide any survival advantage compared to docetaxel in unselected patients or subgroups with clinical characteristics associated with *EGFR* mutations or *EGFR* mutations themselves in the phase III INTEREST trial [47]. However, patients with *EGFR*-mutant tumors had significantly improved objective response rate compared to wild-type tumors when treated with erlotinib plus carboplatin plus paclitaxel in the TRIBUTE study [49]. There was even a non-significant trend toward better response to erlotinib plus carboplatin plus paclitaxel compared to carboplatin plus paclitaxel alone in *EGFR-mutant* patients [49]. Regardless of treatment arm in this study, patients with *EGFR*-mutant tumors had significantly better response rate and longer median time to progression than those with wild-type tumors [49]. The randomized, placebo-controlled INTACT trials did not show added benefit from gefitinib in addition to chemotherapy alone in unselected patients [58]. In at least one study, higher objective response rate was observed in *EGFR* mutation-positive patients treated with gefitinib compared to docetaxel, although mutation status did not predict better survival [70].

4.4.8 *Impact of* EGFR *Mutation on Survival*

Impact of *EGFR* mutation status on treatment response is of great importance, but may have little meaning to patients unless there is a concomitant impact on survival. Longer median survival (but not progression-free survival) after first-line chemotherapy was observed in patients with *EGFR* mutations [71]. Overall and progression-free survival following first-line chemotherapy were also significantly longer in patients with *EGFR*-mutant tumors than in those with wild-type *EGFR* in the study by Hotta et al. [15]. With respect to TKI (gefitinib) treatment, use of this agent as first- or second-line therapy resulted in significantly longer progression-free survival in *EGFR*-mutant patients than in those treated with chemotherapy [71]. In a series of 397 patients by Kosaka et al. in 2009, patients with *EGFR* mutations survived significantly longer than those with wild-type mutations, with no survival difference between exon 19 deletion and L858R substitution [51].

4.4.9 *Molecular Prognostic Value of* EGFR *Mutations*

Because subset analyses of the TRIBUTE and INTACT trials have shown that better response was observed in patients with *EGFR* mutations patients even on chemotherapy alone (not just with chemotherapy plus TKI), it has been argued that *EGFR* mutations are prognostic factors instead of predictive [35, 49, 58]. Other studies have confirmed that patients with *EGFR* mutations (or clinical characteristics classically associated with *EGFR* mutations) have longer survival on chemotherapy even without TKIs [47, 49, 55, 58, 71]. Marks et al. found a trend toward longer overall survival in patients with *EGFR* mutations and stage I disease compared to patients with *EGFR* wild-type or *KRAS*-mutant tumors [72]. On the other hand, both Kosaka et al. and Shigematsu et al. showed that *EGFR* mutation was not a significant prognostic factor in patients treated with surgery alone [35, 51, 57]. In 2009, Kosaka et al. again showed in their series of 397 patients that neither the exon 19 deletion nor the L858R substitution mutation was an independent prognostic factor [51]. It has been suggested that, because of the characteristics of patients who tend to have *EGFR* mutations, they have better prognosis regardless of treatment given [7, 47].

4.4.10 *Sensitivity of* EGFR *Mutation Detection Methods*

As important as detection of *EGFR* mutations may be for patients with NSCLC, currently implemented detection methods may be missing a large portion of positive cases. Hirsch et al. compared two methods to detect *EGFR* mutations: direct sequencing and amplification refractory mutation system analysis (ARMS). ARMS uses allele-specific PCR to detect the exon 21 L858R point mutation and the most

common exon 19 deletion (del G2235-A2249). They concluded that ARMS is more sensitive than sequencing (for detecting the most common mutations) because 10 of the 17 mutations detected by ARMS were missed by sequencing. However, since sequencing is not restricted to detecting specific known mutations, it identified overall 10 different mutations that were missed by ARMS; therefore, it is useful in detecting novel or uncommon mutations [37].

4.5 *EGFR* Gene Copy Number

4.5.1 *Background and Clinical Associations with* EGFR *Amplification*

EGFR gene copy number has also been investigated for its possible predictive and prognostic values in NSCLC. In general, a gene's copy number may be increased as a result of true amplification or it may accompany polysomy of its entire chromosome. Gene copy number in either case is determined using fluorescence in-situ hybridization (FISH) [73, 74]. The frequency of FISH positivity for *EGFR* ranges from 30 % to 50 % in NSCLCs and is more frequent in older patients and women in some studies [22, 37, 50, 58, 60, 63, 70]. In the IDEAL and INTACT trials, the demographic characteristics that are similar between patients with *EGFR* mutations and those that respond to TKIs (female, never-smokers, adenocarcinoma, Asian origin) were not associated with *EGFR* amplification [58]. In one study, *EGFR* FISH positivity was observed significantly more often in African Americans than whites, which was entirely attributable to the cases with high polysomy [60]. Never-smokers have been shown to more often have high *EGFR* gene copy numbers than smokers [22, 63, 70].

4.5.2 *Relationship Between* EGFR *Mutation and Amplification*

Scholl et al. reported on a significant relationship between *EGFR* gene amplification and exon 19 deletions [75]. In a study of 99 pulmonary adenocarcinomas from chemotherapy-naïve, never-smoking east Asian women, significantly more exon 19 deletions were present in tumors with *EGFR* amplification than in those with *EGFR* disomy or low or high polysomy [75]. Further, only the mutated, not the wild-type allele, was amplified [75]. They concluded that *EGFR* amplification only occurs in tumors with exon 19 deletions of *EGFR* and not other types of mutations or wild-type *EGFR* [75]. Other studies have found other mutations associated with *EGFR* amplification; however, in patients treated with prior chemotherapy [75], gene amplification is more often associated with *EGFR* mutations than is polysomy [73].

They further reported that, in tumors with exon 19 deletions, constitutive activation of EGFR occurs only in the presence of amplification [75]. In cells with the L858R substitution, constitutive activation was observed regardless of gene amplification [75]. Other studies have confirmed association between *EGFR* mutation of unspecified type and *EGFR* FISH positivity [63, 66, 67]. Increased EGFR expression by immunohistochemistry (IHC) was also significantly associated with FISH positivity but not mutation status [63, 76].

4.5.3 EGFR *FISH: Criteria and Special Considerations*

Varella-Garcia et al. have proposed the following criteria to define FISH positivity for *EGFR*: at least 40 % of the cells must have at least 4 copies of the *EGFR* signal, or the *EGFR* to centromere enumeration probe for chromosome 7 (CEP7) ratio must be at least two (averaged over all nuclei), or there must be gene clusters (at least 4 spots) in at least 10 % of tumors cells, or there must be at least 15 copies of the *EGFR* gene in at least 10 % of the tumor cells [74]. The result is equivocal if none of the above criteria is met but at least 25 % of the cells have at least four signals; in this case, if a second reviewer calls it positive, it is reported as positive; otherwise the test is reported as negative [74].

Several reports state that TKIs may benefit patients with either *EGFR* amplification or 7p polysomy. However, gene amplification is more often associated with *EGFR* mutations than is polysomy [73]. Since the two mechanisms of increased *EGFR* gene copy number may have different predictive values, a method has been proposed to more reliably distinguish true gene amplification from chromosome 7 polysomy. The *EGFR* gene itself lies on chromosome 7p12, which is close enough to the centromere of chromosome 7 that gene amplification of this region may also amplify parts of the centromere, giving the false impression that the whole chromosome has been duplicated (polysomy 7). The 7q31 region is sufficiently far from both the centromere and the *EGFR* locus that identification of this region may help distinguish signals from *EGFR* amplification and signals from the centromere [73].

4.5.4 *Molecular Predictive Value of* EGFR *Gene Copy Number Versus Mutation*

Most of the studies assessing the predictive value of *EGFR* amplification compare this to the predictive value of *EGFR* mutation. Capuzzo et al. were the first to report that survival following gefitinib was best predicted by increased *EGFR* gene copy number compared to *EGFR* mutation status or EGFR expression by IHC [29, 44, 63]. At least three other groups found *EGFR* gene copy number to be a better predictor of response to TKIs than *EGFR* mutation status [29, 35, 37, 42, 44, 71, 77]. In one study, *EGFR* FISH positivity was even associated with significant survival benefit

from erlotinib, which was not observed for tumors with wild-type *EGFR*, mutant *EGFR*, or those that were *EGFR* FISH-negative [50]. A significantly better response to gefitinib compared to docetaxel was seen in patients with high *EGFR* gene copy number in another study, although gene copy number was not associated with better survival in this study [70].

In the Hirsch study, patients whose tumors were *EGFR* negative by both FISH and IHC had poor response to gefinitib regardless of *EGFR* mutation status. Inversely, patients positive for *EGFR* by both FISH and IHC had excellent outcome independent of *EGFR* or *KRAS* mutations. These findings suggest that *EGFR* gene copy number is a better predictor of response to gefitinib than *EGFR* mutation status [22, 35, 37, 71]. Hirsch et al. determined that the combination of *EGFR* gene copy number and protein expression by IHC is the most predictive of response to gefitinib and concluded that "*EGFR* gene copy number is a predictor of survival benefit with gefitinib compared with placebo" [22, 37]. Takano et al. found after multivariate analysis that response to gefitinib in Japanese patients could be predicted by either high *EGFR* gene copy number or *EGFR* mutation alone but pointed out that the significance of increased copy number is clouded by the observation that mutation presence predicts response whether increased copy number is present or not [67].

On the other hand, at least three groups found *EGFR* mutation status to be a better predictor of response to TKIs than *EGFR* gene copy number [43, 66, 68, 71]. Variations in the demographics of the study populations may be partly responsible for the conflicting results. In fact, Gerber has pointed out that *EGFR* gene copy number seems to have more predictive value in Caucasian populations (who have low *EGFR* mutation frequency) [42, 63], whereas *EGFR* mutation status holds more predictive value in East Asian populations (who have higher *EGFR* mutation frequency) [36, 42, 58, 63, 67].

Not all studies have found evidence to support the predictive role of *EGFR* FISH positivity. Both the INTEREST and INVITE phase III studies failed to show association of *EGFR* FISH positivity with prediction of benefit from gefitinib compared to chemotherapy [47, 60, 78]. Although *EGFR* FISH positivity was associated with TKI response in univariate analysis in the studies by Han et al. in 2006 and Ichihara et al. in 2007, in multivariate analysis, the only factor that independently predicted both response and survival from gefitinib was gefitinib-sensitive *EGFR* mutation [35, 43, 66, 71]. Further, while *EGFR* FISH positivity may predict response to TKIs, it has no predictive value for response to chemotherapy [76].

4.5.5 Molecular Prognostic Value of EGFR *FISH Positivity*

In contrast to its association with favorable response to TKIs, *EGFR* FISH positivity may be a negative prognostic factor [7, 63, 76]. Zhu et al. in 2008 found that *EGFR* FISH positivity was a poor prognostic marker by multivariate analysis [50]. Sholl et al. also found significantly worse outcome (shorter disease-free survival and higher stage at presentation) in patients with *EGFR* amplification [75]. In the Hirsch study,

there was a nonsignificant trend toward worse outcome in patients with high *EGFR* gene copy number compared to low gene copy number in the placebo group [22]. To reiterate, this is in contrast to the better outcome predicted by EGFR FISH positivity in patients treated with gefitinib [37]. Tsao et al. and Dziadziuszko et al. did not find *EGFR* gene copy number to have prognostic value [42, 76]. However, at least one study has suggested a contradictory prognostic implication of *EGFR* FISH positivity [77].

4.6 EGFR Protein Expression Levels

4.6.1 Background

The last mechanism of increased EGFR activity to be discussed is protein overexpression, determined using IHC. Up to 90 % of NSCLCs strongly express EGFR protein by IHC [37, 70, 79], more frequently in squamous cell carcinomas than in adenocarcinomas [39, 40, 76], which is notably the opposite histologic association found with *EGFR* mutations. Although the overexpression of wild-type EGFR does not result in the malignant phenotype [31, 53], and many studies have not demonstrated a relationship between EGFR protein expression and response to TKIs [27, 39, 47, 79, 80], other studies have shown better response to TKIs in tumors expressing EGFR by IHC [7, 22, 37, 42, 44, 50, 63].

Ethnicity-associated EGFR genotypes may underlie some of the predictive value of EGFR overexpression. *EGFR* gene transcriptional activity and EGFR expression are related to the number of CA repeats contained in intron 1, with a higher number of repeats correlating with lower *EGFR* transcription, lower levels of EGFR mRNA, and lower EGFR protein expression [7, 81, 82]. The 20-repeat allele has only approximately 20 % the EGFR transcription activity of the 16-repeat allele [7, 81, 83]. Assuming for a moment that EGFR protein expression may affect response to TKIs, differences in the number of CA repeats may account for some of the racial differences in response to TKIs in EGFR-positive tumors; for example, higher response rates in Japanese patients treated with TKIs may be because in Asia the 20-repeat allele is the most common, whereas Caucasians and African Americans more commonly have the 16-repeat allele [7, 61, 83].

4.6.2 Molecular Predictive and Prognostic Value of EGFR Overexpression

Based on evidence that activating mutations or amplification of *EGFR* predicts response to TKIs, one may suspect that EGFR overexpression may have similar predictive value. Indeed, Tsao et al. found expression of EGFR by IHC to be

associated with response to erlotinib but not with survival [42]. Parra et al. also found that, within a subset of patients with adenocarcinoma, response to gefitinib was significantly associated with high EGFR expression by IHC [39]. Whereas some authors have suggested that EGFR overexpression by IHC may predict response to TKIs, the evidence that overexpression may be related to response to conventional chemotherapy is limited [76, 84]. Comparing response to a TKI and conventional chemotherapy, there was no differential survival between gefitinib treatment and docetaxel treatment with respect to EGFR expression status in the phase III INTEREST trial [47]. Evidence for any predictive value of EGFR overexpression is sparse; in fact, it has been reported that EGFR overexpression actually correlates more with the occurrence of skin toxicity resulting from TKIs than to response of the tumor [7]. Although at least one study demonstrated significant poor prognostic value of high EGFR expression by IHC [39], neither the predictive nor the prognostic value of EGFR protein expression has been established [39, 42, 63, 76, 79, 80, 85].

Results of studies comparing EGFR expression to therapy response are dependent on the method of protein detection (i.e., the specific antibody used), and such differences between studies may account for some of the conflicting results [39, 79]. Comparing results from four different antibody kits, Mathieu et al. found three kits to yield 80 % positivity in a series of cases, while the fourth kit yielded only 68 % positivity [79]. Additionally, biopsies were more often positive than surgical or cytology specimens using one particular kit, while metastases were more often negative than primary tumors using another kit [79].

4.7 *EGFR* Summary

Over time, differing opinions have existed on whether there is enough data to confirm the predictive value of *EGFR* mutations. In 2005, the authors of the TRIBUTE study believed that the use of *EGFR* mutation status as a marker predicting benefit to erlotinib plus chemotherapy was not clear because, although *EGFR* mutants had significantly better objective response rates than wild-type *EGFR* when treated with erlotinib plus carboplatin and paclitaxel, adding erlotinib to carboplatin plus paclitaxel did not improve response in *EGFR* mutants compared to carboplatin and paclitaxel alone [49]. Later publications have stated a more definitive predictive value, which likely arose from more and more data confirming the superiority of *EGFR* mutational analysis over IHC and FISH. In 2006, Han et al. found that, in multivariate analysis, the only factor that independently predicted both response and survival from gefitinib was gefinitib-sensitive *EGFR* mutations [35, 66]. Kalikaki et al. in 2009 went as far as to show that classical activating mutations of *EGFR* are an independent factor predicting response to first-line chemotherapy [55]. Thus, although amplification and overexpression of EGFR may have some predictive value, their analysis has not been employed clinically as often as mutation analysis.

Even though the presence of an activating mutation of *EGFR* may indicate that a patient is more likely to respond to TKIs, some patients without mutations do respond. With this in mind, a patient with a good performance status who does not respond to first-line chemotherapy may wish to pursue TKI therapy regardless of *EGFR* mutation status. EGFR testing can also help with estimating prognosis in some patients.

Testing is not warranted in all patients, especially when the test result (e.g. *EGFR* mutation status) is not going to change clinical management (e.g., administration of a TKI). Other patients who may not require testing for *EGFR* mutations include patients who come for second opinions and are already taking TKIs and showing response, patients with such poor performance status and for whom systemic therapy would not even be considered, and patients in whom initial biopsy material is insufficient for testing and risk of re-biopsy outweighs benefit. In addition, difficulties arise for patients who lack insurance or have insurance issues and thus may have difficulties paying for testing and/or treatment (Dr. Jhanelle Gray, telephone interview). For these types of issues, individuals should seek help from assistance programs.

In summary, patients with activating *EGFR* mutations are more likely to respond to TKI therapy and probably have improved prognosis independent of therapy.

4.8 Tyrosine Kinase Inhibitors

4.8.1 Adverse Effects of Tyrosine Kinase Inhibitors as Predictors of Response

As might be expected from its name, epidermal growth factor is critical for mediating differentiation and proliferation of keratinocytes, explaining why a rash often develops with its inhibition [31, 86, 87]. In some studies, the development of acneiform rash has been quite convincingly correlated with better tumor responses and improved overall and progression-free survival with TKI treatment, likely because skin toxicity is related to systemic drug concentration and activity [5, 80, 86, 88, 89]. There has also been a significant correlation between grade of rash and degree of tumor response to erlotinib [89]. Accordingly, some authors consider skin toxicity to be a predictive marker of response to TKIs [88].

4.8.2 Molecular Alterations Conferring Resistance to TKIs

Although many tumors with *EGFR* mutations initially respond to TKIs, disease progression invariably occurs (usually within 6–12 months), commonly by selective survival of tumor cells with acquired mutations conferring resistance to these agents

[29, 35, 90, 91]. Even small clones of tumor cells containing a mutation that confers resistance to TKIs may have a significant clinical impact [91, 92]. The following section discusses the mechanisms behind several molecular alterations that unfortunately predict resistance to TKI therapy.

4.8.2.1 T790M

The most common secondary *EGFR* mutation leading to TKI resistance is substitution of methionine in place of threonine at codon 790 (T790M), independently identified by Pao et al. and Kobayashi et al. in 2005 [35, 90, 93–95]. After identifying the presence of the T790M point mutation in a patient who was initially sensitive to gefitinib but later became resistant, Kobayashi et al. demonstrated in vitro that introducing the T790M mutation into previously gefitinib-sensitive NSCLC cells harboring either wild-type, exon 19 deletion, or L858R substitution *EGFR* genotypes induced resistance to gefitinib [94]. Pao et al. also demonstrated in vitro that NSCLC cells harboring the T790M mutation with otherwise wild-type or mutated *EGFR* (either exon 19 deletion or L858R substitution) did not respond to either erlotinib or gefitinib [93].

Structural analysis performed by Kobayashi et al. showed that the T790M point mutation of *EGFR* changes the tyrosine in the catalytic domain to methionine, which is bulkier and prevents erlotinib (and presumably gefitinib) from binding [7, 35, 94, 96]. Not only does the T790M mutation alter TKI binding, but it also enhances kinase activity of EGFR (when coupled with the L858R mutation), offering cells with this mutation a survival advantage [11, 29, 52, 97]. Another missense mutation, D761Y, has been reported to confer a milder degree of TKI resistance, which may still be clinically relevant [98]. Greater resistance to gefitinib is produced when the T790M mutation occurs on the same DNA strand as the *EGFR* activating mutation (L858R or exon 19 deletion) [99], which is the most common scenario [100]. This can be explained by understanding that only the mutant allele will code for the mutant, constitutively active EGFR; likewise, binding of TKIs to these mutant, constitutively active EGFRs will only be altered if the resistance mutation has occurred on the mutant allele.

The T790M point mutation has been identified in approximately 50 % of patients who had activating *EGFR* mutations and later acquired resistance to EGFR TKIs [35, 44, 91, 95, 96, 98]. Engelman et al. demonstrated in vitro that malignant cells with activating mutations of *EGFR* that are initially sensitive to gefitinib become resistant after prolonged exposure to gefitinib and may acquire the T790M mutation in *EGFR* [44, 99]. Even so, the question arises as to whether TKI treatment itself induces the resistance mutation or if it merely results in selection of resistant clones [96]. Considering that the T790M mutation is found in 0.5–3.6 % (depending on the sensitivity of the assay; highest with mutant-enriched PCR) of patients who have never been treated with TKIs [29, 91], it is likely that TKIs merely result in selection of resistant clones rather than induce the T790M mutation themselves [29, 91].

4.8.2.2 Exon 20 Insertion Mutation

The exon 20 insertion mutation of *EGFR* (D770insNPG) has been demonstrated by Greulich et al. to confer erlotinib and gefitinib resistance in vitro. Response was obtained, however, from the irreversible EGFR inhibitor CL-387,785 [54]. Two patients harboring this mutation (who incidentally were both never-smoking women with adenocarcinomas) both progressed despite gefitinib treatment [66]. Intermediate sensitivity to erlotinib or gefitinib was observed in G719S of exon 18. The authors suggested that sensitivity may be related to the specific mutation present [35, 44, 54].

4.8.2.3 Low PTEN Expression

Low phosphatase and tensin homolog (PTEN) expression is a major reason why tumors become resistant to TKIs [101, 102]. PTEN normally functions to down-regulate the PI3K pathway (downstream of EGFR; mediates growth, proliferation, and survival). Loss of PTEN leads to persistent activation of the PI3K pathway independent of EGFR signaling, making this proliferation signal unresponsive to EGFR blockade [101, 102]. Bianco et al. demonstrated that, in gefitinib-resistant cells lacking PTEN, reconstitution of PTEN reversed EGFR-independent PI3K pathway activity and restored gefitinib sensitivity [102]. Zhuang et al. demonstrated that irradiating TKI-resistant tumors with low PTEN expression may help reverse the resistance by increasing PTEN expression levels [101]. Although low PTEN expression may induce resistance to TKIs, there may not be a relationship between PTEN expression and survival [27].

4.8.2.4 MET Amplification

Some authors have reported acquired resistance to EGFR TKIs in tumors with MET amplification [29, 99, 103]. It has also been suggested that MET activation (which may be induced by hepatocyte growth factor binding, overexpression, or structural alterations) may be used in place of MET amplification to determine sensitivity to TKIs [103]. The results of an in vitro study by Rho et al. suggested that sensitivity to TKIs was not associated with MET activation in the absence of MET amplification [103].

4.8.3 Methods to Overcome Resistance Mutations

Methods to overcome T790M-induced EGFR TKI resistance are of clinical interest. Murine and in vitro studies reveal some agents that may be beneficial. Although T790M makes cells resistant to gefitinib and erlotinib (which are reversible TKIs),

the effect of irreversible TKIs (which covalently bind the EGFR kinase domain) is not changed by T790M. Irreversible TKIs include EKB-569, HKI-272, CI-1033, CL-387,785, and BIBW2992 [29, 94, 96, 98, 99]. The irreversible EGFR inhibitor CL-387,785 inhibits EGFR and downstream molecules more potently than gefitinib or erlotinib in cells harboring the T790M mutation, although not nearly to the degree that would be observed in cells without the T790M mutation [94, 99, 103]. Regales et al. demonstrated in mice that tumors harboring the T790M TKI resistance mutation had the most shrinkage (and complete response in most tumors) when treated with a combination of two drugs: BIBW-2992 (which is an irreversible EGFR TKI) and cetuximab (which is an EGFR-specific antibody). This combination resulted in lower total and phosphorylated EGFR. Neither agent alone induced complete response in any of the tumors in the study [90]. Another study has shown that, in mouse xenograft models of NSCLC with *EGFR* T790M mutations, CUDC-305 (a heat-shock protein 90 inhibitor) inhibits tumor growth [104].

Blocking signaling molecules downstream of EGFR has the potential to overcome resistance induced by a variety of mutations that alter binding of drugs to EGFR [53, 105]. Src is one such downstream signaling molecule that is vital to maintaining the malignant phenotype of *EGFR* mutant cells [53]. In vitro, Src inhibitors have been shown to prevent oncogenesis caused by *EGFR* mutations, suggesting that these may be of clinical benefit in TKI-resistant *EGFR*-mutated NSCLCs [53]. Faber et al. demonstrated in vitro that blocking two major pathways downstream from EGFR resulted in tumor shrinkage even in cell lines with various TKI resistance mutations including T790M [105].

4.9 Excision-Repair Cross-Complementation Group 1 (ERCC1)

4.9.1 Background

Whereas most of the discussion on molecular predictive factors in NSCLC has focused on response to TKIs, the ERCC1 enzyme may predict resistance to platinum-based therapy [7, 106–109]. Overexpression of ERCC1 occurs in 31–68 % of NSCLCs and is significantly associated with older age, squamous cell rather than adenocarcinoma histology, and pleural invasion [2, 13, 106, 110–112]. ERCC1 repairs platinum-DNA adducts (which cause inter- and intra-strand cross links) caused by platinum-based agents in cancer cells [7, 13, 106, 112–114]. This enzyme first recognizes the DNA damage and then removes the damaged nucleotides [115]. If the defects caused by the cytotoxic agent are not repaired, the cell will undergo apoptosis, which is, of course, the goal of administration of anti-cancer therapy. Through its DNA-repair action, ERCC1 reverses damage caused by certain chemotherapeutics on cancer cells, thus providing resistance to their anti-cancer effects [13, 113, 114, 116]. It is the expression level of ERCC1 that has been studied for its predictive value, since it has been established that ERCC1 mRNA levels correlate with DNA repair capacity [117].

4.9.2 Evidence for Molecular Predictive Value of ERCC1

Based on its normal biological function, ERCC1 has been studied for its relationship to chemotherapy resistance. In a phase III trial, patients were prospectively randomized to either a control arm, where all patients were treated with docetaxel plus cisplatin regardless of ERCC1 mRNA level, or a genotypic arm, where patients with low ERCC1 mRNA received docetaxel plus cisplatin. Those with high ERCC1 mRNA received docetaxel plus gemcitabine. The objective response rate was significantly higher in patients assigned to the genotypic arm than in those in the control arm group [65, 118]. It is interesting to note that patients in the genotypic arm responded better, even though gemcitabine was used for patients with high ERCC1 expression, which is correlated with high RRM1 expression. High RRM1 expression has been shown to predict resistance to gemcitabine treatment (to be discussed further in the following section) [118]. The results of this study clearly demonstrate the clinical utility of ERCC1 mRNA levels for determination of appropriate chemotherapy regimen for patients with advanced NSCLC [118].

Many other studies have confirmed better response or survival following chemotherapy for NSCLC in patients with low ERCC1 expression. The IALT study demonstrated that patients with ERCC1-negative tumors had prolonged survival with adjuvant cisplatin, whereas those with ERCC1-positive tumors did not, suggesting that adjuvant cisplatin-based chemotherapy may be beneficial in completely resected ERCC1-negative NSCLCs [1, 2, 106]. Ikeda et al. drew similar conclusions from their study [2]. Ceppi et al. also found higher median survival time in chemotherapy-treated patients with low ERCC1 mRNA than in those with high [85]. Azuma et al. found longer median progression-free and overall survival in carboplatin/paclitaxel-treated patients with ERCC1-negative tumors compared to those with ERCC1-positive tumors [110]. Further, significantly longer progression-free survival was seen in patients with tumors negative for both ERCC1 and class III beta tubulin compared with tumors positive for either or both of these markers [110]. A randomized, phase III trial found a significant association between expression levels of ERCC1 and response to gemcitabine, although no relation to survival was appreciated [119]. Wang et al. negatively correlated ERCC1 expression with tumor response to platinum-based chemotherapy, concluding that it may be a useful marker to predict response to platinum-based chemotherapy in Chinese patients with advanced NSCLC [120]. Lee et al. found ERCC1 expression to be significantly associated with shorter overall survival in patients with advanced NSCLC treated with platinum-based chemotherapy [111]. At least two groups found low ERCC1 mRNA levels to correlate significantly with longer median overall survival but not with tumor response to platinum-based chemotherapy in patients treated with these chemotherapeutics [30, 65, 121]. Other studies have failed to find a relationship between expression of ERCC1 and response to platinum-containing agents altogether [108, 121–123]. The collective evidence suggests ERCC1 expression may be a useful predictive factor indicating resistance of NSCLCs to platinum-based chemotherapy [1]. On the contrary, many other studies [1, 106, 124] have shown that high rather than low ERCC1 mRNA correlated with longer survival in untreated patients [30].

4.9.3 Molecular Predictive Value of ERCC1 Polymorphisms

Some polymorphisms in the *ERCC1* gene are associated with differential response to platinum-based agents [7, 106–108, 117]. Such differences may be attributable to variation in ERCC1 mRNA levels, mRNA stability, or protein function, depending on the genotype [107, 108]. It has been demonstrated that patients with polymorphic *ERCC1* genes (harboring either CT or TT genotype at codon 118) had significantly shorter survival and time to progression following docetaxel-cisplatin treatment compared to those with the wild-type (CC) genotype [108, 117]. Response to docetaxel-cisplatin, on the other hand, was not associated with *ERCC1* genotype [108]. On the other hand, Zhou et al. did not find any relationship between polymorphisms in codon 118 and survival following treatment with platinum-based chemotherapy [107]. In their study, a different polymorphism, C8092A, was associated with lower survival in patients with stage III disease treated with platinum-based chemotherapy, with significantly lower survival seen for each 8092A allele present [107]. The total number of variant alleles between *ERCC1* and *XRCC1* (possible 0–4) also correlated with overall survival among non-smoking Han Chinese women with stage III or IV adenocarcinoma (the greater the number of variant alleles, the shorter the median survival time) [117]. Tibaldi et al. found that patients with the *ERCC1* C118T genotype and treated with cisplatin-gemcitabine had significantly better clinical benefit, but there was no association with treatment response, time to progression, or survival [125].

Evidence supporting the role of *ERCC* genes in NSCLC carcinogenesis comes from the observation that some *ERCC* genotypes impart a higher risk for development of adenocarcinomas. Yin et al. found that non-smoking women who are heterozygous or homozygous for the 751C variant allele of *ERCC2* have a significantly higher risk of developing lung adenocarcinoma compared to homozygous wild-type. Heterozygous variant alleles of *ERCC1* 118 did not correlate with increased risk of development of adenocarcinoma but combining the heterozygous/homozygous group did reach significance. Some other studies have either not confirmed these results or have suggested the opposite relationship [115].

4.9.4 Molecular Prognostic Value of ERCC1 Expression

In contrast to the negative impact that high ERCC1 expression has on platinum-based-chemotherapy-treated patients, higher ERCC1 expression is actually significantly associated with *longer* median survival in patients not treated with chemotherapy [1, 106, 124]. These data suggest that high ERCC1 expression may be a favorable prognostic marker [1]. Other groups have also found positive prognostic value from ERCC1 expression [13, 85, 126]. A possible explanation for the favorable prognostic association is that patients with higher DNA-repair capacity may be less likely to

relapse following surgical treatment, as fewer oncogenic mutations may be allowed to occur [13, 124]. In some studies, ERCC1 expression did not have any molecular prognostic value [1, 106, 127].

4.9.5 ERCC1 Summary

High expression of ERCC1, which functions to repair platinum-DNA adducts, predicts resistance of NSCLCs to platinum-based chemotherapeutics (which are first-line agents). Determination of ERCC1 levels would seem to hold the most value in patients with poor performance status who would be likely to suffer the most risk from taking systemic therapy that is unlikely to help. Polymorphisms in the *ERCC1* gene, which alter the expression levels or function, correlate with resistance of NSCLCs through their relationship to the level of repair activity by ERCC1. Although high expression of ERCC1 counteracts the effects of platinum-based chemotherapeutics, a high degree of DNA repair activity should naturally be beneficial and could conceivably reduce the risk of development of additional tumors in the future. This may account for the favorable prognosis observed in NSCLC patients with high ERCC1 expression who are not treated with chemotherapy.

4.10 Ribonucleotide Reductase (RRM1)

4.10.1 Background

Ribonucleotide reductase, constitutively expressed in all dividing cells, is responsible for converting ribonucleotides into deoxyribonucleotides via a reduction reaction [128], which is the rate-limiting step in *de novo* deoxyribonucleoside triphosphate (dNTP) biosynthesis [129, 130]. Ribonucleotide reductase is composed of two subunits, designated R1 and R2, each with a specific function in nucleotide synthesis. The R1 subunit is encoded by the *RRM1* gene, and the two closely-related R2 subunits are encoded by the *RRM2* and *RRM2b (p53R2)* genes, respectively [129, 131, 132]. The larger R1 subunit is considered the catalytic and regulatory site, whereas the smaller R2 subunit contains two iron moieties used in the generation of free radicals, important in the reduction of the hydroxyl group of the ribose sugar [67, 128, 129, 132]. The *p53R2* gene is induced by p53 expression, which occurs in the presence of DNA damage; thus, p53R2 is an important mediator of p53-regulated DNA repair [67, 129, 132]. As the rate-limiting step in dNTP synthesis, activity of ribonucleotide reductase ultimately determines the relative intracellular concentrations of the four dNTPs, which is controlled through a specificity site on the R1 subunit [129, 133–136]. As such, ribonucleotide reductase is critical to maintaining replication fidelity, considering that higher rates of base misinsertion and lower

proofreading activity during DNA replication occur when nucleotide concentrations are abnormal [129, 135, 136]. This suggests that overexpression of ribonucleotide reductase may cause abnormal nucleotide concentrations resulting in mutations, which may occur in growth regulatory genes [129, 135].

4.10.2 Role in Carcinogenesis

Overexpression of *RRM1* (which acts as a tumor suppressor gene) [129, 137–139] has been shown to reduce chemical carcinogenesis in the lung [129, 140] and prevent lung cancer metastases [137, 139, 141]. In contrast, overexpression of either *RRM2* or *p53R2* results in higher mutation rates in cultured cells and in the development of lung neoplasms in mice [129]. RRM2 overexpression in particular is associated with the largest tumor size, greatest tumor multiplicity, and the most pathologically advanced neoplasms [129]. The higher incidence of lung neoplasms in transgenic mice with *RRM2* or *p53R2* overexpression in the study by Xu et al. was accompanied by a particular activating *KRAS* mutation (codon 12 G to T transversion) in some of the neoplasms that was not found in neoplasms of control mice or transgenic mice overexpressing *RRM1* [129]. This suggests a different mechanism of mutagenesis and carcinogenesis in tumors that arise in the setting of *RRM2* or *p53R2* overexpression compared to *RRM1* overexpression or spontaneous development in wild-type mice [129]. Free radicals, a normal product of RRM2 activity, may have contributed to the development of the G to T transversions seen in the neoplasms of mice overexpressing these proteins in the Xu study [129]. p53R2, on the other hand, has antioxidant activity [141, 142] and might be expected to be protective against malignancy. In fact, it has been shown that p53R2 binds MAPK/ERK kinase 2 (MEK2) (and much less MEK1), preventing phosphorylation of MEK1/2 and likewise its downstream effector extracellular signal-regulated kinase-1/2 (ERK1/2), thereby inhibiting tumor cell transformation and invasion (considering that ERK1/2 activates cell motility and disrupts adhesions) [141].

4.10.3 Molecular Predictive Value of RRM1 Expression

RRM1 is expressed in approximately 40 % of NSCLC [143], and there has been no correlation between RRM1 mRNA levels and age, gender, performance status, or stage [144], although expression levels of RRM1 are significantly associated with expression of ERCC1 [85, 145]. Gemcitabine is an antimetabolite chemotherapeutic agent that competitively inhibits DNA polymerase as well as ribonucleotide reductase, thereby preventing DNA synthesis and cell division [47, 85, 144–148]. High levels of RRM1 expression overcome the antimetabolite effect of gemcitabine and therefore may be used as a predictive marker of gemcitabine resistance [1, 139, 146, 149, 150].

Conversely, lower expression has been associated with better survival after cisplatin plus gemcitabine combination [7, 109, 145, 151]. Indeed, higher expression of RRM1 by IHC has been associated with shorter overall and progression-free survival as well as lower disease control following gemcitabine treatment in cases of locally advanced or metastatic NSCLC [85, 144, 146]. This is in contrast to the survival-enhancing effect that high RRM1 expression has on early stage NSCLC following curative surgery, presumably for its ability to repair DNA damage and prevent recurrence [139, 146, 152]. Bepler et al. in 2006 also showed that there is a positive relationship between the expression of RRM1 and resistance of NSCLC to gemcitabine plus platinum combination therapy [150]. Since expression of RRM1 and ERCC1 are correlated, it is worthy of mention that the relationship between ERCC1 and resistance to this chemotherapeutic regimen was similar, although to a lesser degree [150]. Bepler et al. confirmed the results of previous studies that tumor shrinkage in response to gemcitabine was significantly inversely correlated with RRM1 expression [17]. In a study by Rosell et al., high RRM1 expression correlated with worse survival in patients treated with the gemcitabine/cisplatin combination, but had no significant impact on survival when vinorelbine was added to this combination [145]. Therefore, a gemcitabine/cisplatin combination could be used in patients with low RRM1 expression levels while vinorelbine could be added when RRM1 expression levels are high [145].

Many other studies have detected significant relationships between RRM1 expression and response or survival but did not find this to be an independent molecular predictive or prognostic factor. Wang et al. found RRM1 expression to correlate with poor tumor response, but this was not an independent predictive factor [143]. In cisplatin/gemcitabine-treated patients, longer median survival time was observed with low RRM1 expression compared to high RRM1 expression, although RRM1 expression was not an independent prognostic factor in this study [85]. Other studies have not demonstrated a relationship between RRM1 expression levels and response to cisplatin, carboplatin, or gemcitabine [122]. Souglakos et al. found RRM2 expression to be an independent predictor of response to docetaxel/gemcitabine in lung adenocarcinomas, but RRM1 had neither predictive nor prognostic value in this study [144]. Overall, the evidence is in support of the molecular predictive value of RRM1 expression-level determination.

4.10.4 Molecular Prognostic Value of RRM1 Expression

Bepler and colleagues have extensively researched the use of the RRM1 component of ribonucleotide reductase (located on chromosome 11p15.5) as a molecular predictive and prognostic marker for NSCLC [42, 149, 153]. Given the tumor suppressor activity of RRM1, it becomes clear how loss of heterozygosity of 11p15.5 (which is reportedly common in NSCLC) contributes to carcinogenesis and survivability of this cancer [42]. Sure enough, patients with NSCLC who only have one copy of the *RRM1* gene (instead of two) have shorter survival [1].

In addition to the above synthetic functions of RRM1, it has been proposed to also suppress cell migration, invasion, and metastasis through induction of PTEN expression [36, 81, 137, 139, 151–154]. In a 2004 study, Bepler et al. found that high RRM1 expression was strongly correlated with a better overall and disease-free survival in resectable NSCLC [153]. They concluded that the mechanism behind improved survival was that RRM1 acted as an inducer for, and in concert with, PTEN, a known tumor suppressor gene [153]. Therefore, high levels of both RRM1 and PTEN may impart a more favorable outcome in patients with resectable NSCLC [153].

In a related study in 2007, Zheng et al. examined tumor specimens from 187 patients with resectable, stage 1 NSCLC [139]. They quantitatively determined the levels of RRM1, PTEN, and ERCC1 and found that both the median disease-free and overall survival were significantly higher in patients with high expression of either RRM1 or ERCC1 [139]. Expression of RRM1 and ERCC1 are correlated, and patients with high expression of both proteins had significantly longer median overall and disease-free survivals than patients with high expression of only one or neither of the proteins [139, 151]. Of note, there was a lack of association between RRM1 and PTEN expressions in this study, which is in contrast to a prior study by Bepler et al. as described above [139, 151, 153].

4.10.5 RRM1 Single-Nucleotide Polymorphisms

As was discussed for ERCC1, single-nucleotide polymorphisms (SNPs) that have an impact on expression levels of a protein that has predictive or prognostic implications may be used as a surrogate to expression level determination. In one study, although there was no relationship between RRM1 mRNA levels or RRM1 SNPs and response to gemcitabine, one SNP located within the promoter region (C(−)37A) was associated with significantly longer progression-free survival following gemcitabine treatment [155]. Curiously, higher RRM1 expression levels have previously been associated with this particular SNP [155, 156]. The highest response to gemcitabine treatment has been associated with the RR37AC-RR524CT allelotype (found in 29.9 % of cases), which has been shown to be associated with low RRM1 expression in vitro [47, 157, 158]. With respect to platinum-based chemotherapy, the (−)524CT allelotype is associated with the highest response rate; again, this finding may be related to the expression level of RRM1, which was not assessed in this study [157]. In patients with resectable NSCLC, the allelotype associated with the highest promoter activity and thus RRM1 expression levels (RR37CC- RR524TT) was associated with the longest disease-free and overall survival [47, 158]. Although these results suggest that RRM1 genotype may have predictive or prognostic value, the above-mentioned SNPs do not seem to correlate with RRM1 expression levels in vivo, and therefore may harbor their predictive or prognostic value independent from correlation with expression levels [158].

4.10.6 RRM1 Summary

RRM1 catalyzes the rate-limiting step in nucleotide synthesis (which will be highly active in NSCLCs) and is competitively inhibited by the antimetabolite gemcitabine. Competitive inhibition is overcome by increased levels of RRM1 (found in 40 % of NSCLCs), which may be related to polymorphisms in the *RRM1* gene. Studies have shown that NSCLCs with high RRM1 levels respond poorly to gemcitabine. RRM1 is thought to induce expression of the tumor suppressor PTEN, and this may account for the observation that high levels of RRM1 correlate with better survival in resectable NSCLC. Expression of RRM1 and ERCC1 are correlated, so it comes as no surprise that NSCLCs with high levels of RRM1 (and thus ERCC1) respond poorly to the combination of gemcitabine (which inhibits RRM1) and cisplatin (which causes DNA damage that ERCC1 can repair). It is promising that, in at least one study, adding vinorelbine to the gemcitabine/cisplatin combination eliminated the association of high RRM1 levels with poor tumor response [145].

4.11 KRAS

Ras proteins are plasma membrane-associated 21-kDa GTPases encoded by the *RAS* gene on chromosome 12p12.1 [159]. The family of *RAS* genes includes three subtypes: *HRAS*, *KRAS*, and *NRAS* [159]. Initially discovered as genes from human cancer cell lines that transformed mouse cells in vitro [159, 160], *HRAS* and *KRAS* were later found to be homologous to retroviral oncogenes (of the Harvey-rat sarcoma virus and Kirsten-rat sarcoma virus, respectively) [159, 161]. *NRAS* was later identified in human neuroblastoma cells [159, 162]. *KRAS* mutations, found in approximately 16 % of NSCLCs, account for the majority of *RAS* mutations in human lung cancers [11, 22, 37, 49, 50, 66, 70, 159, 163, 164]. In contrast to *RAS* genes present in normal tissue, those present in cancer usually possess a missense mutation in codon 12 or 13 (accounting for 97 % of *KRAS* mutations) or less commonly codon 61 [10, 159, 165]. These mutations impair the GTPase activity of the RAS protein, resulting in constitutive activation and perpetual transmission of downstream signals [10, 159, 166].

RAS proteins, initially activated following activation and autophosphorylation of EGFR, transmit signals through a variety of pathways with a wide array of seemingly antagonistic endpoints [159]. For example, the RAS-RAF-MEK-ERK pathway culminates in cell proliferation [159] and the PI3K/Akt, STAT3, and STAT5 pathways have anti-apoptotic effects [159], whereas RAS-related activation of p38, p53, and p16/INK4a leads to senescence [159, 167, 168]. It seems that p16 is not activated when Ras is only moderately activated (i.e., expressed at levels comparable to wild-type *KRAS*) [159, 169], allowing pathways favoring survival and proliferation to dominate, explaining the oncogenic effects of mutant *KRAS* [159, 167].

4.11.1 KRAS *Copy Number Gain*

As is the case with *EGFR*, *KRAS* is frequently amplified in NSCLC [159, 170, 171], with mutant allele-specific imbalance (MASI) occurring in 58 % of tumors with *KRAS* mutations [100, 159]. However, whereas the mechanism of MASI with *EGFR* is typically copy number gain, MASI with *KRAS* usually results from uniparental disomy of the mutant allele without copy number gain [100, 159]. Similar to *EGFR*, copy number gains of *KRAS* are found more frequently in tumors with mutant *KRAS* [100]. Found in only 10 % of 288 lung adenocarcinomas, *KRAS* copy number gains in the absence of *KRAS* mutations showed only a non-significant trend toward association with worse prognosis in the study by Soh et al. [100]. Although few studies have examined the impact of KRAS amplification, copy number gain, or MASI on treatment response or survival, it is known that higher levels of active, GTP-bound KRAS are found in tumors with either *KRAS* mutations or MASI; GTPase activity is highest when both alterations are present [100, 159].

4.11.2 KRAS *Mutation Background*

In 1984, the association between somatic *KRAS* mutations and lung carcinoma was discovered [159], and as early as 1990 it was reported that activating mutations of the *KRAS* oncogene indicated poor prognosis in NSCLC [29]. Mice with induced *KRAS* mutations prematurely develop tumors in many organs, especially the lung, where the tumors resembled well-differentiated human papillary adenocarcinomas of alveolar type II pneumocyte origin [172]. In contrast to *EGFR* mutations, which are identified in never-smoking women of Asian origin, *KRAS* mutations and copy number gains [100] tend to occur in Caucasians with smoking histories, although mutations in either oncogene are most commonly found in adenocarcinomas [7, 11, 29, 37, 49, 50, 159, 163]. With respect to histology, mucinous or goblet-cell patterns of bronchioloalveolar carcinoma more frequently harbor *KRAS* mutations than the non-mucinous type [159, 164, 173–175]. Tumors that are KRAS-dependent tend to be well-differentiated and have *KRAS* copy number gain and KRAS overexpression [159, 176]. Even though they did not find a significant association between *KRAS* mutation status and smoking history or cumulative smoke exposure, Riely et al. found that *KRAS* mutations in smokers were significantly more often G to T or G to C transversions rather than the G to A transitions found in never smokers, providing further evidence of distinct molecular alterations in lung cancers of smokers [10].

4.11.3 *Molecular Predictive Value of* KRAS *Mutations*

Strategies to block mutant *KRAS* signaling in NSCLC have not been met with success [100, 176], because unlike the case of *EGFR* mutations, tumors with mutant *KRAS* are not always dependent upon KRAS for signaling so that targeted blockade

of KRAS results in little to no tumor shrinkage [159, 177]. Consequently, the molecular predictive value of *KRAS* mutations lies in their association with resistance to TKIs [55]. The first report of EGFR-TKI resistance in NSCLC patients with *KRAS* mutations was by Pao et al. in 2005 [35, 97]. Recalling the position of KRAS as a second-messenger of EGFR signaling, it becomes clear why constitutive activation of KRAS (such as by mutation) leads to proliferation independent of blockade of EGFR signaling at the level of EGFR itself. However since mutations of both *KRAS* and *EGFR* are found in the same patient [7, 11, 37, 50, 61, 66], it is debatable whether it is the presence of the *KRAS* mutation or the absence of an *EGFR* mutation that is responsible for the lack of response to TKIs in *KRAS*- mutant tumors [159]. In fact, among patients without *EGFR* mutation treated with first-line EGFR-TKIs, the *KRAS* status has no impact on overall survival [159, 177].

Supporting the role of *KRAS* mutation status in determining responsiveness clinically, in vitro evidence provided by Park et al. demonstrated that NSCLC cells with *KRAS* mutations are resistant to gefitinib. Furthermore, down-regulation of *KRAS* through lovastatin administration led to significantly more cytotoxicity compared to gefitinib alone in these resistant cells [38]. Most clinical studies have found lower response to TKIs and even conventional chemotherapy in *KRAS*-mutant tumors as well as decreased overall and progression-free survival in these patients [7, 10, 66, 92, 96]. Miller et al. demonstrated a significantly higher response rate to EGFR TKIs for wild-type *KRAS* patients compared to *KRAS* mutants [44, 68]. Patients with wild-type but not mutant *KRAS* derived significant survival benefit from erlotinib in the BR.21 study [50]. Adjuvant chemotherapy significantly prolonged survival in patients with tumors harboring wild-type *KRAS*, but it did not prolong survival in patients with mutant *KRAS* [2]. Many other studies showed nonsignificant trends toward decreased survival or decreased response to chemotherapy or TKIs in patients harboring *KRAS* mutations [37, 49, 77]. Other studies revealed no association between *KRAS* mutation status and response to TKIs or conventional chemotherapy whatsoever [22, 50, 51, 55, 70, 178].

Although there are conflicting opinions, *KRAS* mutation is generally accepted as a predictor of resistance to TKI therapy [10, 163]. Further, there is evidence that detection of *KRAS* mutations by methods more sensitive than direct sequencing has an even higher predictive value [92]. In the study by Marchetti et al., *KRAS* mutation detected by mutant-enriched sequencing was an independent predictive factor of response to TKIs [92]. The power of the results from the Marchetti study suggests that failure of some other groups to detect the predictive ability of *KRAS* mutations may lie in their failure to detect a large portion of the actual mutations themselves. In their own study, Marchetti et al. found that *KRAS* mutation status as detected by direct sequencing alone (the standard, less sensitive method) was not an independent predictive factor for resistance to TKIs; independent predictive value was only held by *KRAS* mutation status as detected by the more sensitive mutant-enriched sequencing (which in this study detected *KRAS* mutations in approximately twice as many tumors compared to direct sequencing). Highlighting the importance of mutations in minor clones, patients with tumors harboring *KRAS* mutations had the same survival outcome whether the mutation was detected by direct sequencing or by mutant-enriched sequencing alone, indicating that mutations detected by mutant-enriched

sequencing alone are equally as clinically significant as those detected by both methods. The clinical impact of selection of minor clones harboring resistance mutations was discussed earlier [92].

4.11.4 KRAS *Summary*

In summary, *KRAS* mutations are useful in predicting resistance of NSCLCs to TKIs; further studies are required to determine the impact on response to conventional chemotherapy. Although tumors with *KRAS* copy number gain have higher levels of activated KRAS, suggesting that copy number gain may have molecular predictive value similar to that of *KRAS* mutation, the predictive value needs to be validated clinically. Finally, *KRAS* mutations are thought to hold negative prognostic value [11, 42, 49, 68, 96].

4.12 *ALK* Gene Rearrangements

4.12.1 Background

First linked to lung cancer when it was identified as a transforming agent from a pulmonary adenocarcinoma in 2007, the *ELM4-ALK* fusion gene (found in approximately 3.8 % of NSCLCs) has become of interest as a possible therapeutic target in NSCLC [179, 180]. It is interesting to note that the ELM4-ALK rearrangement in NSCLC was discovered in the same manner as Ras proteins originally (as transforming agents) [179]. The anaplastic lymphoma kinase (ALK; originally discovered in anaplastic lymphomas in 1994) [179, 181, 182] is a receptor tyrosine kinase that is a member of the insulin receptor family [179, 181, 183]. Similar to other receptor tyrosine kinases including EGFR, ligand binding triggers dimerization of ALK receptors followed by autophosphorylation and precipitation of downstream signals through the RAS-ERK, JAK3-STAT3, and PI3K/Akt pathways, which, as mentioned previously, affects cell proliferation, survival, and migration [179, 184]. A common rearrangement partner with ALK, echinoderm microtubule-associated protein-like 4 (ELM4) is a cytosolic protein critical to microtubule formation [179, 185, 186].

ALK (on chromosome 2p21) and *ELM4* (on chromosome 2p23) are located only 12.7 megabases apart [179]. Inversion of this part of the short arm of chromosome 2 [Inv(2)(p21p23)] creates a fusion between these two genes, with resultant disregulation of transcription and constitutive activation of ALK [179, 180]. At least 11 other variants of the ELM4-ALK rearrangement have been described [187], and *ALK* can also fuse with *TFG* and *KIF5B* in NSCLC [187–189]. Rearrangements of the ALK gene can be detected by FISH, IHC, or real-time polymerase chain reaction [179].

4.12.2 Demographic and Histologic Associations

Similar to the case with EGFR mutations, ALK rearrangements are usually found in adenocarcinomas of non- or light-smokers [179, 180, 187, 190–192], although the patients tend to be younger and with more advanced tumors [187, 190, 193]. Despite these similar clinicopathologic characteristics between EGFR mutations and ALK rearrangements, these molecular alterations are almost always mutually exclusive, which is also the case between ALK rearrangements and KRAS mutations [179, 187, 190–194]. Certain histologic patterns have been reported to be associated with ALK rearrangements in NSCLCs. In Asian populations, ALK-rearranged lung adenocarcinomas usually have an acinar pattern [187, 188, 193]. Approximately 60 % of ALK-rearranged lung adenocarcinomas in Western populations harbor solid growth patterns containing at least 10 % signet-ring cells, a pattern uncommonly found in ELM4-ALK wild type tumors [187, 191, 195].

4.12.3 Role in Carcinogenesis and Molecular Predictive Value

As is the case with constitutively active EGFR and KRAS, constitutively activated ALK resulting from the ELM4-ALK fusion leads to the development of adenocarcinomas in mice, which regressed upon administration of ALK inhibitors [179, 180, 194]. Recognition of ALK rearrangements in NSCLC led to the trial of the ALK inhibitor PF02341066 (crizotinib), which in one study led to partial response in 17 of 29 patients [179, 187, 196]. In contrast to EGFR, where the targeted therapy (TKIs) was first discovered followed by identification of EGFR mutations as the predictive factor, the discovery of ELM4-ALK rearrangement in NSCLC was followed by trials of targeted therapies. In vitro studies have shown crizotinib to stop growth in cancers harboring ALK translocations [187, 197, 198]. ALK rearrangements do not seem to predict response to platinum-based chemotherapy. In 12 patients with NSCLC containing ALK rearrangements, both response and survival following platinum-based chemotherapy were similar to results experienced by patients with *EGFR* mutations only or double-wild-type tumors [187, 191]. Response to erlotinib in patients with tumors containing ALK rearrangements was similar to that in patients lacking *EGFR* mutations [187, 191].

4.12.4 ALK Rearrangement Summary

Certain ALK rearrangements lead to constitutive activation of ALK, triggering downstream pathways that disregulate cellular growth and survival. Just like activating *EGFR* mutations, activating ALK rearrangements have been shown to cause pulmonary adenocarcinomas in mice. After the discovery of ALK rearrangements

in some NSCLCs, trials with the ALK inhibitor crizotinib were initiated, the results of which are promising that ALK rearrangements may predict response to crizotinib.

4.13 Multidrug Resistance Proteins

Multidrug resistance proteins (MRPs) have been investigated for their possible predictive value in lung carcinoma. It is not yet clear whether MRPs have predictive or prognostic implications. Although Li et al. found that response to cisplatin correlated with expression of MRP1 and that higher MRP1 expression levels correlated with shorter progression-free and overall survival, MRP1 expression was not an independent prognostic factor [126]. In contrast, MRP1 did not predict response to cisplatin nor did it correlate with overall survival in the study by Filipits et al. [199]. Other reports have found worse outcome associated with MRP1 overexpression and at least one found longer overall survival [199]. RRM2 expression levels were an independent prognostic factor for completely resected NSCLC in one study, but it still did not predict response to cisplatin [199].

4.14 BRCA1

BRCA1 has been shown to correlate with risk of breast and ovarian cancer development in women [1, 7]. Much like ERCC1, BRCA1 is involved in nucleotide excision repair, one of the mechanisms for repairing platinum and alkylating agent-induced DNA adducts. In accordance with their related functions, expression of BRCA1 is significantly associated with expression of both ERCC1 and RRM1 (all of which are expressed at higher levels in squamous cell carcinoma) [1, 7, 127]. Dysfunctional BRCA1 may fail to repair damage induced by these chemotherapeutics, leading to drug sensitivity. This suggests that decreased BRCA1 function may serve as a predictive marker [7, 111, 139, 200, 201]. Some studies have demonstrated that patients with low BRCA1 expression have better response to neoadjuvant cisplatin-gemcitabine [1, 7, 120, 200, 201]. Other studies have not found any association between BRCA1 expression and tumor response to cisplatin-gemcitabine or epirubicin-gemcitabine [123]. The association of BRCA1 overexpression with poor prognosis was confirmed by Rosell et al. [127]. The true predictive and prognostic values of BRCA1 in NSCLC are not yet established.

4.15 p53

TP53 is the gene encoding the p53 tumor suppressor gene. Studies report varying associations of p53 expression and *TP53* mutations with treatment response and prognosis in NSCLC. In the JBR.10 study, p53 overexpression (demonstrated in 132/253 cases) was associated with significantly shorter overall survival. However, p53-positive tumors

did benefit significantly from adjuvant chemotherapy, whereas p53-negative tumors did not [2]. In the study by Kosaka et al., *TP53* mutations were not associated with prognosis in Japanese patients with lung adenocarcinomas treated surgically [51].

4.16 Gene Expression Profiling

Given the wide array of molecular alterations that may impact on treatment response, gene expression profiling may hold the most promise for a comprehensive estimate of a given patient's response to treatment and prognosis. Gene expression profiling uses databases of expression profiles that are associated with sensitivity or resistance to various agents [7]. Potti et al. have developed a gene expression profile that estimates prognosis even more accurately than clinical or pathologic stage, which helps identify specifically high-risk patients from within stage 1A who may benefit from adjuvant chemotherapy [1, 202]. Chen et al. also found a five-gene panel (consisting of *DUSP6*, *MMD*, *STAT1*, *ERBB3*, and *LCK*) independently associated with both progression-free and overall survival in NSCLC [1, 203].

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Chapter 5
Molecular Pathology and Diagnostics of Colorectal Cancer

Rodney E. Shackelford, Aejaz Nasir, Ardeshir Hakam, David Shibata, Timothy Yeatman, and Domenico Coppola

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R.E. Shackelford
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, SL-79, New Orleans, LA 70112, USA

A. Nasir
Diagnostic & Experimental Pathology & Tailored Therapeutics Program, Eli Lilly & Company, Indianapolis, IN, USA

A. Hakam • D. Coppola, M.D. (✉)
Department of Anatomic Pathology and Oncological Sciences, H. Lee Moffitt Cancer Center & Research Institute and University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: Domenico.coppola@moffitt.org

D. Shibata
Department of Surgical Oncology, H. Lee Moffitt Cancer Center & Research Institute and University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612, USA

T. Yeatman
Gibbs Research Institute and Cancer Center, Spartanburg, SC 29303

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Abstract Colorectal carcinoma (CRC) is the third most common malignancy in the United States and is the second leading cause of cancer deaths. Over the past 20 years, our knowledge of this CRC carcinogenesis has enormously increased; presently, several molecular diagnostic tests are commonly employed to analyze stage IV CRC and familiar CRC syndromes to determine treatment options and CRC inheritance and risk. More recently the number of CRC diagnostic tests has enormously expanded as our knowledge of CRC carcinogenesis has increased. Here, we review the standard tests employed in CRC, such as KRAS and Braf analyses, and also include less commonly employed tests related to familiar CRC and microsatellite instability. Last, we focus on possible future CRC genetic tests and testing modalities, focusing on techniques such as microarray and mass spectrophotometry.

Keywords Colorectal carcinoma • KRAS • BRAF • FAP • UGT1A1 • NHPCC • PJS • APC • Mutation • Hamartomatous polyposis syndromes

Abbreviations

8-oxo-G	7,8-dihydro-8-oxo-deoxyguanosine
AFAP	Attenuated familial adenomatous polyposis
APC	Adenomatous polyposis
ARMS	Amplification resistant mutation system
CRC	Colorectal carcinoma
CSGE	Conformation strand gel electrophoresis
EGFR	Epidermal growth factor receptor
FAP	Familial adenomatous polyposis
GTP	Guanosine-5′-triphosphate
HNPCC	Polyposis hereditary nonpolyposis colon cancer
HRM	High-resolution melting
JPS	Juvenile polyposis syndrome
MAP	MYH-associated polyposis
mCRC	Metastatic CRC
MLH1	MutL homolog 1
MLH3	MutS homolog 3
MLPA	Multiplex ligation-dependent probe amplification
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSI	Microsatellite instability
NCCN	National Comprehensive Cancer Network
PCR	Polymerase chain reaction

PJS	Peutz-Jeghers syndrome
PMS2	Postmeiotic segregation increased 2
PTT	Protein truncation test
SSCP	Single strand conformation polymorphism
T-ARMS-PCR	Tetra-primer amplification refractory mutation system PCR

5.1 Introduction

Colorectal carcinoma (CRC) is the third most common malignancy in the United States, with nearly 150,000 new cases annually, accounting for roughly 15 % of all US malignancies, and is currently the second leading cause of cancer-related deaths for both sexes [1]. CRC shows a peak incidence between the ages of 70 and 79 years and by 70 years roughly 50 % of the population in high-risk areas for CRC will have some form of colorectal neoplasia, spanning the continuum from adenomatous polyps to CRC [1, 2]. The average life-time risk for CRC in the US for individuals who lack a personal or family history of this malignancy is ~6 %, ~20 % for individuals with an effected first-degree relative, and 80–100 % for individuals with a hereditary CRC syndrome [1–3].

5.2 Environmental Versus Genetic Factors in Colorectal Cancer Risk

CRC shows a 20-fold variation among different geographic locations, with low incidences in India and Africa and a higher incidence in the US and Western Europe. Migration studies from low-risk to high-risk areas indicate that environmental factors play an important role in CRC risk [1, 2, 4]. Several studies have addressed the relative roles of heritable factors vs. shared and nonshared environmental factors in CRC risk and estimate the percentage of CRCs resulting from statistically significant heritable causes to range between 15 and 35 % [5–8]. The largest of these studies analyzed 44,788 mono-and dizygotic twin pairs and found heritable causes accounted for approximately 35 % of the risk for CRC, with the remaining risk due to environmental factors. Interestingly, hereditary factors played a slightly greater role in younger individuals with CRC than in older individuals [5].

Environmental risk factors for CRC include a diet high in red meat and fat, increasing age, a family history of CRC, the Metabolic Syndrome, a personal history of inflammatory bowel disease or a heritable CRC syndrome, smoking, physical inactivity, heavy alcohol consumption, a history of nightshift work, and a diet low in fiber and plants [9–13]. Conversely, physical activity, a diet high in vegetables

and fruits, and nonsteroidal anti-inflammatory drug use are associated with a lower CRC risk, with nonsteroidal anti-inflammatory drugs reducing colonic adenomatous polyp numbers [9].

5.3 Molecular-Genetic Testing in CRC

Over the past 15 years, the diagnosis, analysis, and treatment of CRC have dramatically changed. Until relatively recently, the establishment of a CRC diagnosis and prognostic indicator analysis depended on hematoxylin-eosin stained slide examination supplemented with immunohistochemical analysis. Within the past few years, molecular-genetic testing has become increasingly important in the diagnosis and management of hereditary CRC syndromes and in determining the treatment options for de novo metastatic CRC (mCRC), particularly as related to panitumumab and cetuximab use [14–17].

5.4 EGFR, *KRAS,* and De Novo CRC

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein and member of the ErbB family of growth factor receptor tyrosine kinase family, which consists of four closely related receptors. The EGFR has six major ligands that are expressed to various degrees in different tissues: heparin binding EGF, transforming growth factor-α, betacellulin, amphiregulin, heregulin, and EGF [18, 19]. EGFR is overexpressed in a variety of tumors, including ~70 % of individuals with mCRC, with its overexpression linked to a poorer prognosis [20–23]. Not surprisingly, EGFR overexpression is often associated with EGFR ligand overexpression by CRC cells, forming a malignancy-promoting autocrine regulatory loop [24–26].

EGFR binding leads to receptor homodimerization, subsequent tyrosine kinase domain activation with receptor cytoplasmic tail autophosphorylation, the recruitment of intracellular signaling proteins such as growth factor receptor-bound protein (GRB2), son of sevenless (SOS), with eventual KRAS activation [27]. *KRAS* is the human homologue of the Kirsten rat sarcoma virus [28, 29]. *KRAS* encodes the KRAS protein, a member of the Ras small guanine nucleotide-binding proteins, which functions in signal transduction cascades initiated by the ligation of several cell surface receptors, including EGFR, hepatocyte growth factor (c-Met), and insulin-like growth factor receptors [30, 31].

Upon activation, KRAS binds guanosine-5′-triphosphate (GTP) and activates a number of down-stream signaling proteins, including Raf, Braf, mTOR, MEK, ERK, and PIK3CA, resulting in the expression of gene products promoting cell growth, differentiation, and proliferation and which exert anti-apoptotic and pro-angiogenic effects [19, 32]. KRAS is a GTPase that hydrolyzes GTP to guanosine-5′-diphosphate resulting in KRAS inactivation. The GTPase activity of KRAS is accelerated

by the GTPase-activating proteins, which attenuate KRAS signaling. Thus, the growth-promoting effects of EGFR and KRAS are tightly regulated under normal conditions, while constitutive EGFR and KRAS signaling promotes CRC growth and neoplastic progression [19, 23, 32–34].

Recently, two anti-EGFR monoclonal antibodies, panitumumab (Vectibix) and cetuximab (Erbitux), have demonstrated efficacy in the treatment of mCRC. Cetuximab is a chimeric mouse-human monoclonal IgG1 antibody with proven second-or third-line efficacy for treating CRC and head and neck cancers [35–38]. Cetuximab binds the EGFR with an affinity ~fivefold higher than physiological EGFR ligands, preventing EGFR homodimerization, tyrosine kinase activation, and subsequent signaling [39, 40]. Cetuximab binding also causes the cetuximab-EGFR complex to be internalized and degraded, thus both EGFR signaling and receptor availability are lowered [41, 42]. Last, cetuximab mediates antibody-dependent cellular cytotoxicty against the EGFR in a murine tumor model; this mechanism of action may also contribute to its efficacy [43].

Panitumumab is a fully humanized IgG2 monoclonal antibody that binds the EGFR with high affinity ($Kd = 5 \times 10^{-11}$ M). Upon binding, panitumumab blocks EGFR, EGF, and transforming growth factor-α binding, blocking tyrosine kinase activation, attenuating tumor cell activation and proliferation, and stimulating receptor internalization. In vitro panitumumab administration causes cell cycle arrest at the G_0/G_1 interphase, inhibiting tumor colony formation. Last, like cetuximab, panitumumab also initiates complement-dependent and antibody-dependent cell-mediated cytotoxicities [43, 44].

Immunohistochemical EGFR positivity was initially employed as an entry criterion for studies evaluating the efficacy of these antibodies in CRC treatment. However, many studies demonstrated that EGFR expression is a poor biomarker for anti-EGFR antibody response, with responses seen in individuals with low, high, or even no EGFR expression [35, 45–47]. These results necessitated alternative biomarker testing for the evaluation of clinical cetuximab and panitumumab use. Since EGFR signaling promotes and maintains the CRC malignant phenotype [20–26], mutations causing constitutive activation of a down-stream EGFR signaling proteins are likely to be involved in CRCs refractory to cetuximab and panitumumab treatment. Several studies demonstrated constitutive activating *KRAS, BRAF,* and *PIK3CA* mutations in CRC, with *KRAS* mutations being most frequent at 27–45 % [35, 47–50]. Interestingly, activating *KRAS* mutations appear early in the genesis of CRC, with mutations found in adenomatous polyps [50]. Most activating *KRAS* mutations exhibit GTPase activity loss combined with GTPase-activating protein insensitivity [32]. Roughly 90 % of *KRAS* activating mutations occur in codons 12 and 13 of exon 2, with rare mutations seen in codons 59 and 61 of exon 3 [32, 51–53]. Interestingly, not all *KRAS* mutations are the same; CRCs with microsatellite instability (MSI) combined with the G13D *KRAS* mutation have a significantly improved prognosis [54]. Additionally, a recent study has demonstrated that patients with the G13D KRAS mutation still respond well to cetuximab [55]. Thus, evidence is accumulating that not all activating KRAS mutations are the same and not all render patients refractory to anti-EGFR antibodies.

5.5 KRAS Mutation Testing in De Novo CRC

Activating *KRAS* mutations are strongly linked to loss of anti-EGFR antibody response, and individuals carrying these mutations experience no survival benefit from cetuximab or panitumumab. Additionally, this treatment may be detrimental to individuals with *KRAS* mutations. Last, cetuximab and panitumumab treatment is costly at roughly $10,000 per month [47, 56–59]. Thus molecular testing for *KRAS* mutations is important in patient care and for cost-effective healthcare delivery; it is also currently recommended by the National Comprehensive Cancer Network (NCCN) for all patients with mCRC being considered for anti-EGFR therapy [60]. Currently, there are several different molecular protocols employed in *KRAS* mutation testing. Three common ones are amplification resistant mutation system (ARMS) for identifying specific mutations, various sequencing methods, and high-resolution melting analysis (HRM). Each of these techniques has specific advantages and disadvantages, and they are often combined.

5.5.1 Tissue Preparation

Sample preparation for *KRAS* testing in mCRC is more elaborate than for many other molecular tests. Mutation analysis typically begins with formalin-fixed or formalin-fixed, paraffin-embedded tissue. DNA from these samples require special preparation given that (1) it is usually fragmented and cross-linked to other biomolecules, making amplification (especially of longer DNA sequences) difficult, (2) it contains polymerase chain reaction (PCR) inhibitors, which must be thoroughly removed prior to amplification, (3) formalin fixation can introduce DNA sequence alterations, and (4) tumor tissue containing possible *KRAS* mutations is usually intermixed with areas of benign tissue and/or benign stromal elements, often necessitating histological examination and microdissection of the slide to increase the portion of tumor within the sample [50–63].

DNA purification usually begins with histological identification of CRC on a glass slide, often followed by removal and purification of DNA from the malignant component, from an unstained recut or core taken from a tumor-bearing portion of the paraffin block. Removal with a needle or scalpel is possible where a clear boundary exists between malignant and benign tissue. Alternatively, laser microdissection allows the isolation of small cell clusters or even single cells if extremely pure tumor DNA is required [63]. The resulting tumor-enriched sample is often deparaffinized in xylene, digested with protinase K, and the DNA is isolated via robotic instrumentation or commercial DNA purification columns [63]. Once purified DNA is obtained, *KRAS* mutational status may be analyzed by several different molecular tests. Where an instrument such as a microtome is used to cut and dissect slides, the instrument should be carefully cleaned between runs to prevent cross contamination (30 min with 30 % bleach, followed by washing with dH_2O and ethanol has been used to clean all cutting surfaces).

5.5.2 Direct Sequencing

Direct DNA sequencing is commonly used in *KRAS* mutation analysis for mCRC. Typically purified temple DNA is amplified via PCR employing forward and reverse primers spanning the activating mutations in codons 12 and 13 of exon 2. The amplified product is purified and sequenced usually via a commercial automated sequencer and analyzed with special attention given to any changes in codons 12 or 13. While pyrosequencing is commonly used, Sanger sequencing is considered the gold standard and requires that the tumor-derived DNA template contains at least 20 % mutant *KRAS* DNA for detection [64–67]. Most automated sequencers employ software programs to assist in the analysis of the statistical significance of low level base pair changes [64–66]. A clear advantage of direct DNA sequencing is that all base pairs are examined within the amplified sequence.

5.5.3 ARMS

The *Taq* DNA polymerase initiates the PCR reaction poorly from mismatched template-primer 3′-termini; with a 10^{-3}–10^{-6} lower amplification efficiency depending on the mismatch type [68]. ARMS PCR takes advantage of this *Taq* property by using multiple primers with 3′ ends, which match *KRAS* codons 12 and 13 mutations. Increased *KRAS* mutations are detected via technologies such as labeled probe degradation in real-time PCR or via the use of similar Scorpion primers [69, 70]. An advantage of ARMS is that it can detect very small concentrations of mutant *KRAS* since it employs mutation-specific primers. In some systems, ARMS technology can detect one mutant allele in a background of 10^4 wild-type alleles [71]. Additionally, since most tumors with mutations will have some component of unmutated *KRAS*, amplifying unmutated *KRAS* can serve as a useful PCR positive control. One disadvantage of ARMS PCR is that 3′ mismatched primers will initiate amplification at a low rate from a wild-type *KRAS* template [68], thus "*KRAS* mutations" that appear only at very high Cp values should not be considered valid mutations. Comparison to a wild-type *KRAS* amplification controls can be useful. Positive ARMS PCR results have been used where the Cp value is 35 or lower [70].

5.5.4 HRM

HRM involves PCR amplification of the KRAS genomic DNA with primers spanning codons 12 and 13 of exon 2. The amplified DNA is mixed with a fluorophore that emits more strongly bound to dsDNA than ssDNA. The amplified dsDNA is then heated from a low to a high temperature and fluorescent intensity is measured and compared to a wild-type control. Multiple data acquisitions (~25) are taken per degree temperature increase [70]. As the temperature increases, the dsDNA

denatures to ssDNA, lowering fluorescent signal intensity, producing a sigmoid curve. Amplified KRAS sequences with denaturation points different than the wild-type control are mutated [71]. HRM is quick to perform but also relatively expensive and does not identify specific mutations [72].

5.5.5 Comparing Direct Sequencing, ARMS, and HRM

In general, KRAS mutation analysis via DNA sequencing, HRM, ARMS, and other PCR-based detection methodologies give similar results and all are clinically useful [70–72]. Franklin et al. [70] compared these techniques and found some interesting differences. HRM gave the highest frequency of KRAS mutations with 54 % of samples showing denaturation point abnormalities, while DNA sequencing and ARMS gave 36 % and 43 % mutation rates, respectively. Sequencing of the HRM samples with denaturation point abnormalities revealed that only 61 % actually had KRAS mutations. ARMS identified mutations where 100 % detected by HRM, indicating 100 % sensitivity. Additionally, ARMS was 100 % accurate with single round testing. DNA sequencing detected 17 % fewer mutations than ARMS due to lower sensitivity and required a 20–50 % greater concentration of mutant DNA to achieve reliable mutation detection. The authors concluded that (1) HRM samples with abnormal denaturation points should be sequenced to confirm the presence of mutation (making testing cumbersome), (2) DNA sequencing sensitivity may be suboptimal for clinical testing, and (3) ARMS is the most sensitive and rapid of these techniques and probably the most clinically useful technique. Other studies have found similar results with real-time PCR-based assay techniques having greater sensitivity than DNA sequencing [73]. Additionally, many of these techniques, such as ARMS, do not test for the rare KRAS exon three codons 59 and 61 mutations or uncommon codon 13 mutations [32, 70–73]. While these mutations are rare, not testing for them may result in a low number of patients receiving cetuximab or panitumumab inappropriately. For example, individuals with exon 3 codon 61 mutations do not respond to cetuximab therapy, despite having unmutated codons 12 and 13 [74]. Last, differences exist within different sequencing methods. For example, Sanger sequencing requires a ~20 % mutant DNA concentration for mutation detection, while pyrosequencing requires 5 %. Sanger sequencing also yielded 11.1 % and 6.1 % false positive and negatives, respectively, while pyrosequencing gave no false positives and negatives [67, 70]. Interestingly, Tsiatis et al. [67] demonstrated that the same tumor can have different activating KRAS mutations.

5.6 Other Mutations in De Novo CRC

While KRAS is most frequently mutated in cetuximab/panitumumab resistant CRC, other mutations in proteins downstream of the EGFR also confer resistance [48, 74].

5.6.1 Braf

Braf, also known as V-raf murine sarcoma viral oncogene homolog B1 (B-raf-1), is a serine/threonine kinase downstream of KRAS. Activated KRAS phosphorylates and activates Braf, increasing cyclins D1-3, mdm2, and c-myc expression, promoting cell cycle entrance, increasing cell growth potential, and inhibiting apoptosis [48, 74, 75]. Braf is mutated and constitutively activated in several human malignancies, including de novo CRC, malignant melanoma, papillary thyroid cancer, and low-grade ovarian serous carcinomas [48, 74–78]. In CRC, Braf mutation is often associated with MSI [74]. Approximately 8.7–40 % of CRCs carry activating Braf mutations that confer cetuximab/panitumumab resistance [23, 48, 74, 75, 79, 80]. Braf mutations are frequently identified in sessile serrated adenomas, mixed polyps, high-level MSI CRC, and high CpG island methylator phenotype CRC and serrated adenomas. Braf mutations are less common in low CpG island methylator phenotype low and negative CRC, hereditary non-polyposis CRCs, hyperplastic polyps, conventional adenomas, and sporadic non-MSI-high CRCs [81]. Although not yet common, Braf mutation testing is done as reflex testing in some labs, following the determination of unmutated KRAS status. The exact role of Braf mutation testing for determining EGFR inhibitor sensitivity remains somewhat inconclusive. Currently, in the 2012 NCCN guidelines, Braf genotyping remains optional in the setting of Kras wild-type tumors [60].

5.6.2 Molecular Testing for Braf Mutations

Braf is found on 7q34 and consists of 19 exons. Activating mutations occur in exons 11 and 15, with over 90 % of mutations involving a T → A transversion at position 1,779, causing a valine → glutamate substitution at residue 600 (V600E), resulting in 500-fold increased kinase activity compared to wild-type Braf [82]. Braf mutation testing has been performed by several different techniques, including direct sequencing, ARMS, single strand conformation analysis, and restriction fragment length polymorphism analysis [83–85]. While direct automated sequencing is widely accepted as standard, Benlloch et al. [83] compared V600E mutation detection via ARMS vs. automated sequencing and found ARMS to be less costly, labor intensive, require a shorter assay time, and have 100 % sensitivity and specificity, demonstrating that this technique has value for clinical testing. Molecular testing for Braf mutation is complicated by the Braf pseudogene (B-raf-2) located at Xq13, necessitating careful primer design to avoid pseudogene amplification [83, 86]. Interestingly, B-raf-2 is often expressed in B-raf-1 negative papillary thyroid carcinomas. Additionally, B-raf-2 expression is tumorigenic in nude mice, activates

the MAP kinase, and can transform NIH3T3 cells, demonstrating oncogenic potential [87]. Presently, there is no evidence for a role in B-raf-2 expression or mutation in CRC.

5.6.3 NRAS

NRAS mutations are found in ~3 % of CRC [88]. *NRAS* functions in a pathway similar to *KRAS*, but exerts apoptosis suppressive effects and its mutations arise much later in CRC genesis than *KRAS* mutations [53]. *NRAS* is commonly mutated at codons 2, 3, 12, 13, and 61, conferring cetuximab therapy resistance [53, 89, 90]. Multiplex PCR has been used to identify NRAS codon 2 and 3 mutations [88]. Presently, reflex *NRAS* testing is offered by Quest Diagnostics following identification of unmutated *KRAS* and *Braf* status.

5.6.4 PIK3CA and PTEN

PIK3CA, a member of the phosphatidylinositol 3-kinase family, is mutated in 9.9–32 % of CRCs [23, 86]. Activated PIK3CA promotes cell proliferation, motility, and cell invasion, via AKT and PDK1 activation [91–94]. PIK3CA is mutated in CRC; however, the significance of these mutations in anti-EGFR therapy is presently controversial [91, 95, 96].

PTEN, a phosphatidylinosital trisphosphate phosphatase that antagonizes *PIK3CA* activity, is lost in 42 % of CRCs [23]. PTEN loss and *PIK3CA* mutation are likely to contribute to malignant progression [23, 91–94, 97]. However, Tol et al. [23] demonstrated that PTEN loss has no predictive value for cetuximab response.

5.6.5 Future Directions

Only 10 % of EGFR-positive mCRCs respond to cetuximab or panitumumab therapy, while those with indentified unmutated *KRAS* show a ~22 % response [49]. Mutations in *KRAS*, *BRAF,* or *NRAS* are largely mutually exclusive of each other, while *PIK3CA* mutation may coexist with *KRAS*, *BRAF,* or *NRAS* mutations and PTEN loss may coexist with *KRAS* or *BRAF* mutations [23, 48, 74, 97–99]. *KRAS*, *BRAF,* and *NRAS* mutation may all result in resistance to anti-EGFR therapy and since they are largely mutually exclusive, molecular testing for mutations of all three may help better identify patients who can benefit from anti-EGFR therapy.

Presently *KRAS* testing followed by reflex *Braf* testing for unmutated *KRAS* status is recommended [59, 81, 100]. *PIK3CA* and PTEN testing is not presently useful in the evaluation of anti-EGFR therapy use.

5.7 Hereditary Colorectal Cancer Syndromes and Molecular-Genetic Testing

Although 15–35 % of CRCs have a statistically significant genetic component, only 3–10 % of these malignancies result from highly penetrant germline mutations identified as hereditary CRC syndromes [5–8]. The first hereditary CRC was described in 1861 and was probably Familial Adenomatous Polyposis (FAP) [101]. Since then, several hereditable CRC syndromes have been described in detail, including FAP, Attenuated Familial Adenomatous (AFAP), Polyposis Hereditary Nonpolyposis Colon Cancer (HNPCC) or Lynch Syndrome, MYH-Associated Polyposis (MAP), and the *I1307K* adenomatous polyposis (APC) mutation in the Ashkenazi Jewish population. In general, most hereditary CRC results from either *APC* gene loss resulting in chromosomal instability or MSI with loss of mismatch repair gene function [5–8]. Molecular testing plays an important role in the diagnosis and management of these syndromes, providing information for genetic counseling. Failure to detect a mutation does not invalidate a clinically definite hereditary CRC syndrome diagnosis, but identification of a mutation is required to perform predictive at-risk testing for relatives presently without symptoms. In general, the more the clinical picture fits a specific hereditary CRC syndrome, the more likely a positive molecular testing result [102, 103].

5.7.1 Sample Preparation

For hereditary CRC syndrome testing, genomic DNA can be obtained from peripheral blood, formalin-fixed, or formalin-fixed, paraffin-embedded tissue. In general peripheral blood is a better DNA source as it is easier to process, less fragmented/cross-linked, and contains the possible germline mutation.

5.8 FAP

FAP is an autosomal dominant hereditary CRC syndrome, which accounts for slightly less than 1 % of all CRCs with an incidence of 1:7,000–10,000 [9, 104]. FAP is the second most common hereditary CRC and characterized by the development of hundreds to thousand of colorectal adenomas of different sizes. It typically has few symptoms until the mid-to-late teens (average age of 16 years), when the adenomas grow large enough to cause adenoma-related symptoms, such as rectal bleeding, anemia, palpable abdominal masses, weight loss, constipation, and diarrhea.

In FAP, CRC usually develops ~10 years following the clinical appearance of adenomatous colonic polyps, resulting in nearly 100 % of FAP individuals having CRC by their early 1940s, with a mean age of 39 years in untreated individuals and a range of 34–43 years. Other gastrointestinal manifestations include gastric fundic gland polyps and adenomatous polyps in the 2nd and 3rd portion of the duodenum and periampullary region [105]. Interestingly, 40 % of the fundic gland polyps show adenomatous changes, although an increased risk for gastric cancer is none to slight in Western populations, but significantly higher in Asian populations [106, 107]. FAP confers a 100–330-fold higher risk for duodenal cancer, compared with the general population, resulting in a life-time risk for duodenal and periampullary cancers of 10 % and 5 %, respectively [108, 109].

Extraintestinal FAP manifestations include osteomas, desmoid tumors, congenital hypertrophy of the retinal pigmented epithelium (CHRPE), nasopharengyeal angiofibromas, pancreatic mucinous adenocarcinomas, hepatoblastomas, sebaceous and epidermal cysts, and brain tumors, including medulloblastomas and glioblastomas [110]. The clinical diagnosis of FAP rests upon endoscopic identification of more than 100 adenomatous colonic polyps, extraintestinal manifestations of FAP, and a family history of an autosomal dominant inheritance pattern [105].

The genes mutated in FAP and APAP are *APC* located at 5q21-22 and *MUTYH* located at 1p34.3-p32.1. *APC* mutations account for 70–95 % of FAP and 20–30 % of AFAP, while *MUTYH* mutations account for ~6.6 % of FAP and ~18 % of AFAP cases [105, 111–114]. In general, the more severe the FAP symptoms are, the more likely molecular testing will locate a specific *APC* mutation [104, 111–115]. *APC* spans 108,353 bp consisting of 16 exons of which exons 2–16 are coding. Interestingly, exons 2–15 code for 653 amino acids, whereas exon 16 codes for 2,190 [105, 111–115]. APC function is lost in de novo CRC genesis and some cancers of the stomach, pancreas, thyroid, and ovary [116]. It is also, among the early genes, lost in the Vogelstein model of CRC genesis [117–121]. The APC gene product has a molecular weight of 310 kDa and is localized in the cytoplasm and nucleus [122–125]. APC exerts numerous functions, including acting as a negative regulator of β-catenin function, indirectly suppressing *c-myc* levels, maintaining cytoskeletal integrity, cellular adhesion, functioning in Wnt signaling, and regulating the $G_0 \rightarrow G_1$ transition in part via regulating cyclin D1 expression [126–130]. APC also binds the ends of microtubules embedded within kinetochores, forming a complex with the checkpoint Bub1 and Bub3 proteins, with APC being a high-affinity substrate for the Bub kinases. Truncating APC mutations, common in FAP, may alter normal chromosome-spindle attachment, disrupting the mitotic spindle cell checkpoint, causing genomic instability [131].

5.9 *APC* Molecular Testing Methods

Over 700 different *APC* have been reported, with greater than 98 % either being frameshift (68 %) or nonsense mutations (30 %) [111, 131]. Most individuals with FAP will have a family history of FAP with accompanying *APC* mutations;

however, 25–30 % will have de novo *APC* mutations and lack a family history of FAP [132, 133]. The specific mutation patterns show variation, with germline mutations common 5′ at codons 1,061 and 1,309 and somatic mutations common at codons 1,309 and 1,450 within the mutation cluster region [116]. Interestingly, there is a strong, but not complete correlation between the specific *APC* gene region mutated and the FAP phenotype. For example, congenital hypertrophy of the retinal pigment usually only appear with mutations 3′ to exon 9, while individuals with profuse FAP (more than 5,000 polyps) commonly show frameshift mutations between codons 1,255 and 1,467 [134, 135]. The number of mutations and relatively large size of the APC gene make molecular testing complex. Several protocols are currently employed in testing:

5.9.1 APC Protein Truncation Test

Over 98 % of FAP *APC* mutations result in truncated APC protein expression [116]. Based on this, a molecular assay has been developed, the protein truncation test (PTT), which measures disease-inducing *APC* mutations via analyzing protein truncation. The assay involves three steps:

1. Patient nucleic acid is isolated, usually from peripheral leukocytes. Either RNA or genomic DNA is isolated, with RNA transcribed into DNA via reverse transcription PCR. The resulting DNA is amplified via PCR into one or several DNA templates, depending on the size of the original.
2. The templates are transcribed in vitro into RNA, which is subsequently translated into protein, in a reticulocytelysate system.
3. The translated proteins are analyzed with gel electrophoresis, and any changes in the molecular weight of the APC protein are detected by comparison to a wild-type control.

The PCR primers employed introduce a promoter sequence allowing the DNA template to be transcribed into RNA, and subsequently translated into protein in a coupled reaction. Often a T7 promoter sequence is employed for transcription by T7 RNA polymerase, as well as a consensus sequence for the initiation of translation [136, 137]. The protein is visualized by several techniques, such as radiolabeling with ^{35}S-cysteine, ^{35}S-methionine, or ^{3}H-leucine in the reticulocytelysate system. An example of a PTT gel is shown in Fig. 5.1 [136].

PTT has good sensitivity, a low-false-positive rate, and detects disease-inducing APC protein truncations, but not silent mutations, or the more than 20 *APC* gene polymorphisms, none of which cause disease [116, 136, 137]. PTT can also detect promoter mutations that lower *APC* mRNA expression and mutations, which create unstable mRNA (for those assays that amplify *APC* RNA). Disadvantages of the PTT are that it does not identify all *APC* gene mutations and does not identify the specific *APC* gene mutation, which is useful for directed mutation testing within effected kindreds. Last, PTT is superior to Western blotting, having a significantly higher

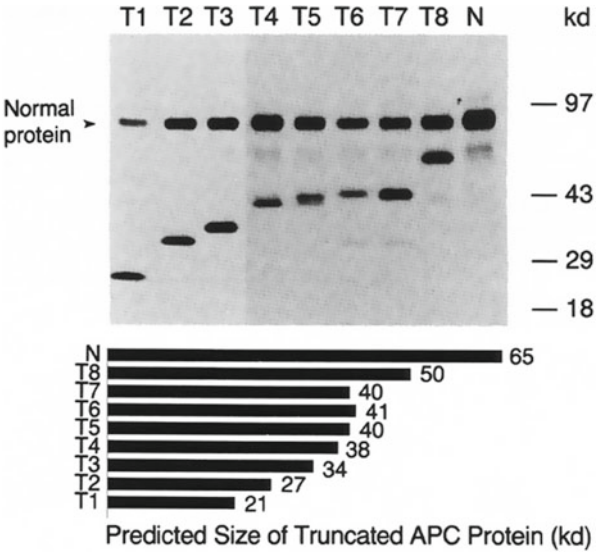

Fig. 5.1 An example of a PTT from Ref. [136]. Eight representative samples from de novo CRCs (T1 → T8), with truncating mutations identified by sequencing analysis. A substantial amount of normal full-length APC protein is seen from remaining normal alleles. The proteins were transcribed and translated in a reticulocytelysate system with ^{35}S-methionine as a label. The kDa of the truncated proteins is predicted of the right

APC protein detection rate [134]. Using this assay, Powell et al. [136] successfully identified APC mutations in 87 % 62 individuals with FAP tested. The PTT test is also used to identify mutations in other genes, such as *BRCA1* and *dystrophin* [126, 138].

5.9.2 Direct APC Gene Sequencing and Multiplex Ligation-Dependent Probe Amplification

Direct DNA sequencing of the *APC* gene is considered the gold standard and detects *APC* gene mutations in ~70 % of individuals with FAP. The advantage of this technique is that all amplified bps are analyzed. However, the length of the APC coding region requires that many primers (~30) be used, making sequencing expensive, labor intensive, and time consuming [139]. Additionally, submicroscopic deletions have been detected in 6–55 % of *APC* kindreds, with larger deletions seen in 2 % and insertions seen in 6 % [115, 139, 140]. Since primer-dependent DNA sequencing is poor at detecting deletions/insertions, techniques such as multiplex ligation-dependent probe amplification (MLPA) have been employed to detect these *APC* mutations [140–142]. In MLPA, genomic DNA is interrogated with two probes that

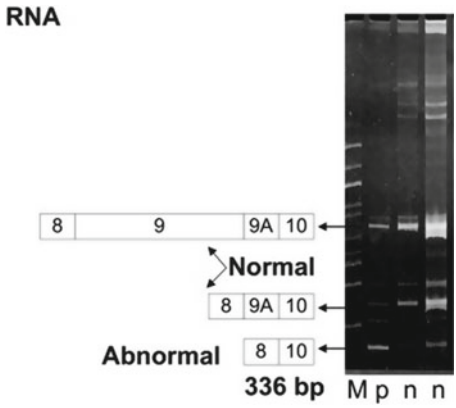

Fig. 5.2 An example of an MLPA analysis run on 6 % PAGE. **M** is a 100 bp ladder. The two **n**'s are normal splice variants of the APC gene; exons 8-9-9A-10 and 8-9A-10. **p** is from an individual with FAP showing a deletion resulting in a shorter 336 bp amplicon. The patient's RNA was amplified by reverse transcription PCR and visualized with ethidium bromide staining

bind adjacent to each other. Each probe has one primer sequence that will amplify the two probes and intervening sequences only if the two probes are ligated. Once the two probes are hybridized to the genomic DNA, a ligation step is performed. If the two probes are hybridized and near enough to each other, the ligation step will produce one probe with an intervening sequence possibly revealing an insertion or deletion. Additionally, the probe will now have two primer sites allowing PCR amplification. The molecular weight of the amplified/ligated region is often measured via gel electrophoresis or by automated systems that measure a labeled and incorporated primer [140–144]. An example of MLPA is shown in Fig. 5.2.

5.9.3 Conformation Strand Gel Electrophoresis and Single Strand Conformation Polymorphism Testing

Conformation strand gel electrophoresis and single strand conformation polymorphism testing (CSGE and SSCP, respectively) have both been used to detect *APC* gene mutations [105, 145]. In CSGE, control homoduplex wild-type dsDNA is compared to dsDNA, which may carry a point mutation. The two DNA duplexes are subjected to electrophoresis under mildly denaturing conditions. Under such conditions, heteroduplexes will often adopt a more complex three-dimensional conformation than homoduplexes and as a result migrate more slowly. In SSCP, wild-type and possibly mutant ssDNAs are compared. The two DNAs are denatured, rapidly renatured, and subjected to electrophoresis. A ssDNA segment with a point mutation will assume a different secondary and tertiary structure, resulting in a different migration pattern compared to the wild-type control. In general, CSGE and SSCP are less efficient and accurate than DNA sequencing; hence, sequencing has largely supplanted these testing methods. However, both still have value and have been used to screen for mutations that are then further analyzed via PTT or DNA sequencing [105]. Some data indicate that CSGE is more sensitive than SSCP [145].

5.10 FAP Management

FAP has an autosomal dominant inheritance pattern, and affected individuals have a virtually 100 % chance of developing CRC by their 1940s, making FAP detection and kindred analysis important in the management and prevention of FAP-associated CRC [105, 146, 147]. Once diagnosed, individuals with FAP should undergo annual lower endoscopy and have as many polys removed as possible. Upper endoscopy should be performed every 6 months to 3–4 years to detect duodenal, gastric, and periampullary adenomas, with the frequency determined by the polyp burden [146, 147]. Prophylactic colectomy is recommended by the end of the second decade, when there is usually a large polyp burden. A total protocolectomy with ileoanal J-pouch is recommended, although an ileorectal anastomosis can be performed on individuals with low rectal polyp numbers. With the latter procedure, yearly endoscopic rectal surveillance is still required [146–149]. Known individuals with FAP while under the age of 10 years should have annual α-fetoprotein levels and abdominal ultrasound to monitor for hepatoblastoma, 1 in 20 of which occur due to FAP [150].

Individuals with FAP given 400 mg/day celecoxib for 6 months showed a 28 % reduction in colorectal adenomas compared to a 4.5 % reduction in the control placebo group. Similarly, 400 mg celecoxib twice per day for 6 months reduced duodenal polyps 14.5 % compared to 1.4 % in placebo controls and 31 % in individuals with a high polyp burden [151, 152]. The US Food and Drug Administration Oncologic Drug Advisory Committee recommends celecoxib as an adjunctive treatment to endoscopic surveillance and colectomy in FAP treatment (New Drug Application Number 21,156).

Following a clinical FAP diagnosis, *APC* mutation testing should be performed. Standard APC mutation testing via DNA sequencing will identify mutations in 70–80 % of individuals clinically diagnosed with FAP [105]. MLPA and other methods will identify a further 2–5 % of mutations involving *APC* insertions/deletions, while *MUTHY* mutations will account for ~6.6 % of cases [105, 111, 141–144]. The remaining ~20 % of FAP have a more severe phenotype and appear to be due to *APC* mutations not identifiable by standard testing [111, 113]. Laken et al. [111] employed monoallelic mutation analysis to independently examine the status of each of the two *APC* alleles without mutations by standard testing. 78 % of the "unmutated" samples tested were found to have significantly reduced expression from one of their two *APC* alleles. Thus ~95 % of FAP is due to APC mutation. Monoallelic mutation analysis is complex and cumbersome, making it difficult to apply to molecular diagnostics.

Once a proband *APC* mutation is identified, all first degree relatives should have genetic counseling and molecular testing for the proband mutation. Individuals with FAP identified by an effected relative have a significantly greater life expectancy than individuals identified by FAP symptoms, warranting presymptomatic testing [153]. Directed mutation testing should be performed to simplify testing and lower costs [154]. Individuals with *APC* mutations should receive endoscopy, celecoxib

treatment, and eventual colectomy. Individuals without mutations should receive endoscopic surveillance recommended for the general population.

When no *APC* mutation is detected in the proband, first degree relatives should receive annual lower endoscopic examines until age 35. If no colonic adenomatous polyps are found by age 35, lower endoscopic examines should be performed every 3 years until age 50, where endoscopic surveillance should match the general population. Where first degree relatives have a high burden of colonic adenomatous polyps, they should be managed as having FAP [105]. Presently, *MUTYH*, MLPA, or monoallelic mutation analyses are not routinely done for FAP testing [105, 111].

5.11 Attenuated FAP (AFAP)

AFAP is clinically defined by an autosomal dominant inheritance pattern, 10–99 adenomatous colonic polyps occurring at a later age than in FAP, and an 80–100 % lifetime CRC risk. Often, the colonic polyps are mostly proximal with little rectal involvement, increasing the likelihood of a misdiagnosis of sporadic polyps [105, 155]. Additionally, gastric and duodenal polyps occur and can rarely become malignant. Breast cancer and hepatoblastoma occur, with other extraintestinal manifestations being rare [155]. *APC* mutations are found in 20–30 % of individuals with AFAP and another 18 % have *MUTHY* mutations [105, 112]. *APC* mutations in AFAP are generally found 5′ or at the 3′ end of the gene, proximal to codon 1,517 or distal to codon 1,900 [105, 156–158]. Molecular testing for *APC* mutations in AFAP are done as for FAP. If testing reveals no APC mutation in individuals with AFAP, *MUTHY* testing should also be performed [105]. Due to the very high CRC incidence in AFAP, effected individuals and their first degree relatives should receive the same counseling and treatment as those with FAP [105].

5.12 Turcot and Gardner Syndromes

Turcot and Gardner syndromes are phenotypic variants of FAP. Gardner syndrome is characterized by colonic, gastric, and duodenal polyposis and soft tissue tumor, including osteomas, lipomas, fibromas, epidermal inclusion cysts, and desmoids tumors [159, 160]. *APC* mutations associated with Gardner syndrome are usually truncating mutations between codons 1,403 and 1,578 [160]. Molecular testing is the same as for other FAP variants [160].

Turcot syndrome is characterized by primary brain tumors accompanied by colorectal adenomas and/or CRC [161, 162]. Interestingly, Turcot syndrome shows molecular heterogeneity, with one form caused by *APC* mutations and the other resulting from HNPCC with mutations in one of the DNA mismatch repair genes

MSH2, MLH1, PMS1, or *PMS2* (see below, [162, 163]). In general, individuals with Turcot syndrome with *APC* mutations will have medulloblastomas, while those with HNPCC will have gliomas [162, 163]. Molecular testing for Turcot syndrome involves *APC* mutation analysis or DNA mismatch repair gene (MMR) analysis (see below).

5.13 I1307K *APC* Mutation in Ashkenazi Jews

Ashkenazi Jews carry a number of ancestral mutations conferring predisposition to several diseases, including CRC. One mutation found in 6–12 % of this population is the *APC:I1307K* mutation, involving a T→A transversion at nucleotide 3,920 converting AAATAAAA to an extended mononucleotide tract (A_8), resulting in a lysine replacing isoleucine at codon 1,307 [164, 165]. While this amino acid substitution does not alter *APC* gene function, the A_8 polymorphism is ~32 times more mutatable than the most mutatable wild-type *APC* sequences and often results in somatic biallelic *APC* inactivation and CRC tumorigenesis [164–166]. Interestingly, Gryfe et al. [166] found that, of the CRCs in *APC:I1307K* mutation carriers, only 42 % had *APC*-specific mutations that could be linked to the CRCs, a very modest mutation rate compared with HNPCC. Additionally, unlike in FAP, *APC:I1307K* must undergo somatic biallelic *APC* inactivation, resulting in only a 1.5–2-fold increased CRC incidence [164]. After adjustment for age, *APC:I1307K* carriers are more likely than noncarriers to have colorectal polyps, with either tubular adenomas or tubulovillous adenomas, but not villous adenomas, significantly more frequent among carriers (37.2 % vs. 23.6 %, P = 0.005) [166].

APC:I1307K mutation testing is limited to measuring a T→A transversion, making analysis via allele-specific oligonucleotide hybridization PCR a logical choice. For example, Niell et al. [167], employed a common reverse primer and two forward primers: one wild-type (5′-CTTTTCTTTTATTCTGC-3′) and one *APC:I1307K* (5′-CTTTTCTTTTTTTTCTGC-3′) specific primer. The 1 bp difference allowed the identification of the *APC:I1307K* transversion incidence in different populations. Other investigators have used PCR to amplify the genomic region around the *APC:I1307K* transversion and employed SSCP analysis, with transversion positive samples confirmed via DNA sequencing [168]. Not surprisingly, PCR has also been used to amplify the genomic region around the *APC:I1307K* transversion followed by direct amplicon sequencing [165].

Individuals with a positive *APC:I1307K* mutation test should undergo colonoscopic surveillance, as they may benefit from the early eradication of pre-cancerous polyps [169]. There is some evidence that smoking may increase CRC in *APC:I1307K* carriers, so smoking cessation should be encouraged [165]. The Ashkenazim population also have a low (0.7 %) carrier incidence of *MSH21906G > C* mutation, which is highly penetrant and causes CRC at an early age [164]. Last, although this population has a low (0.9 %) incidence of a *BLM* exon 10 frameshift mutation; BLM^{ASH}. BLM^{ASH} carriers do not have an elevated CRC incidence [165, 170].

5.14 MYH-Associated Polyposis

5.14.1 MUTYH and the MAP Phenotype

Reactive oxygen species derived from normal aerobic metabolism and exogenous sources readily damage DNA forming a wide variety of lesions, including oxidized bases [171–174]. One well-studied and common oxidized base is 7,8-dihydro-8-oxo-deoxyguanosine (8-oxo-G). 8-oxo-G readily pairs with adenine, leading to G:C → T:A transversion mutations following DNA replication [172–174]. MYH, the *MUTYH* gene product, is a glycosylase that catalyzes the cleavage of adenine mismatched to 8-oxo-G via base excision repair, preventing G:C → T:A transversions [175, 176]. Biallelic *MUTYH* mutation results in glycosylase activity loss and increased G:C → T:A transversions, including in *APC* and *KRAS*, but not *Braf*, leading adenoma development and CRC [177, 178].

MAP is the most important differential diagnosis for FAP and AFAP, occurring in ~1/10,000 individuals, accounting for ~0.6 % of all CRC [179, 180]. Phenotypically, biallelic *MUTYH* mutation (homozygous or compound heterozygous) results in a 93-fold increased CRC risk, with clinical symptoms appearing at 40–60 years, and a variable number of adenomatous colonic polyps, 10–100, rarely up to 1,000 [180]. Extraintestinal manifestations are less common than in FAP, with ~31 % of effected individuals having duodenal adenomas and/or gastric fundic polyps and a 4 % lifetime duodenal cancer risk [180–182]. MAP is autosomal recessive and therefore does not show the strong intergenerational history of FAP [180, 181]. The CRC incidence is high in MAP, with a 100 % lifetime risk and 50 % of effected individuals having CRC at diagnosis [181, 183]. Extraintestinal manifestations of MAP include breast, endometrial, and sebaceous carcinomas [182, 184]. Interesting, neither immunohistochemistry nor histology reveal any significant differences between MAP-associated CRC and sporadic CRC [185].

5.14.2 Molecular Testing for MAP

Unlike the 700+ known *APC* mutations, about 23 pathogenic *MUTYH* mutations are known, with two (Y165C and G382D), accounting for ~82 % of inactivating mutations [114, 115, 186]. Thus initial MAP testing is often directed at these two mutations. If one of these mutations is identified, sequencing is performed to identify the inactivating mutation on the opposite allele. If two inactivating mutations are found, the CRC risk will be very high. Failure to initially identify the Y165C or G382D combined with a strong suspicion for *MUTYH* mutation necessitates testing for less common mutations usually by DNA sequencing, which has the highest clinical sensitivity [187].

Numerous studies have demonstrated that in many cases ARMS is useful in MAP testing. For example, Piccioli et al. [188] employed a tetra-primer amplification refractory mutation system PCR (T-ARMS-PCR) to examine six common *MUTYH* mutations, which would identify at least 85 % of all mutations; Y165C, G382D,

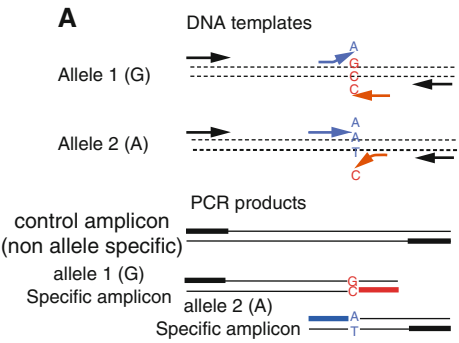

Fig. 5.3 T-ARMS-PCR from Ref. [188]. Two control primers (*black arrows*) which flank the mutation site, amplify one non-allele-specific control fragment, while two allele-specific primers in opposite orientations (*blue and red arrows*) in combination with the control primers, can amplify both smaller wild-type or mutant amplicons. The two allele-specific amplicons have different lengths and can easy be separated and identified via electrophoresis. Thus, the presents of wild-type and/or mutant amplicons can be determined with the longer non-allele-specific control amplicon serving as a positive control

1395_7delGGA, Y90X, 1103delC, and R231H. T-ARMS-PCR amplifies both wild-type and mutant alleles in one PCR reaction. T-ARMS-PCR and ARMS-PCR work well for MAP testing and cover ~85 % of known mutations [188, 189]. It is relatively inexpensive, can identity small deletions, requires no specialized equipment, and importantly is ideal for targeted molecular testing. Thus, once a kindred-specific mutation has been identified, ARMS-PCR and T-ARMS-PCR allow inexpensive, accurate, and rapid molecular analyses. Again ARMS-PCR does not identify ~15 % of known mutations, making DNA sequencing analysis necessary to identify uncommon mutations [187, 189]. The T-ARMS-PCR protocol is described in Fig. 5.3.

5.14.3 MAP Management

For individuals with a significant colonic adenoma burden, *MUTYH* and *APC* mutation testing can be performed simultaneously, particularly if the family history suggests an autosomal recessive inheritance pattern. *MUTYH* testing should also be considered for the 20–30 % and 70–90 % of *APC* mutation negative FAP or AFAP cases, respectively. For FAP, the number due to *MUTYH* mutations is low, accounting for only ~5 % of FAP [111–114]. Once an individual is identified as having inactivating biallelic *MUTYH* mutations, molecular testing of all siblings is recommended as they have a 25 % chance of having the same mutation [112]. Presently, testing the partners of individuals with bilallelic *MUTYH* mutations is controversial, as the risk of MAP for children of an effected parent is ~1 % [181]. However if the partner of a bilallelic mutation carrier has one *MUTYH* mutation, an apparently autosomal dominant CRC inheritance is seen, possibly resulting in a misdiagnosis of FAP. Additionally, the CRC risk for *MUTYH* mutation heterozygotes is low, with

a relative risk of 1.5–2.1 to the general population. Presently, the need for increased lower endoscopic surveillance is this population is undetermined [112, 190, 191].

Individuals with biallelic *MUTYH* mutations should undergo annual or biennial lower endoscopic surveillance beginning as teens. Upper endoscopic surveillance for duodenal adenomas/adenocarcinomas should be performed every 2 years [192]. The high rate of progression to CRC often necessitates a prophylactic colectomy [112, 192].

5.15 HNPCC

Lynch Syndrome, also known as HNPCC, is the most common hereditary CRC, accounting for ~2 % of all CRCs [193]. HNPCC was identified in 1996, when two large kindreds in the Midwestern United States showed excess CRC that lacked multiple colonic adenomas with a constellation of associated malignancies, particularly endometrial cancers [194]. Subsequent researchers named the disorder "Lynch Syndrome" or HNPCC. HNPCC is characterized by an autosomal dominant inheritance, high penetrance (~85 %), early onset CRC (~45 years), proximal CRC predilection, accelerated adenoma → CRC progression, increased survival with CRC, and CRC, which is histologically poorly differentiated, having increased mucinous and signet ring cell-features, and an excess of tumor-infiltrating lymphocytes. HNPCC associated extracolonic tumors include endometrial, ovarian, sebaceous, gastric, small intestinal, pancreatic, brain, hepatobillary tract, and upper urothelial cancers [193–195].

HNPCC results from activity loss of one or more DNA mismatch repair (MMR) genes, resulting in MSI, a specific form of genomic instability: [194, 195]. DNA polymerase activities collectively have a 10^{-7} error rate/bp synthesized. MMR gene activity locates and removes the substitution mismatches and insertion/deletion mismatches that arise during DNA replication. Additionally, the MMR genes play a role in dsDNA break repair (the most harmful DNA lesion), the initial sensing of DNA damage, and the initiation of apoptosis with severe DNA damage [171, 196, 197]. Overall, MMR gene activity increases genomic stability 50–10^3-fold, making MMR activity loss a powerful mutator phenotype [196]. The MMR genes are described below.

5.15.1 MutL Homolog 1 (MLH1)

MLH1 is located at 3p21.3-23 and consists of 19 exons of 57.36 kb, coding for an 84.6-kDa nuclear protein. MLH1 has two interaction domains, one which interacts with MSH2, MSH3, or MSH6 and the other which interacts with PMS2, MLH3, or PMS1. More than 250 different *MLH1* germline mutations in NHPCC have been identified, consisting of missense, nonsense, splicing errors, and insertion/deletions, many of which truncated protein expression [198, 199]. Germline *MLH1* mutations

are seen in ~46 % of HNPCC cases, while 78 % of sporadic CRCs with high MSI (MSI-H) have reduced *MLH1* expression secondary to promoter methylation and *BRAF* mutation. Thus *MLH1* inactivation appears to occur by separate molecular pathways in HNPCC vs. sporadic CRC [200].

5.15.2 MutS Homolog 2 (MSH2)

MSH2 is located at 2p22-p21 and consists of 16 exons of 80.10 kb, coding for a 104.7 kDa nuclear protein. MSH2 has DNA, MSH3/MSH6, and MLH1/PMS2 binding domains. More than 300 different *MLH2* germline mutations in HNPCC have been identified, consisting of mutation types similar to those seen in *MHL1*. *MLH2* mutations are seen in ~40 % of HNPCC cases, thus ~90 % of HNPCC CRCs result from *MHL1* or *MLH2* mutations [198–202].

5.15.3 MutS Homolog 6 (MSH6)

MSH6 is located at 2p16 and consists of 10 exons of 23.8 kb, coding for a 160 kDa nuclear protein. *MSH6* mutations account for 7–10 % of HNPCC [202–204]. 73 % of female *MSH6* mutation carriers develop endometrial hyperplasia and carcinoma, compared to 29 % in *MSH2* and 31 % in *MLH1* carriers, making endometrial tumors more common among female *MSH6* mutation carriers than CRC [205]. Most mutations are truncating mutations producing an inactive protein [205]. Interestingly, individuals with *MSH6* mutations have a lower CRC incidence and a later age of onset than those with *MSH2* and *MLH1*mutations [206].

5.15.4 Postmeiotic Segregation Increased 2 (PMS2)

PMS2 is located at 7p22.2 and consists of 15 exons of 63.14 kb, coding for a 96 kDa nuclear protein. *PMS2* mutations account for less than 2 % of HNPCC kindreds and normally function as heterodimers with *MLH1* forming MutLα [198, 202–204]. MSH2, MSH6, MLH1, and PMS2 associate with BRCA1, ATM, RAD50, MRE11, NBS1, and PCNA, forming a complex suggested to regulate DNA replication and maintain genomic integrity [207].

5.15.5 MutS Homolog 3 (MLH3)

Wu et al. [208] examined 39 unrelated HNPCC families who fulfilled the Amsterdam Criteria for HNPCC. Of 288 individuals examined, 12 had 10 different germline *MLH3* variants, including one frameshift, and nine missense mutations. Normal

immunostaining for MSH2, MLH1, or MSH6 was detected. The authors concluded that *MLH3* mutation probably played a role in a small subset of HNPCC.

The molecular mechanism underlying CRC carcinogenesis in HNPCC appears to be increased genomic instability in ~32 target genes that have mononucleotide repeats of 7 or more within their coding sequences. Many of these genes, such as *RAD50, BLM,* and *PTEN*, are tumor suppressors; others such as *TGFβ1R2* and *BAX* inhibit epithelial cell growth or exert proapoptotic effects. MSI causes frameshift mutations in these genes, leading to loss of function [209, 210]. Interestingly, *MLH3, MSH3,* and *MSH6* have A_9, A_8, and C_8 tracts, respectively, thus loss of one MMR gene may lead to progressive inactivation of the entire MMR system, possibly explaining the rapid adenoma → CRC progression seen in HNPCC [211].

5.16 HNPCC Diagnosis

Several guidelines have been developed to aid in HNPCC diagnosis: the Amsterdam criteria, the Amsterdam II criteria, and recently the Revised Bethesda Guidelines [212]. The Revised Bethesda Guidelines recommend MSI testing and/or immunohistochemical analysis in the following groups:

- Colorectal cancer diagnosed in a patient less than 50.
- Presence of synchronous, metachronous CRC, or other HNPCC associated tumors, regardless of age.
- CRC with the MSI-H histology diagnosed in a patient less than 60.
- CRC diagnosed in one or more first-degree relatives with an HNPCC-related tumor, with one of the cancers being diagnosed under age 50 years.
- CRC diagnosed in two or more first-or second-degree relatives with HNPCC-related tumors, regardless of age.

Additionally, the following patients should also receive the same testing:

- Patients diagnosed with Muir-Torre syndrome (association of CRC with cutaneous sebaceous neoplasms) or Turcot Syndrome (especially glioblastoma brain tumor).
- Family members of individuals with a known MMR gene mutation.

5.16.1 HNPCC Testing Guidelines

Once HNPCC is suspected, specific testing protocols should be followed to obtain a definitive diagnosis. First, MSI or immunohistochemical analyses of CRC and normal tissue should be performed. Should MSI be present, or MMR protein expression lost in CRC, further analyses are performed to diagnose HNPCC [212, 213].

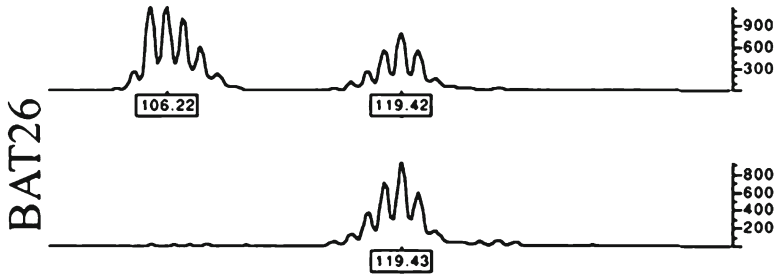

Fig. 5.4 An example of CRC MSI positivity at the BAT26 microsatellite from Ref. [212]. Genomic was PCR-amplified from tumor (*upper*) and normal (*lower*) tissue and run in an ABI PRISM 377 DNA Sequencer. The data were collected automatically and analyzed by GeneScan 3.1 software

5.16.2 MSI Testing for HNPCC

MSI testing involves the analysis of five microsatellite markers standardized by the National Cancer Institute for HNPCC screening: BAT-25, BAT-26, D2S123, D5S346, and D17S250. Typically, both tumor and matching normal patient DNA are compared. MSI testing may be performed without normal patient DNA, but it is recommended that all studies assessing MSI using these markers should use matched normal-tumor DNA pairs [212]. Each microsatellite marker is PCR amplified with primers specific for each marker, and the resulting amplicons are analyzed for length variation via techniques such as gel electrophoresis, automated sizing equipment, or denaturing high-performance liquid chromatography. The primers may be labeled or unlabeled, depending on the requirements of the amplicon detection method [213–215].

MSI is defined as a change of any length due to a deletion/insertion of repeating units within a tumor microsatellite not seen in normal tissue DNA. With five markers, MSI is present and high (MSI-H) when ≥ 2 or more microsatellite markers show MSI. MSI is low (MSI-L) when one marker has MSI, and the tumor is MSI-L or microsatellite stable when one or no markers show MSI. Where more the five markers are analyzed, $\geq$ 30–40 % with MSI equals MSI-H, $<$ 30–40 % is MSI-L, and no MSI is microsatellite stable [213]. Microsatellite stable or MSI-L tumors are unlikely to result from HNPCC [212]. An example of an MSI analysis is shown in Fig. 5.4.

5.16.3 Immunohistochemical Analysis for HNPCC

Immunohistochemical analysis for the loss of MMR genes is as effective as molecular MSI testing for HNPCC [212, 215, 216]. Immunohistochemical analysis of CRC and adjacent normal colorectal epithelium from individuals with HNPCC will often reveal loss of nuclear MSH2, MLH1, MSH6, or PMS2 protein expression. Following

CRC analysis, if staining for each MMR protein is identified, the individual is unlikely to have HNPCC. If staining for an MMR protein is lost in CRC, but present in adjacent normal colonic epithelium, HNPCC is likely to be present [212, 215, 216]. The correlation between immunohistochemical MMR protein loss in CRC and MSI is high, with some studies showing MLH1 or MSH2 loss having 100 % specificity and 92.3 % sensitivity for predicting CRC with MSI-H [216]. Although both MSI and immunohistochemistry will work for initial testing, ideally both should be performed [212, 215]. Interestingly, both MSI-H and loss of MMR protein expression has been identified in a significant portion of colonic adenomas from individuals with HNPCC, demonstrating that these events occur early in CRC genesis [215]. Immunohistochemical testing has the advantage of being both cheaper and faster than MSI testing, with costs estimated to be 14–28 % of MSI testing [217]. There is growing interest in the application of reflex testing for MMR and/or MSI for all patients diagnosed with CRC. In particular, the Evaluation of Genomic application in Practice and Prevention (EGAPP) Working Group has provided genetic testing strategies in newly diagnosed individuals with colorectal cancer aimed at reducing morbidity and mortality from Lynch Syndrome in relative.

5.16.4 Molecular MMR and BRAF V600E Mutation Testing in HNPCC

With no loss of MMR protein immunopositivity, or MSI testing showing MSI-L/MMS, HNPCC is unlikely and no further testing is required [212]. Should MSI-H and/or MMR protein loss be identified, molecular testing should be performed for MMR gene mutations. Most protocols recommend *MLH1* and *MSH2* molecular testing, as these make up ~90 % of MMR gene HNPCC mutations [198–202, 212, 215, 216]. If immunohistochemistry detected loss of a specific MMR protein, molecular testing should be directed to the encoding gene [212, 215, 216].

Ten to fifteen percent of sporadic CRCs are MSI-H, have MLH1 protein loss, and have the specific *BRAF-V600E* mutation [82–86, 218, 219]. In these tumors, the *MLH1* promoter is hypermethylated, leading to loss of protein expression, while the *MLH1* sequence is unmutated [84, 85, 218, 219]. *BRAF-V600E* occurs only in sporadic CRCs and is not seen in HNPCC-associated CRCs [84, 85, 220, 221]. Therefore, *MLH1* molecular testing is unnecessary where a *BRAF-V600E* mutation has been identified, unless there remains a high clinical suspicion for HNPCC [84, 85, 195, 220, 221]. Thus *BRAF-V600E* testing can rule out HNPCC, saving time and avoiding costly MMR gene molecular analysis [82, 84, 85, 220].

Molecular testing for *BRAF-V600E* is described above. Testing typically follows two approaches:

1. With MSI testing, *BRAF-V600E* mutation testing should be performed. With no identified *BRAF-V600E* mutation, *MLH1* and *MLH2* mutation testing should be performed. If no *MLH1* and *MLH2* mutations are identified, *MSH6* and *PMS2* testing can be performed.

2. Where immunohistochemistry for MMR protein expression is employed, the specifically lost MMR gene should be tested for mutations. If the lost MMR protein is MLH1, *BRAF-V600E* mutation testing should be done first and followed by *MLH1* gene analysis if *Braf* is unmutated.

Failure to identify an MMR gene mutation usually rules out HNPCC, while identification of an MMR gene allows an HNPCC diagnosis. In some cases, a gene variation of unknown significance is identified. In other cases, no MMR gene mutation is identified, but the family history and/or individual's presentation still strongly indicate HNPCC. Genetic testing will not identify all mutations leading to a HNPCC phenotype. Where an MMR gene mutation is identified, directed testing should be performed on the probands at-risk relatives and genetic counseling should be offered [195].

MMR gene analysis has been performed in a variety of ways, including Southern blotting, SSCP, DNA sequencing, MLPA, denaturing gradient gel-electrophoresis, quantitative PCR, and combinations of these methods [222–225]. Many germline *MLH1* and *MSH2* mutations are missense, nonsense, and frameshift mutations and are readily detected by these techniques. However, other mutations include large deletions and duplications [225–227]. While Southern analysis will detect large genomic rearrangements, it is also cumbersome, expensive, and time-consuming compared to other methods [222]. Charbonnier et al. [225] employed short fluorescent fragments to analyze *MLH1* and *MSH2* mutations, detecting deletions and duplications. 19 and 16 labeled primer sets were required for *MLH1* and *MSH2* analysis, respectively, with the reactions multiplexed and analyzed on a Gene scanner model 672 fluorescent fragment Analyzer. Control and patient electropherograms were superimposed and amplifications/deletions were detected by observing changes between superimposed control DNA and control and patient DNA. Most *MLH1* and *MSH2* mutations are unique with only a few common and recurring mutations identified, thus ARMS would be an extremely inefficient approach to MMR gene mutation analysis [226]. An example of the technique employed in Vasen et al. [228] is shown in Fig. 5.5.

5.16.5 HNPCC Management

Järvinen et al. [229] compared two at-risk HNPCC cohorts over a 15-year period. Colonoscopic screening reduced the CRC incidence 62 %. Additionally, the CRCs in the screened cohort were localized and caused no deaths, compared to 9 CRC-related deaths in individuals who declined colonoscopy. Interestingly, Vasen et al. [228] found several HNPCC patients with CRC 3.5 years following a negative colonoscopy, demonstrating a need for short screening intervals. Currently, individuals with HNPCC should receive colonoscopy every 1–2 years, beginning at age 20–25 (age 30 years with *MSH6* mutation), or 10 years before the first effected family member presented with CRC.

In HNPCC, an increased incidence of CRC is found arising in flat and diminutive adenomas proximal to the splenic flexure. Interestingly, pan-colonic chromoscopic

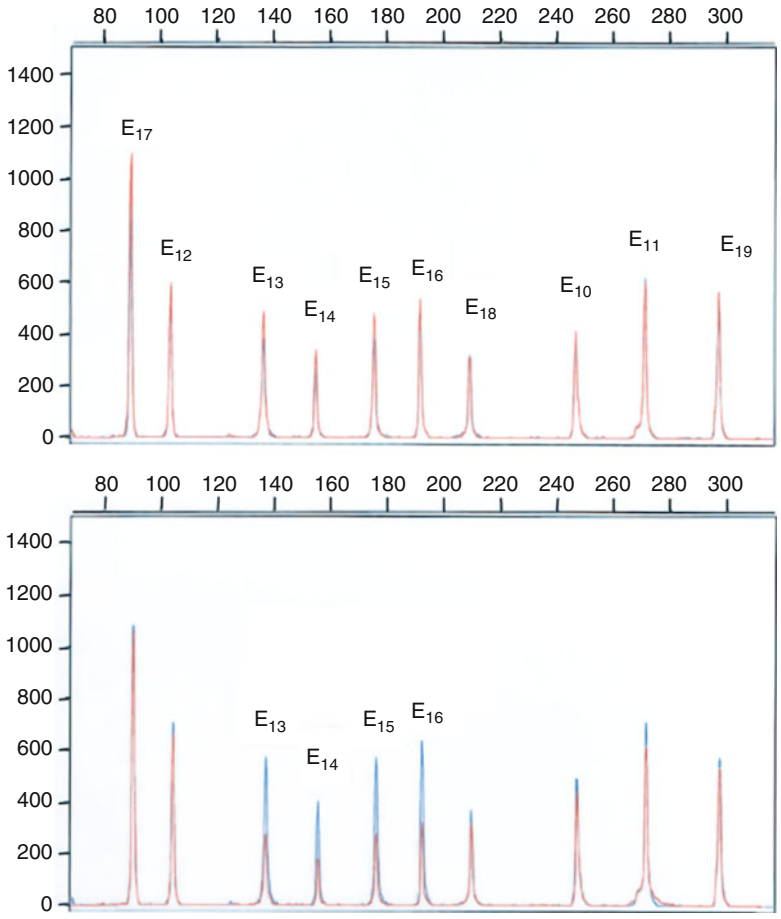

Fig. 5.5 The technique employed by Charbonnier et al. [226] to analyze exon deletions and duplications of the MMR genes in HNPCC CRC families, using multiplex polymerase chain reaction of short fluorescent fragments. Electropherograms from two individuals were superimposed. The *Y* axis displays fluorescence in arbitrary units, and the *X* axis indicates the size in bp. The upper diagram is a multiplex PCR of 10 *MLH1* exons in two controls (*in blue and red*). The lower diagram shows detection of a genomic *MLH1* deletion (*in red*) involving exons 13–16

colonoscopy significantly increased flat and pedunculated adenoma detection in HNPCC. Presently however, pan-colonic chromoscopic colonoscopy is not routinely employed in the endoscopic surveillance of individuals with HNPCC, although it may allow a longer surveillance period prior to colectomy [230]. If endoscopic surveillance cannot be performed, a subtotal colectomy should be considered [231]. Prophylactic colectomy, whether subtotal or complete proctocolectomy, is not recommended if annual endoscopic surveillance can be carried out [231]. When unresectable adenomas or CRC are found, subtotal colectomy may be performed due the increased rate of secondary tumors [231].

Women with HNPCC have a 20–60 % lifetime risk for endometrial cancer and should undergo annual endometrial biopsy beginning at age 30–35 years. Transvaginal ultrasound has little value in screening for endometrial cancer, but can be used for annual ovarian screening [231–233]. Prophylactic hysterectomy/oophorectomy can be performed once the childbearing years are completed, as it results in a significant reduction in endometrial and ovarian cancer [234]. Screening for the more common Lynch Syndrome-associated malignancies, such as gastric cancer and urothelial cancer, is also recommended with frequency dictated by individual and family history (NCCN CRC screening guidelines). Screening for rarer malignancies is not defined by guidelines but is determined by individual and family history.

Most studies on NSAID use and chemoprevention in hereditary CRC have focused on FAP. There are no data on the effects of NSAIDS on chemoprevention in HNPCC, and they are currently not recommended. Additionally, although oral contraceptives are known to lower the incidence of endometrial and ovarian cancer in the general population, no data have demonstrated a chemopreventative effect in HNPCC. Last, individuals with HNPCC show an increased CRC with smoking and should be encouraged not to smoke [231].

5.17 Hamartomatous Polyposis Syndromes

Several heritable CRC syndromes are characterized by a specific constellation of extracolonic manifestations and increased hamartomatous rather than adenomatous polyps. Hamartomatous polyposis syndromes were first described in 1957 when investigators [232] reviewed the literature on pediatric on colorectal polyps and found that most contained mucous-filed glands with retention cysts, abundant connective tissue, and a high number of eosinophils. Presently, two hamartomatous polyposis syndromes with increased CRC are recognized: Juvenile polyposis syndrome (JPS) and Peutz-Jeghers syndrome (PJS). Although both are diagnosed based on clinical manifestations, molecular tests are used to aid diagnosis.

5.17.1 JPS

JPS was first reported by Diamond et al. [235] who described a 30-month-old girl with a prolapsed polyp, accompanied by constipation, hematochezia, and a pedunculated sessile polyp on proctoscopy. In 1975, Stemper et al. [236] described a family with 10 affected members; juvenile polyps were identified in the stomach, small and large intestines, and rectum. Several developed gastric and CRC. Since that time, many studies have found increased gastrointestinal malignancies in kindreds affected by JPS, including gastric, colon, pancreatic, and small intestinal cancers [236–240]. The lifetime risk for malignancy in JPS is estimated to be 9–50 % [241, 242]. Histologically juvenile polyps are characterized by a smooth

appearance, with cystically dilated glands embedded in a hyperplasic stroma which has an inflammatory infiltrate.

JPS is presently defined by any of three manifestations: (1) more than 5 juvenile polyps in the colorectum, (2) multiple juvenile polyps throughout the GI tract, and (3) any number of juvenile polyps and a family history of JPS [243]. JPS has been divided into three distinct categories: (1) JPS where the disease is limited to the colon, (2) JPS of infancy characterized by early onset, failure to thrive, hypoalbuminemia, and a poor prognosis, and (3) generalized JPS, where juvenile polyps are seen in the upper and lower gastrointestinal tract [244]. JPS is rare, occurring in ~1 in 10^5 individuals and shows an autosomal dominant inheritance pattern, with approximately 75 % of individuals having an affected parent and 25 % of cases arising de novo [245, 246]. The diagnosis of JPS is largely clinical, with molecular testing employed for prenatal diagnosis, kindred testing, and clinical diagnosis confirmation.

5.17.2 BMPR1A and SMAD4

Two genes are mutated in PJS: *BMPR1A* and *SMAD4*, both accounting for ~20 % of JPS cases [247].

SMAD4 or mothers against decapentaplegic homolog 4, is located at 18q21.1 and consists of 13 exons, which encode a 60.4-kDa protein of 552 residues. *SMAD4* encodes an intracellular mediator of the TGF-β family and activin type 1 receptor signaling. Ligation of the TGF-β family receptor leads to SMAD1,−5 and−8 phosphorylation and activation, forming a complex with Smad4, activating pathways that mediate growth inhibitory signals [247–249]. Over 60 *SMAD4* mutations have been identified in JPS. Interestingly, one mutation identified in multiple kindreds deletes 4 nucleotides (1244_1247delACAG), creating a truncated protein of 433 residues lacking regions critical for normal protein activity [249, 250].

BMPR1A or bone morphogenetic protein receptor, type IA, is located at 10q22.3, and consists of 13 exons that encode a 60.2 kDA protein of 532 residues. BMPR1A is a serine-threonine kinase, which upon binding TGF-β, phosphorylates and activates SMAD1,−5, and−8. These SMADs bind SMAD4, creating a complex that inhibits cell growth and division [247–249]. Thus loss of either BMPR1A or SMAD4 activity results in cellular release from the TGF-β growth-inhibitory pathway, increasing malignant transformation risk [247, 248]. More than 60 *BMPR1A* have been identified, most of which produce a truncated, nonfunctional protein [247–250]. Together, *BMPR1A* or *SMAD4* mutations account for ~45 % of all JPS cases [250]. Patients lacking *BMPR1A* or *SMAD4* mutations are likely to have undiscovered gene mutations. Therefore, failure to identity *BMPR1A* or *SMAD4* mutations does not exclude a JPS diagnosis. The identities of other genes mutated in JPS are currently unknown, although some data implicate endoglin (*Eng*) gene mutation [251].

5.17.3 Molecular Testing for JPS

Due to the large number of *BMPR1A* or *SMAD4* mutations, DNA sequencing is most often employed in mutation analysis of these genes, although techniques such as PCR based denaturing gradient gel electrophoresis have been employed [249–251]. As for other genes analyzed, a set of specific forward and reverse primers first amplify the genomic DNA, which is purified, analyzed in a commercial sequencer, and compared to the wild-type sequence. Where a mutation is identified, it is often confirmed by sequencing in the opposite direction [249]. Such approaches have been able to identity 18 % and 21 % of *BMPR1A* and *SMAD4* JPS mutations, respectively [249, 250]. As with most direct sequencing techniques, a number of deletions/insertions are missed; hence, techniques such as MLPA have been used to identify 6 % and 7 % more *BMPR1A* and *SMAD4* JPS mutations, respectively [250]. Once identified, directed mutational testing can be performed on individuals related to the proband, who may be at risk for JPS.

5.17.4 JPS Management

Once an individual is identified as having JPS, a thorough history and physical examination should be performed, with particular emphasis on the present or absence of rectal bleeding/prolapse, anemia, constipation, obstruction, diarrhea, and abdominal discomfort. Symptomatic individuals should have upper and lower endoscopies, while asymptomatic individuals should have endoscopic screenings beginning at age 15, with all polyps removed. If the initial endoscopic examinations reveal no polyps, they should be repeated in 3 years. If polyps are found, endoscopies should be repeated every year, with all polyps removed. Once no more polyps are identified, endoscopic screening should performed once every 3 years. Individuals related to the proband without an identified mutation should been screened at age 15 and if no polyps are identified screening should be done 10 years to age 45 and then as the general population if no polyps are found [252].

Children with severe bleeding, iron-deficiency anemia, hypoproteinemia, failure to thrive, and/or a non-reducible intussusception should receive an ileorectal anastomosis [253]. Individuals without these severe symptoms should receive an ileorectal anastomosis by age 20–25, when the cancer risk becomes greater than risks associated with surgery. Individuals receiving an ileorectal anastomosis should undergo flexible sigmoidoscopy every 1–3 years for polyp formation in the rectal remnant [254]. Last, individuals with generalized JPS should undergo upper endoscopy every 3 years or annually if polyps are present, beginning at age 15, due to the increased upper gastrointestinal malignancies seen in generalized JPS [241, 242, 254]. Gastric polyps tend to be diffuse and difficult to endoscopically remove, hence a total of subtotal gastrectomy should be performed where gastric outlet obstruction, adenomatous changes, or adenocarcinoma are identified [254].

5.17.5 PJS

PJS is inherited in an autosomal dominant pattern occurring in ~1 in 60,000 and is characterized by pigmented mucocutaneous lesions, especially on the lips, buccal mucosa, and digits, as well as hamartomatous polyps mainly seen in the small intestine. The first detailed description of PJS was given by Peutz in 1921, who described a Dutch family with familial gastrointestinal polyposis, mucocutaneous lesions, and increased cancer [255]. Individuals with PJS have a ~15-fold increased risk for developing intestinal malignancies compared to the general population, with 50 % of PJS individuals dying of cancer by age 57 [255]. The increased malignancies include small intestinal, colonic, esophageal, gastric, breast, lung, pancreatic, uterine, ovarian, testicular, and cervical cancers. Other manifestations of PJS include intussusception, abdominal pain, intestinal polyp prolapse, and hematochezia [255–258]. Interestingly, women with PJS have a high incidence of cervical adenoma malignum and ovarian sex cord tumors with annular tubules, two very rare, well-differentiated neoplasms [255–258].

PJS polyps are characterized by bundles of smooth muscle between non-neoplastic epithelium, typically having an elongated arborizing, Christmas tree-like pattern**.** Endoscopically PJS polyps do not have specific features and their identification requires histological examination [255–258]. The diagnosis of PJS is based on clinical findings, although molecular-genetic testing is commonly performed for prenatal diagnosis, mutation-specific kindred testing, and clinical diagnosis confirmation. The syndrome is present when any of these conditions are met: (1) presenting with 2 or more PJS polyps, (2) having one polyp and typical PJS pigmented lesions, and (3) having one PJS polyp and a family history of PJS [259].

5.17.6 STK11/LKB1

PJS is caused by mutation of *LKB1* or *STK11* (*serine/threonine kinase 11*), found at 19p13.3. *STK11* consists of 10 exons spanning 23 kb encoding a nuclear 48.6-kDa protein. STK11 is likely regulated by phosphorylation by cAMP-dependent protein kinase A and prenylation [260]. It functions as a tumor suppressor protein, with roles in the G_1 checkpoint, p53-initiated apoptosis, and regulation of Wnt-β catenin-, vascular endothelial growth factor-, and mammalian target of rapamycin (mTOR) signaling pathways [261–265]. More than 140 different *STK11* mutations have been identified in individuals with PJS, with many resulting in inactive truncated kinases. Germline *STK11* mutations have been found in 50–70 % of PJS kindreds and 12–50 % of sporadic PJS cases [266–269]. The reason for the relatively low detection rate is likely due to genes other than *STK11* being mutated in PJS and *STK11* mutations being missed due to the limitations of specific molecular tests [270, 271].

5.17.7 Molecular Testing for PJS

STK11 mutation testing has been performed with a variety of techniques, including DNA sequencing (often limited to exons), CSGE, SSCP, Southern blotting, real-time PCR, quantitative fluorescent PCR, denaturing gradient gel electrophoresis, long range PCR, and MLPA [272]. Volikos et al. [273] analyzed *STK11* mutations via MLPA and found 39 % (11 of 28 individuals with PJS) had large *STK11* deletions ranging from loss of one exon to whole gene deletions. Taken together, ~59 % of *STK11* mutations (range 10–100 %) are detectable by standard methods such as SSCP, CSGE, and DNA sequencing [269, 273–280]. Another 10 % of PJS cases are detectable by RNA based methods only and ~30 % of cases require MLPA testing to detect larger deletions [273, 274, 279, 280]. Thus, current molecular diagnostics allows 80–85 % *STK11* mutation identification in PJS. Currently, most clinical testing employs sequencing of the 10 *STK11* exons and/or MLPA, with the later technique most being effective at detecting larger deletions [272, 274].

5.17.8 Management of PJS

Due to their high risk for carcinomas, all individuals with PJS must be enrolled in lifelong cancer screening programs. While many individuals with PJS develop clinically apparent gastrointestinal lesions as early as their second decade, there are examples of malignancies in young patients and children [281, 282]. Presently there are no organization-approved guidelines for cancer surveillance in PJS patients and different institutions use different protocols [282]. The protocol used at the Johns Hopkins Hospital includes an initial baseline upper endoscopy and small bowel series at 8 years. If polyps are detected, upper endoscopy and small bowel series should be performed every 2–3 years. Upper endoscopy and small bowel series should be done annually from age 18. Colonic endoscopy should be performed every 2–3 years from age 18. At age 25–30, pancreatic endoscopic ultrasound should be done every 1–2 years. CT scanning and CA19-9 levels are offered as options. For women, a pelvic exam with a Pap smear should be performed annually from age 25. At this age, an annual transvaginal ultrasound and serum CA-125 should be done to screen for ovarian cancer. Last, at age 18, monthly breast exams should be done and at 25 semiannual clinical breast exams and annual mammography should be performed. Prophylactic mastectomy should be discussed with the patient. Males should have testicular exams and/or ultrasound of the testicles every 2 years until age 12 [283]. Recently, double balloon enteroscopy and intra-operative enteroscopy have been used to both facilitate better removal of upper gastrointestinal polyps and improve small bowel surveillance [282].

The macrolide compound rapamycin is an effective inhibitor of mammalian target of rapamycin, a cell growth-promoting kinase hyperactivated in PJS [265].

Rapamycin decreases polyp formation in PJS *lkb1* +/– mice [284]. Currently, there are several clinical trials examining the effect of rapamycin on polyp formation in PJS patients [282]. Similarly, PJS *lkb1* +/– mice treated with the COX-2 inhibitor celecoxib show reduced new polyp formation and a decreased size of preexisting polyps [285]. Two of 6 PJS patients given 200 mg celecoxib twice daily for 6 months showed a significant reduction in gastric polyps, suggesting that COX-2 inhibition may have some value in treating PJS [285].

5.18 Chemotherapy Testing for CRC

Irinotecan is a standard chemotherapeutic agent for mCRC treatment, often used in the FOLFIRI regimen: infused 5-fluorouracil, leucovorin, and irinotecan [14, 15]. The effective irinotecan dose depends on the initial amount of drug infused and rate of drug elimination by uridine diphosphate glucuronosyltransferase [*UGT1A1*, 286]. Patient response to irinotecan has been difficult to predict, and toxic reactions are relatively common [287]. For example, fatal events of up to 5.3 % during single-agents irinotecan treatment have been reported [288]. Recently, molecular diagnostics have been used to prospectively identify patients who may be at risk for toxic irinotecan reactions.

5.18.1 UGT1A1

Irinotecan is a topoisomerase 1 inhibitor approved by the FDA in 1994 [14, 15]. Irinotecan stabilizes the complex between topoisomerase 1 and DNA, causing DNA breaks, DNA synthesis inhibition, and apoptosis [289]. Irinotecan is itself relatively inactive, but is converted to the 100–1,000-fold more active metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) by tissue and serum carboxylesterase-converting enzymes [286, 290]. SN-38 is glucuronidated and inactivated by hepatic *UGT1A1* [286]. The dose-limiting toxicities of irinotecan are severe diarrhea and myelosuppression [287, 288, 291]. The relative activity of UGT1A1 determines the effective in vivo concentration and exposure time to SN-38 [292]. Thus, decreased UGT1A1 expression may lead to a toxic reaction secondary to increased SN-38 levels and longer exposure time [287, 288, 291, 292].

UGT1A1 is located at 2p37, spans ~160 kb, and has at least 9 promoters and first exons that can be spliced to four common expressed exons, resulting in 9 different UGT1A1 enzymes, with the most common "wild-type" gene/protein designated UGT1A1*1 [293, 294]. More than 60 *UGT1A1* coding and promoter variations have been identified, which lessen UGT1A1 enzyme activity [295]. The most common polymorphism, *UGT1A1*28*, contains 7, rather than 6, TA promoter repeats resulting in 70 % lower UGT1A1 expression [296, 297]. TA_7 homozygotes are relatively slow to glucuronidate/inactivate SN-38, TA_6 homozygotes are more efficient,

and TA_7/TA_6 heterozygotes show intermediate activity. As expected, individuals with TA_7 homozygotes are more likely to have severe diarrhea and neutropenia following irinotecan therapy, compared to TA_6/TA_6 and TA_7/TA_6 individuals [298]. Interestingly, individuals homozygous for TA_5 and TA_8 promoter repeats occur in African populations, with TA_8 associated with Gilbert syndrome and lower in vitro glucuronidation rates [299].

Other polymorphisms, such as variants at codons 71, 486, 229, and 233, have amino acid substitutions that significantly reduce enzymatic activity and SN-38 clearance. For example, a T → G transversion at codon 486 (*UGT1A1*7*) substitutes a tyrosine moiety for an aspartic acid, resulting in 93 % reduce enzyme activity [300, 301]. Following irinotecan therapy, UGT1A1*28 homozygotes show an increased incidence of irinotecan toxicity compared to *UGT1A1*1* homozygotes, with other variants such as $G^{71}R$ (*UGT1A1*6*) predictive of severe irinotecan-induced neutropenia and poor chemotherapeutic response in Asian populations [302–304]. Many other polymorphisms exist that alter irinotecan therapy responses, often showing population-related distribution [303, 304]. Not surprisingly, individuals heterozygous for two low-activity *UGT1A1* alleles often exhibit irinotecan toxicity. For example, Takane et al. [305] reported severe irinotecan toxicity in a UGT1A1*6/*28 patient.

5.18.2 UGT1A1 Molecular Testing

UGT1A1 allelic variations have been detected through a variety of techniques, including analysis of single base extension of PCR-amplified products, DNA sequencing, HRM, radioactive PCR, denaturing high performance liquid chromatography, and Invader technology. Currently, Invader technology is FDA-approved for *UGT1A1* molecular testing and is commonly used [297, 302, 306–309].

Invader technology typically distinguishes between two different alleles within one non-PCR-based reaction. The reaction involves two steps. First, two allele-specific oligonucelotides, termed Invader oligonucleotides, bind to an unlabeled primary probe. The two Invader oligonucleotides hybridize in tandem with the primary probe forming a specific overlap structure. The 5′-end of the primary probe contains a non-complementary sequence that does not hybridize with either Invader oligonucleotide. The 3′-end of the Invader oligonucleotides are designed to overlap the primary probe such that one of the Invader oligonucleotides will form a complementary base pair with the primary probe. This bp (or pairs for the *28 TA_7) correspond to the wild-type and polymorphism being tested. The complementary base pairing does occur, the resulting structure is recognized and cleaved by the Cleavace® enzyme (Flap endonuclease), releasing the 5′ portion of the primary probe. Should the 3′ portion of the Invader oligonucleotide not be complementary, the Cleavace® enzyme will not cut the structure.

In the second reaction, a second probe binds to the cleaved 5′primary probe sequence, forming a similar structure that is cleaved by the Cleavace® enzyme.

The second probe is designed such that the second cleaving reaction separates a fluorophore from a quencher, creating an easily measured signal. This system can incorporate two primary probes that will eventually cleave either one of two secondary probes, each labeled with a spectrally distinct fluorophore corresponding to a specific allele. The ratio of one fluorophore to the other allows allele identification.

The accuracy of assay comes from the extreme specificity of the Cleavace® enzyme and the sequence specificity of the oligonucleotide hybridization. Purified genomic DNA may be used without PCR amplification and the system has the advantage of being completely closed once the assay has begun. Invader technology is a medium through-put, relatively low-cost technology that commonly allows up to 96 patient samples and controls to be simultaneously interrogated [308, 309].

Despite the large number of different techniques employed in *UGT1A1* genotyping, the analytic sensitivity was 100 % for the *UGT1A1*28* and 98–100 % for the **6* allele, where the referent test was DNA sequencing [310]. The reproducibility of Invader technology was 98.8 % [309]. Additionally, estimates of the first run failure *UGT1A1*1* and *UGT1A1*28* analysis was 5.0 % for sequencing, 1.7 % for PCR/capillary electrophoresis, and 6.7 % for Invader® technology, with most discrepancies solved by repeat testing [309].

5.18.3 UGT1A1 Polymorphism Management

The risk of a toxic irinotecan reaction is roughly 50, 12.5, and 0 % for **28/*28*, **28/*1*, and **1/*1* individuals, respectively [297]. However, there is significant overlap between these groups, and despite a large number of studies, the association between the **28* homozygotic genotype and irinotecan toxicity is not statistically significant [309]. Additionally, at standard irinotecan doses, the tumor response rate is 41 % for wild-type, 45 % for **28* heterozygotes, and 70 % for **28* heterozygotes [309]. Thus the wild-type individuals may be under dosed, while the **28* heterozygotes show a better response. Based on this, The Evaluation of Genomic Applications in Practice and Prevention Working Group found insufficient evidence to recommend for or against *UGT1A1* genotyping guiding irinotecan treatment protocols [309].

Recently however, one Phase I study was performed on 27 patients with mCRC, examining irinotecan toxicity and the **28* polymorphism: 18 patients were **1/*1* and 9 were **1/*28*. A maximum tolerated dose was not observed up to a 150 mg/m^2 dose in the **1/*1* patients, while the **1/*28* patients showed toxicity at a 100 mg/m^2 irinotecan dose [310]. The study concluded that **1/*1* individuals should receive 150 mg/m^2 dose irinotecan, while **1/*28* individuals should receive 70 mg/m^2. The study was limited to 27 patients and included no **28/*28* individuals. Most likely, further studies will be performed and may demonstrate clinical utility for *UGT1A1* genotyping [310].

5.19 Future Directions

Molecular diagnostic testing in CRC is becoming increasingly important in the diagnosis and management of de novo and hereditary CRC, a trend that will probably continue [14–17, 60]. Several trends are readily apparent.

5.19.1 Increased Molecular Diagnostic Testing

Murff et al. [311] found that only 17 % of individuals who met the criteria for early-onset breast cancer testing were actually referred for genetic testing. Presently, many individuals who would benefit from molecular testing do not receive it. As the clinical utility of molecular testing becomes more apparent and testing costs fall, this diagnostic modality will be increasingly employed.

5.19.2 Expansion of the Number of Genes Tested in CRC

Presently, *UGT1A1* genotyping to guide irinotecan use is not routinely performed, although an initial study suggests that it might have use in determining the optimal irinotecan dose [310]. Additionally, Bedoya et al. [312] found that Dukes stage C and D patients were significantly more likely ($P = 0.05$) to have a position 1,359 G→A nucleotide change in the cannabinoid receptor-1. They also had a shorter survival time and increased lymph node metastasis. While not currently a part of molecular diagnostics, increased understanding of mutations/polymorphisms in other genes effecting CRC genesis and progression, such as the cannabinoid receptor-1, will likely become important in the molecular diagnostics of this malignancy [14–17, 311].

5.19.3 Specific Molecular Techniques in CRC Testing and Analysis

Several molecular techniques, while not often employed in clinical testing, have proven to be very useful in the analyses of CRC and are very likely to significantly contribute to the molecular diagnostics of CRC in the near future.

Microarray. Micoarray consists of a solid support, often a glass slide, to which DNA probes of known sequence are fixed in an orderly arrangement. The slides are hybridized under high-stringency conditions with labeled nucleic acids (often cDNAs derived from RNAs), extensively washed, and the relative amount of probe-bound target sequence is measured via label detection. The number of gene/nucleic acid sequences that can be simultaneously measured can range from 10 to 1 million.

Comparisons of normal colonic mucosa and CRC have shown a large number of gene products over-or under-expressed in CRC compared to normal colonic tissue [313]. Microarray has allowed the development of specific gene expression panels with diagnostic (Pathwork test for tissue of origin) or prognostic (OncotypeDX) abilities. For CRC, specific gene products have proven useful in predicting clinical behavior. For example, osteopontin RNA expression is a well-documented biomarker of CRC progression [314].

Proteomics. Proteomics involves the analysis and identification of proteins produced by cells in normal and pathologic conditions. Often these analyses examine protein modifications, such as phosphorylation and protein-protein interactions. The specific techniques used ranges from antibody-based protein chips resembling microarray techniques, to yeast two-hybrid systems to identify protein-protein interactions, high throughput crystallography screens for structural analyses, two-dimensional electrophoresis, and mass spectrometry. Although preliminary, these analyses have proven very useful in identifying proteins, whose expression correlates with chemotherapy response, metastatic potential, and degree of clinical aggressiveness. With time, specific proeomic profiles can be developed to predict the clinical behavior of specific CRC cases [313].

Metabolomics. Metabolomics is the study of the chemical footprints unique to specific fluids or tissues and comparing these chemical footprints to others that have been treated in some way or are involved by a pathologic process. Typically, small molecules are analyzed, such as lipids, sugars, and amino acids. Most often, metabolomic analyses are performed with various forms of mass spectrometry following initial separation by techniques such as high-performance liquid chromatography or by nuclear magnetic resonance spectroscopy (NMR). Although preliminary, encouraging results have been obtained. For example, Bezabeh et al. [315] used NMR to analyze the metobolomic profile of fecal samples diluted in a phosphate-buffered saline/D_2O buffer. 412 controls without and 111 with CRC were analyzed. The fecal samples from individuals with CRC showed a unique NMR profile with an 87 % sensitivity and specificity. Interestingly, the cost of this analysis was about $50/sample, significantly lower than many other molecular techniques. Although not yet employed clinically, metabolomic analysis may prove useful in detecting de novo and recurrent CRC and in predicting clinical behavior.

Whole Genome Sequencing. Since 2005 “next generation” DNA sequencing platforms have become increasingly available. Many of these newer sequencing platforms have reduced the cost of sequencing roughly fourfold, compared to standard Sanger sequencing techniques. With time, our rapidly expanding ability to sequence large DNA sequences at lower costs is likely to allow the molecular analysis of an individual’s entire genome and/or tumor genome for molecular diagnostics [316, 317]. Obviously such technology would give a vastly increased amount of molecular data for clinical analysis and be a significant tool in the era of personalized medicine.

One interesting new approach that is currently available is “exome sequencing”, also known as “targeted exome capture” In this technique genomic DNA is randomly sheared and the resulting DNA fragments are enriched for exomic DNA by

aqueous phase hybridization to short labeled (magnetic beads or biotin) DNA or RNA oligonucleotides that correspond to exonic sequences. The labeled hybrids are captured and washed, removing all unbound DNA, leaving only the exonic portion of the genome—which accounts for less than 2 % of the genome, but carries roughly 85 % of disease-causing mutations [318]. The exomic DNA is then PCR amplified and subjected to massively parallel DNA sequencing methods. The advantage of exomic sequencing is that it reduces the volume of DNA to be analyzed by ~98 %, but still allows analysis of the majority (~85 %) of the DNA containing disease-causing mutations [318]. Presently Agilent, Illumina, and Nimblegen offer kitted reagents for exome capture [317].

MicroRNA (miRNA) Profiling. miRNAs are small (~22 nucleotides) posttranscriptional regulators that function by binding to the 3′ untranslated regions of target mRNAs, repressing their expression. miRNAs in some ways represent "a genome within the genome" as each miRNA commonly regulates multiple mRNA targets, functioning as master controls of gene expression. Bioinformatic analysis has suggested that miRNAs regulate about 30 % of human gene expression [319]. CRCs often show specific patterns of increased and decreased miRNAs, especially those miRNAs known to regulate cellular functions such as metastasis, proliferation, DNA repair, apoptosis, and the epithelial-to-mesenchymal transition. Although in its early stages of analysis, miRNA profiling may eventually have clinical use as a diagnostic marker and as a predictor of clinical outcome [320].

Epigenetics. Epigenetics is the study of heritable changes in gene expression that involve mechanisms other than changes in DNA sequence. These changes can include many different biochemical changes, including aberrant DNA methylation, dysregulated miRNA expression (see above), histone modifications such as acetylation and prenylation, and even such bizarre entities as prions. Analysis of the gene methylation patterns found in CRC has shown that nearly all CRCs carry hundreds to thousands of aberrantly methylated genes [321]. Although too complex to review here, epigenetic changes in CRC are becoming increasingly important in the analysis of this tumor. Additionally, with time pharmacologic treatment modalities involving changing the methylation patterns of the tumor CRC genome to restore non-malignant gene expression patterns may become possible in the near future [321, 322].

5.20 Conclusions

In the future the rapidly increasing ability to sequence large DNA sequences at low cost is likely to lead to molecular analysis by sequencing an individual's genome and/or tumor genome for molecular diagnostics [316]. Obviously such technology would give a vastly increased amount of molecular data for clinical analysis and be a significant tool in the era of personalized medicine.

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Chapter 6
Molecular Pathology and Diagnostics in Esophago-gastric Cancer

Rodney E. Shackelford, Maisoun Qassim Abdelbaqi, Khaldoon Almhanna, Kenneth Meredith, and Domenico Coppola

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R.E. Shackelford, DO, Ph.D.
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, SL-79, New Orleans, LA 70112, United States

M.Q. Abdelbaqi
Department of Pathology, LSU Health Sciences Center Shreveport, 1501 Kings Highway, Shreveport, LA 71130, USA

K. Almhanna, M.D. • K. Meredith, M.D.
Department of GI Oncology, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, United States

D. Coppola, M.D. (✉)
Department of Pathology, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, United States
e-mail: Domenico.Coppola@moffitt.org

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Abstract Esophageal and gastric cancers are the eighth and fourth most common cancers worldwide, respectively. Because of their often-aggressive clinical courses and high fatality rates, they represent the sixth and second most common causes of cancer deaths worldwide. The diagnosis of these malignancies is mainly established by their clinical presentation, combined with biopsy and subsequent histologic analysis of hematoxylin-eosin-stained tissue sections. Recently, however, molecular studies have become increasingly important in establishing a diagnosis and prognosis and guiding the clinical management of these malignancies. For some subtypes of these malignancies, such as hereditary diffuse gastric cancer, molecular diagnostics is central in establishing a diagnosis and in determining appropriate clinical management. Here, we review current methods and recommendations for molecular-genetic testing for esophageal and gastric cancers and briefly discuss future possible applications of this technology to these malignancies.

Keywords Esophageal cancer • Gastric cancer • Gastrointestinal stromal tumor • CDX2 • miRNA • Microarray • *H. pylori* • Diffuse gastric carcinoma

Abbreviations

BE	Barrett's esophagitis
DHPLC	Denaturing high-performance liquid chromatography
EAC	Esophageal adenocarcinoma
ESCC	Esophageal squamous cell carcinoma
ESD	Esophageal squamous dysplasia

GIST	Gastrointestinal stromal tumors
miRNA	MicroRNA
NSAIDS	Non-steroidal anti-inflammatory drugs
POD	Days post-operatively

6.1 Esophageal Cancer

Esophageal cancer is the eighth most common malignancy worldwide and the sixth leading cause of cancer-related deaths due to its often-aggressive clinical course and fatal outcome. Overall, esophageal cancer has a poor survival rate, with 16 % and 10 % 5-year survival rates seen in the United States and Europe, respectively. Like colorectal carcinoma, esophageal cancer shows a striking geographical variation, with its incidence varying by over 20-fold in areas such as China, with an incidence of 27.4 cases/100,000 in Chinese men compared to 1.3/100,000 seen in Western African men. In general, the sex ratio of esophageal cancer is skewed strongly toward men, with the male-to-female ratio in Eastern Europe being 7:1 for example. In places where the esophageal cancer incidence is lower, such as Western Africa, the sex ratio is much closer to one [1]. Although genetic factors play a role in the incidence of esophageal cancer [2], much of the variation seen in esophageal cancer rates is due to differential carcinogen exposure, particularly as related to tobacco and alcohol use [3].

6.2 Esophageal Cancer Types

Although esophageal anatomy is relatively simple, the esophagus is host to many different malignancies, including salivary gland tumors (such as adenoid cystic and mucoepidermoid carcinomas), stromal tumors (such as gastrointestinal stromal tumors, leiomyosarcoma, and liposarcomas), signet ring cell esophageal adenocarcinomas, hematopoietic malignancies, melanoma, and small cell and lymphoepithelioma-like carcinomas. However, most of these esophageal malignancies are relatively rare. The most common esophageal malignancies are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), which together account for more than 90 % of all esophageal cancers [4]. These malignancies differ significantly in their histology, precursor lesions and development, epidemiology, clinical behavior, and anatomic origin, with ESCC most commonly arising in the proximal esophagus and EAC arising in the distal esophagus. Unfortunately, both ESCC and EAC are aggressive diseases, typically presenting with early lymphatic and hematogenous dissemination, making their surgical resection difficult and sometimes not an option, particularly for disseminated disease.

Interestingly, the epidemiology of esophageal cancer has been changing. For example, before the 1970s, ESCC constituted over 90 % of all esophageal cancer

worldwide [3–6], while in some Western countries EAC presently accounts for ~ 50 % of all esophageal cancers [3]. Interestingly, one study demonstrated that in westernized areas, the ratio of adenocarcinomas of the lower esophagus and gastric-esophageal junction to ESCC has changed from 1:4.7 in 1975 to 1:0.43 in 1998; within the last decade, the incidence of lower esophageal adenocarcinoma has exceeded that of ESCC in several Western countries, including the United States [7–9]. In this review, we will mainly focus on esophageal ESCC and EAC.

6.3 Environmental Risk and Protective Factors for Esophageal Cancer

Multiple environmental toxins have shown a strong association with esophageal cancer, with esophageal ESCC and EAC often showing stronger or weaker associations following exposure to specific environmental toxins. Because of the often-aggressive clinical course of esophageal cancer, the association of esophageal cancer with specific exposures has been documented for over a century [10, 11]. Below are some of these toxic agents.

6.3.1 Alcohol Abuse

Alcohol abuse has been identified as a significant risk factor for ESCC since the 1930s [10, 11]. The risk for ESCC is elevated three- to fivefold with excessive alcohol consumption of more than three drinks per day [12–14]. Interestingly, however, alcohol abuse appears to have no effect on the incidence of EAC [4, 12–14]. The mechanism by which alcohol causes ESCC is presently poorly understood; however, several factors are likely involved. First, the initial metabolite of ethyl alcohol is acetaldehyde, a known highly toxic carcinogen [15]. Acetaldehyde binds strongly to DNA, forming adducts and interstrand cross-links, many of which block DNA synthesis, causing replication errors and polymerase errors resulting in mutations, and can eventually activate proto-oncogenes and inactivate tumor suppressor genes leading to malignancy via the accumulation of unrepaired mutations [16]. Additionally, chronic alcohol consumption induces certain gene products involved in alcohol detoxification, some of which actually promote malignancy. For example, Millonig et al. [17] examined 37 patients with a history of alcohol abuse and upper aerodigestive tract cancer and compared this group to 16 control patients without cancer. Patients with cancer and a history of alcohol abuse showed significant induction of cytochrome P-450 2E1 levels, which strongly correlated with the carcinogenic etheno-DNA adducts and cell proliferation. Interestingly, cell proliferation indices were highest in those who both smoked and drank. Non-smokers and drinkers had the lowest levels of cytochrome P-450 2E1, etheno-DNA adducts, and Ki-67. The authors concluded that chronic alcohol abuse leads to increased DNA damage

and cell proliferation, thus likely to contribute to cancer. Alcohol may further contribute to carcinogenesis by increasing the solubility of other carcinogens, such as those found in tobacco smoke, and may contribute to nutritional deficiencies, which play a role in carcinogenesis [18].

6.3.2 Tobacco

Approximately 78 % of male and 75 % of female deaths are due to esophageal cancer [19]. Unlike alcohol, tobacco use increases the risk for both ESCC and EAC, about three- to sevenfold for ESCC and twofold for EAC, depending on the amount and duration of tobacco use [13, 19]. Tobacco is largely carcinogenic due to the wide variety of toxic agents produced by the combustion of the tobacco during smoking, including acetylaldehyde, nitrosamines, and polycyclic aromatic hydrocarbons [20]. The mechanisms of tobacco-induced carcinogenesis are complex, although they are partially understood and include the formation of mutagenic DNA adducts, intercalation of polycyclic aromatic hydrocarbons causing frame-shift mutations, and mutagenesis resulting from DNA replication forks stalled by tobacco toxin-induced DNA modifications [20]. Interestingly, some smoking-induced cancers may arise from radioactive agents in tobacco and tobacco smoke, including ^{210}Pb and ^{210}Po. These agents may be preferentially deposited in specific areas of epithelium and cleared slowly, contributing to carcinogenesis [21]. Within the squamous epithelial lining of the esophagus, both smoking and excess of alcohol induce esophageal squamous dysplasia (ESD), the precursor lesion of ESCC. ESD can be mild, moderate, or severe. ESD confers an elevated risk for ESCC of 3-, 10, and 30-fold, depending on whether the dysplasia is mild, moderate, or severe [22]. Together, alcohol abuse and tobacco account for over 90 % of all esophageal cancer cases worldwide and together synergistically increase the risk for esophageal cancer [23]. Several other recognized causes of esophageal cancer include are summarized below.

6.3.3 Mycotoxins

Mycotoxins are found in many sources, such as pickled vegetables and spoiled foods, and include aflatoxins, ochratoxin A, fumonisins, trichothecenes, and zearalenone. They exert a range of effects, including allergic reactions, immune suppression, mutagenesis and taratogenesis, and cancer. The fumonisins were discovered in the 1980s and consist of 20 carbon aliphatic chains with two ester-linked hydrophilic side chains that structurally resemble sphingosine, a common and essential phospholipid found in cell membranes [24, 25]. The toxicity of the fumonisins arises from its inhibition of ceramide synthase, resulting in the accumulation of sphinganine, some of which are metabolized into highly toxic-free sphingoid bases [26]. Fumonisin B_1 is found at relatively high levels in maize, where it is produced by the fungal pest *Fusanum*

monilifornte and related species. Fumonisin B_1 is found only on maize and is thought to cause high levels of esophageal cancer in areas of Southern Africa and parts of China [27, 28]. The molecular actions of Fumonisin B_1 are poorly understood, but it appears to cause cancer, in part by altering cellular proliferative and apoptotic responses [29].

6.3.4 Opium

By itself, opium is not a mutagen [30, 31]. However, opium is often smoked, a process that produces a large array of mutagenic agents, particularly polycyclic aromatic hydrocarbons that are potent carcinogens [20]. One study in the Golestan Providence of Northeastern Iran, where both opium and tobacco are commonly smoked, showed an odds ratio for esophageal cancer of 1.70 for tobacco use and 2.35 for opium smoking compared to individuals who did not smoke either substance. Three hundred esophageal cancer cases compared to 571 controls were matched on neighborhood of residence and age [32]. Interestingly, 2 % of the individuals studied used alcohol, which was not associated with an increase in esophageal cancer.

6.3.5 Maté

Maté, also known as yerba maté, an infusion of the herb *Ilex paraguayensis,* is commonly consumed in relatively large quantities in areas of South America known to have an elevated esophageal cancer incidence [33–35]. Several studies have shown a correlation between maté use and ESCC, showing odds ratios of 3.81 and 1.87 for ESCC among maté drinkers compared to non-maté drinkers [33–35]. The mechanism(s) underlying the carcinogenic activity of maté appears to be the relatively high levels of polycyclic aromatic hydrocarbons found in maté [36–38]. Interestingly, maté use does not appear to increase the risk for EAC [33–35].

6.3.6 Helicobacter pylori

H. pylori is a helical, gram-negative bacterium that infects the stomach, often producing chronic gastritis, which confers an increased risk for MALT lymphomas and gastric adenocarcinoma [39]. Unlike these malignancies, *H. pylori* exerts a protective effect on EAC risk and lowers its incidence ~ 50 %. It does not appear to affect ESCC risk [40, 41]. The mechanism(s) underlying the protective effects of *H. pylori* appears to come from lowered gastric acid secretion and hence lower esophageal irritation, due to *H. pylori*-induced parietal cell apoptosis and lowered H^+-K^+-ATPase parietal cell expression by increased NF-κB activity following exposure to bacterial endotoxins [42, 43].

6.3.7 *Non-steroidal Anti-inflammatory Drugs*

Non-steroidal anti-inflammatory drugs (NSAIDs) have been demonstrated to reduce multiple cancer types, including EAC but not ESCC [44]. The overall reduction on EAC is ~ 40 %, although the degree of reduction varied with the frequency of NSAID use; interestingly, as little as 75 mg/day of aspirin significantly reduced the risk for EAC [44, 45]. The mechanism underlying this risk reduction is unknown, although one meta-analysis of 14 studies on aspirin use and EAC did show a protective effect that became evident following appearance of Barrett's esophagitis [46].

6.3.8 *Gastro-esophageal Acid Reflux, Obesity, and Barrett's Esophagitis*

Barrett's esophagitis (BE) is a metaplastic change in response to reflux of bile and acid across the gastro-esophageal junction, possibly functioning as a protective response to caustic effects of reflux [42, 47]. In BE, the normal lower esophageal squamous epithelium is replaced by intestinalized columnar epithelium. Clinically, this change is significant because BE has a propensity to develop dysplasia that can later progress to EAC. A diagnosis of BE requires that two criteria be met. First, there should be cephalad displacement of the squamocolumnar junction seen via upper endoscopy (Fig. 6.1a). Second, intestinalized epithelium or epithelium containing goblet cells should replace the normal squamous epithelium of the lower esophagus, being histologically identified from areas showing cephalad displacement of the squamocolumnar junction (compare Fig. 6.1b, c) [48].

The definition of BE shows some variation, with goblet cells in the metaplastic epithelium required for a diagnosis of BE in the United States but not in the United Kingdom [48–50]. The risk of EAC with BE is relatively low, conferring a risk of

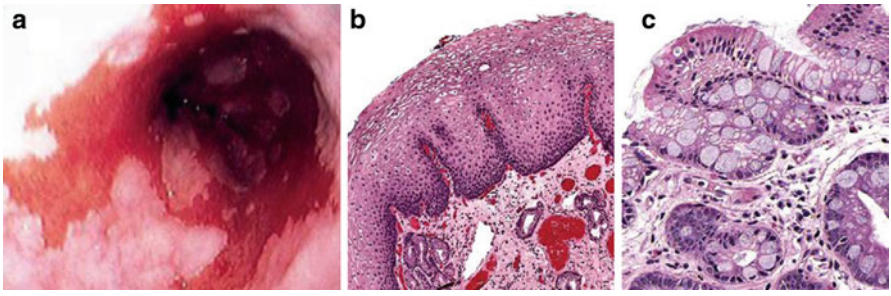

Fig. 6.1 Lower esophageal changes seen in Barrett's esophagitis (BE). Irregular cephalad protrusions of Barrett's esophagitis gastro-esophageal junction (**a**). Normal lower esophageal squamous epithelium compared to Barrett's esophagitis (compare panel **b** to **c**) (From Ref. [48])

about 0.5 % per year, such that most individuals with BE will die with BE and not from EAC. Where EAC does arise in a background of BE, a specific sequence of histologic changes from normal squamous epithelium to BE and from BE to low-grade or high-grade dysplasia and finally invasive EAC has been identified [48]. Not surprisingly, the risk for EAC increases with the dysplastic changes, such that individuals with high-grade dysplasia have an EAC risk of ~ 10 %/year [51]. The risk for BE and EAC is increased by obesity, which increases gastro-esophageal reflux via increasing intra-abdominal pressure, thus weight loss decreases both intra-abdominal pressure and the risk for BE and EAC [52]. Interestingly, specific molecular changes have been identified in the progression from normal lower esophageal epithelium to BE to EAC (discussed below).

6.4 Genetic Risk Factors for Esophageal Cancer and Molecular-Genetic Testing

Although environmental factors are major determinants of esophageal cancer risk, about 30 % of esophageal cancers have a heritable genetic component [53, 54]. Additionally, several genetic factors have been identified as risk factors for esophageal cancer, including enzymes involved in the detoxification of xenobiotics and Tylosis, a rare autosomal dominant characterized by hyperkeratosis of the palms and soles associated with a high incidence of esophageal cancer [55, 56] (also discussed below).

Both ESCC and EAC have defined histologic precursor lesions [3, 4, 48–51]. Recent studies have demonstrated that these precursor lesions are accompanied by specific molecular changes. Although many of these changes are currently studied at the basic research level, they are likely to be incorporated into molecular diagnostic testing in the near future. Others, such as molecular testing for Tylosis, are currently being performed [55]. Here, we will review our understanding of the molecular pathology of esophageal cancer and current molecular diagnostics in this area.

6.4.1 Microarray Studies

Microarray allows the massive, parallel, semiquantitative analysis of the relative gene expression levels between two different cell populations, often comparing different, but related, cell populations such as primary and neoplastic cells or primary and metaplastic cell populations. Anywhere from ten to several hundred thousand expressed RNA species can be simultaneously analyzed, allowing detailed comparisons of global gene expression between two samples. The drawbacks to microarray are its relatively high costs, its high complexity of implementation and validation, its "snapshot" presentation of the transcriptome, the lack of information given on an expressed sequence's ultimate function, and the sometimes poor correlation between mRNA expression and protein expression, which microarray does not measure [57].

One of the earliest microarray analyses of BE and esophageal cancer was performed by Selaru et al. [58]. Thirteen esophageal specimens were compared, consisting of seven BEs, and six esophageal cancers. Eight thousand cDNA clones were analyzed, and hierarchal clusters analysis was performed. The esophageal samples clustered into two predominant groups, each expressing unique gene expression profiles. Comparison to histology revealed that one group consisted of all seven BE samples, whereas the other six were all malignancies. Interestingly, the malignant samples showed sub-clusters consisting of EAC, ESCC, and one signet ring cell esophageal adenocarcinoma. Although the specimen sample size was small, microarray combined with bioinformatics was demonstrated to be able to differentiate malignant and premalignant esophageal disease using small biopsy-sized tissue samples.

Later, Greenawalt et al. [59] employed microarray to examine 128 tissue samples from normal esophageal epithelium, BE, EAC, and ESCC, taken from patients between ages 28 and 86, with a mean age of 63 years. The tumor stage and degree of dysplasia in BE were recorded and incorporated into the analysis. Tissue samples were collected during diagnostic endoscopy and immediately placed in RNAlater solution and stored at −20 °C. Biopsies were divided in half; with one part subjected to independent histologic analysis and the other prepared for RNA extraction. All tumor samples contained 75 % or more tumor. Additionally, biopsies of normal mucosa were taken from greater than 5 cm proximal to any tumor margins and were conformed 100 % normal by histologic examination.

RNA was extracted from each sample via phenol-chloroform followed by column chromatography. Four micrograms of total RNA were amplified with T7 RNA polymerase to linearly amplify the total RNA. The resulting RNA amplicon was annealed to the total RNA, and dsDNA was made in the presence of dNTPs, DNA polymerase, *E. coli* ligase, and RNase H. Transcribed RNA form the dsDNA was purified and labeled with cyanine-5. Reference cDNA was extracted from nucleic acids from a pool of 11 cell lines (MCF7, Hs578T, OVCAR3, HepG2, NTERA2, MOLT4, RPMI-8226, NB41ATRA, UACC-62, SW872, and Colo205) and labeled with cyanine-3. The sample and reference cDNAs were competitively hybridized to cDNA printed microarray slides containing 10,500 elements, representing 9,400 unique cDNA clones. The hybridized slides were scanned with a confocal laser scanner, and the data were processed using GenePix Pro Software 4.1 to calculate the mean intensity of each spot. Hierarchal clustering was done with a Pearson's similarity metric using a Gene Cluster 3.0 and viewed with an R statistical package.

After normalization, 9,386 genes separated the tissue samples into four distinct clusters, ESCC, normal squamous epithelium, BE, and EAC. To qualify as distinct, a fourfold difference in gene expression was required. Consistent with their phenotype, ESCC and normal squamous epithelium clustered closely together and were distinct from EAC and BE, which are columnar lesions. Additionally, the normal and BE samples were clearly distinct, whereas the BE and EAC sample clustered closely together (Fig. 6.2). A large group of cDNA clones were differentially expressed between any two tissues: 2,158 between normal and BE, 2,913 between normal and EAC, and 1,306 between EAC and SCC. Thus a large degree of overlap was identified.

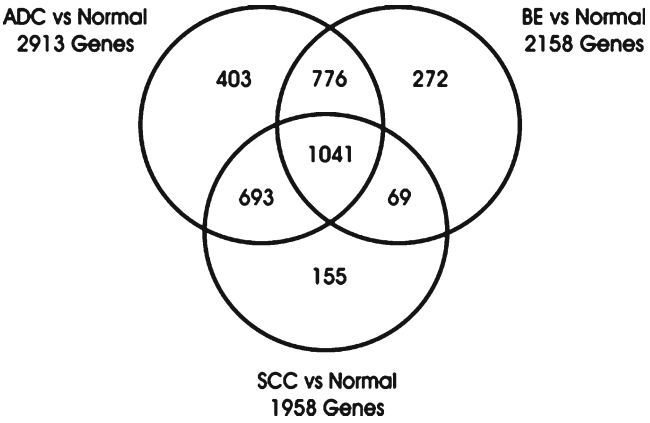

Fig. 6.2 A diagram of differentially expressed genes from the microarray study by Greenawalt et al. [59]. The differentially expressed genes between esophageal *SCC*, *AC*, and *BE* are presented as a Venn diagram. The number of genes expressed in more than one tissue appears in the overlapping regions of the diagram

Several patterns of differential gene expression were identified that correlated with the differentiation state of the esophageal biopsy. For example, genes involved in tissue development, including keratinization, intercellular junction formation, calcium ion binding, and endopeptidase activity, were overexpressed in BE and EAC. EAC overexpressed genes involved in immune and inflammatory responses and those associated with responses to external signaling and collagen metabolism and proteolysis. Interestingly, BE, but not EAC, overexpressed genes involved in digestion and alcohol metabolism. ESCC overexpressed genes involved in cell death and apoptosis, regulation of NF-κB expression metalloprotease activity, and plasminogen activator activity. Last, specific gene expression cluster groups were found to be associated the different malignancies. Malignancy was associated with the expression of genes involved in extracellular matrix formation, collagen binding, immune response, the cell progression and checkpoints, spindle organization, and signal transduction cascades. Thus Greenawalt et al. [59] were able to differentiate EAC and ESCC via molecular profiling and demonstrate both a different cellular origin for each malignancy (squamous vs. columnar), but also show clear molecular differences between benign, premalignant, and malignant esophageal tissues.

In a similar study, Helm et al. [60] employed the Human Hu U133A GeneChip to perform a microarray analysis of ten endoscopic biopsy specimens from nine patients with esophageal adenocarcinoma. Six patients had adenocarcinoma without BE, while three had adenocarcinoma arising in BE. Ten biopsies were taken three patients in which BE cancer had arisen and a further ten biopsies were taken from six additional patients with BE, but with no dysplasia or malignancy. Each biopsy specimen was carefully examined and microdissected by a pathologist to remove stroma, necrotic debris, and normal adjacent tissue in cases of BE or malignancy. Total RNA was prepared by Trizol extraction and analyzed for integrity. Five micrograms RNA were hybridized to the Hu U133A GeneChip,

which contained 22,214 probe sets. The RNA was prepared by standard Affymetrix protocols [61].

Analysis of gene expression patterns revealed that even in BE without histologic signs of dysplasia genes associated with cell differentiation were under-expressed. Adenocarcinoma overexpressed genes associated with tissue remodeling and invasion. Interestingly, cancers that arose without an identifiable BE precursor lesion showed higher expression of these genes.

Many other microarray studies have been preformed comparing various common esophageal lesions and a number of candidate genes have been identified which may have value in esophageal cancer staging, recurrence prediction, prognosis, and eventually in the selection of different treatment modalities [62]. Although microarray is presently too costly, time-consuming, and technically difficult for most clinical applications, it has been a very valuable tool for identifying specific genes that may be examined by simpler molecular techniques for esophageal cancer molecular diagnostics.

6.4.2 Prognostic Significance of Serum mRNAs

One goal of molecular diagnostics is to predict treatment response and establish a prognosis, optimize the management of a patient with a specific malignancy, and accomplish this with the least invasive procedure possible. For esophageal cancer, the measurement of serum RNA profiles is relatively non-invasive. Although not currently used in esophageal cancer molecular diagnostics, some success has been achieved with serum analysis. For example, Takahashi et al. [63] measured the serum levels of MMP9, CRP, HMGB1, MUC1, EGR1, PBEF1, PDGFA, TGF-β1, TNF-α, Von Willebrand factor, and IL-6 mRNA in 27 patients who underwent radical surgery for esophageal cancer. mRNA was obtained for 14 days post-operatively (POD), with collections taken during surgery and at PODs 1, 3, 5, and 14. mRNA was extracted, and real-time RT-PCR was performed following DNase treatment. RNA from 200 μL of serum was dissolved in 200 μL of H_2O. RT-PCR was performed with 1 μg of the extract and 2 μg SYBT Green I employing a one-step RT-PCR kit. RT-PCR conditions were incubation at 50 °C for 30 min, 12 min at 95 °C, and 50 cycles at 95 °C (0 s), annealing at 50–55 °C (10 s), and 72 °C (15 s), followed by extension at 40 °C (20 s). All primers were optimally designed and shown in Table 6.1. The final concentration of the primers was 1 μmol. β-actin was employed as a relative expression-level control. All reactions were done twice, and IL-6 and CRP protein levels were verified by Western blotting.

mRNA expression profiles over 14 days revealed that MMP9 and NAMPT were both up-regulated at POD 5 and onward. Von Willebrand factor and TGFβ1were both up-regulated from POD 3. MUC1 and PDGFA were up-regulated at POD 3, with levels falling at later dates. IL-6 was up regulated at POD 5 and thereafter fell to basal levels. EGR1 and HMGB1 slowly decreased from intraoperative levels to baseline at POD 14. Increased TNF-α mRNA levels correlated with increased time spent under anesthesia. CRP mRNA expression correlated with the length of the

Table 6.1 Primer sets used for each gene investigated by Takahashi et al. [63]

Gene	GenBank accession no.	Forward primer	Reverse primer
VWF	NM_000552.2	TGA CCA GGT TCT CCG AGG AG	CAC ACG TCG TAG CGG CAG TT
TGFβ1	MN_000660.3	GAC TAC TAC GCC AAG GAG GT	GGA GCT CTG ATG TGT TGA AG
PDGFA	MN_002607.5	GGG AGT GAG GAT TCT TTG GA	AAA TGA CCG TCC TGG TCT TT
NAMPT	MN_005746.2	CTG TTC CTG AGG GCA TTG TC	GGC CAC TGT GAT TGG ATA CC
CRP	MN_000567.2	ACA GTG GGT GGG TCT GAA AT	TAC CCA GAA CTC CAC GAT CC
EGR1	MN_001964.2	TTC TTC GTC CTT TTG GTT TA	CTT AAG GCT AGA GGT GAG CA
HMBG1	MN_002128.4	AAC CAC CCA GAT GCT TCA GT	TCC GCT TTT GCC ATA TCT TC
TNF-α	MN_000594.2	TGC TTG TTC CTC AGC CTC TT	GCA CTC ACC TCT TCC CTC TG
MUC1	MN_001018016.1	CCA TTC CAC TCC ACT CAG GT	CCT CTG AAG GAG GCT GTG AT
MMP9	NM_004994.2	TTG ACA GCG ACA AGA AGT GG	CCC TCA GTC AAG CGC TAC AT
IL-6	MN_000600.3	ATG CAA TAA CCA CCC CTG AC	TAA AGC TGC GCA CAA TGA GA
β-actin	MN_031144.2	ACC TGA CTG ACT ACC TCA TG	GCA GCC GTG GCC ATC TCT TG

ICU stay and was associated with the 6-month mortality. Interestingly, high IL-6 mRNA levels on POD 0 was an independent indicator of a poor prognosis, as were days spent on a ventilator or days spent in the ICU, whereas a high POD three levels predicted the onset of pneumonia. Up-regulation of Von Willebrand factor and TGFβ1 mRNA intraoperatively correlated with mortality ($P = 0.0021$). A worse prognosis was also found with POD 1 up-regulation of PDGFA, ERG1, and HMGB1 mRNAs. At POD 3, NAMPT and MUC1 mRNAs were independent prognostic factors for 1-year mortality. High NAMPT mRNA at POD 14 correlated with mortality at 30 days and 1 year. The authors proposed that profiling serum inflammatory gene expression could have value in stratifying patients according to their relative risk for mortality and the onset of severe inflammatory conditions. Although not presently commonly employed in molecular diagnostics, such serum mRNA profiling could have value in patient care, but requires further validation.

6.4.3 MicroRNA Expression Analysis

MicroRNAs (miRNAs) are endogenous, single-stranded, 20–23 base-pair non-coding RNA species that regulate protein expression at the posttranscriptional level by forming base-pair-specific interaction between the 5′portion of the miRNA and sites

within the coding and un-translated regions of mRNAs. Typically, these interactions lower mRNA stability, attenuating mRNA translation and, hence, protein synthesis. miRNAs play an important role in multiple cellular pathways, including embryogenesis, apoptosis, cellular differentiation and proliferation, hematopoiesis, immune function and regulation, and carcinogenesis [64, 65] miRNAs are dysregulated in variety human malignancies, including colorectal, thyroid, breast, prostate, lung, and pancreatic malignancies, and in fact are dysregulated in every tumor type so far examined [66–72], miRNAs regulate ~ 30 % of global protein translation, with some miRNAs functioning as tumor suppressors and other as oncogenes. In human malignancies, the former are suppressed and the latter are overexpressed [64, 73–75]. Interestingly, profiling of miRNA expression patterns provides more accurate tissue and tumor classification than does global mRNA expression profiling [76, 77].

In esophageal cancer miRNA expression is dysregulated in BE, EAC, and ESCC [78–87]. In many cases, the down-stream target gene products of specific miRNAs have been identified. For example, miR-93, miR-25, and miR-106b are coordinately expressed from a polycistron at 7q22.1. During the transition from the normal squamous epithelium to BE to EAC, their expression is increased seven- to ninefold [88–90]. miR-93 and miR-25 down-regulate the cyclin-dependent kinase inhibitor 1 (p21), a potent inhibitor of CDK2 and −4, which together move cells though the G_1 and S phases of the cell cycle, promoting cell division. Additionally, Bcl-2-like protein 11 (Bim) levels are also suppressed, inhibiting apoptosis [89–91]. Thus activation of the 7q22.1 polycistron promotes malignant transformation. Many other miRNAs dysregulated in esophageal cancer suppress tumor suppressor genes and cause over-expression of oncogenes [reviewed in 92]. Interestingly, many miRNA species are expressed from polycistrons, possibly forming a basic regulatory network coordinating mRNA translation patters [78–90, 92].

6.4.4 Molecular Analysis of miRNA Expression

miRNA expression has been analyzed with multiple molecular techniques, with RT-PCR and microarray analyses being commonly employed, with Northern blotting sometimes used to confirm PCR results. For example, Chen et al. [79] employed qRT-PCR to analyze miRNA expression in 107 ESCC cases using normal adjacent esophageal squamous epithelium as control tissue. All tissue samples were snap frozen in liquid nitrogen, and total miRNA was isolated from the frozen tissue using a mirVana miRNA isolation kit. The first strand of cDNA was synthesized with a RevertAid first strand cDNA synthesis kit and later amplified with mirVana qRT-PCR primers sets in a TaqMan gene expression master mix. An Applied Biosystems 7,300 Real-Time PCR system was used for amplification, following the manufacturer's instructions. miR-92a was significantly up-regulated in the ESCC compared to the control normal epithelium. Interestingly, the relative expression of mi-92a was significantly increased in lymph node metastases compared to patients without node metastases ($p = 0.001$). miR-92a expression also correlated with the patients TNM stage ($p = 0.049$), but not with age, gender, differentiation grade,

tumor position type, and T classification. Additionally, 65 patients were followed for 5 years. Those with higher mi-92a expression had a shorter survival and higher TNM and N stages than those with low expression.

Last, the CDH1 mRNA (E-cadherin) 3′ un-translated region contains a highly conserved binding region from 499 to 524 for mi-92a. Chen et al. [79] found a strong inverse correlation between E-cadherin expression of mi-92a levels. Further proof for a role in mi-92a-regulating E-cadherin expression was shown in a cell line where removal of the mi-92a binding domain in the CDH1 mRNA abrogated the inhibitory effects of mi-92a on E-cadherin expression. The authors concluded that mi-92a overexpression likely promotes metastasis and a poorer clinical course, in part, via down regulating E-cadherin. The advantages of employing qRT-PCR in miRNA analysis is that the technique is cost effective, lends itself to high-throughput analyses, and readily detects low abundance nucleic acid species [93]. While mi-RNA analyses have been often performed on esophageal cancer tissue samples, qRT-PCR has also been successfully employed in the analysis of circulating serum miRNAs, a technique that is relatively uninvasive and that will eventually have clinical applications (for review, see [94]).

*6.4.5 Acetylaldehyde Dehydrogenase*2*

Ethanol is metabolized by a two-step process, being first converted into acetylaldehyde by alcohol dehydrogenase, and subsequently acetylaldehyde is metabolized to acetate by acetylaldehyde dehydrogenase (ALDH2), which is attached to acetyl coenzyme A and enters the citric acid cycle [95]. Acetylaldehyde is a mutagen and potent carcinogen and, like most carcinogens, a longer exposure at higher concentrations results in increased cellular damage [15–18]. ALDH2 is found at 12q24, is 53 kbp, and consists of 13 exons and 12 intones, which encode a 501 amino acid residue protein [96, 97]. ALDH2 has multiple polymorphisms, one of which profoundly affects the enzymes catalytic efficiency [98, 99]. In the ALDH2*2 polymorphism, a lysine at residue 487 replaces a glutamic acid residue found in the more common ALDH2*1 allele. ALDH2*2 has little ability to metabolize ethanol and has a very low affinity for NAD, a cofactor required for enzymatic function and the metabolism of ethanol [99]. Following alcohol consumption, individuals with ALDH2*2 homozygotes show 18 times higher blood alcohol levels and those with ALDH2*1/ALDH2*2 heterozygotes show a five times higher blood alcohol levels than individuals with the wild-type allele [100]. As a result of the very poor enzymatic activity of the ALDH2*2 polymorphism acetylaldehyde, levels are higher in ALDH2*2 hetero- and homozygotes than in ALDH2*1/ALDH2*1 individuals following alcohol consumption [99–101] (Fig. 6.3). Not surprisingly, ALDH2*2 homozygotes and ALDH2*1/ALDH2*1 individuals exhibit higher levels of acetaldehyde-derived DNA damage following alcohol consumption than ALDH2*1 homozygotes [102].

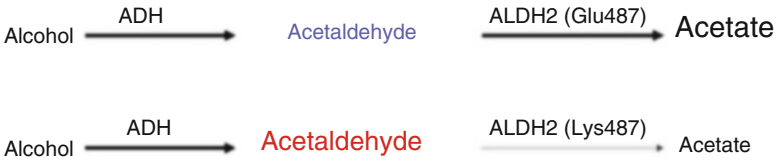

Fig. 6.3 With similar alcohol dehydrogenase (*ADH*) activities, the very poor ability of ALDH2 (Lys487, ALDH2*2) allele results in the accumulation acetylaldehyde that can cause DNA damage [102] (The figure is from Ref. [101])

Lewis et al. [103] performed a meta-analysis of seven articles that reported on ALDH2 polymorphism and esophageal cancer. Compared to ALDH2*1 homozygotes, ALDH2*2 homozygotes had lower rates of esophageal cancer (odds ratio 0.36), while ALDH2*1/ALDH2*1 individuals had elevated esophageal cancer rates (odds ratio 3.19). The reason for the lower esophageal cancer rates seen in the ALDH2*2 homozygotes was attributed to the uncomfortable "alcohol flushing response" seen in ~ 36 % of Asians, where ethanol consumption causes facial flushing, nausea, and tachycardia. These individuals were found to avoid alcohol and therefore have lower esophageal cancer rates than ALDH2*1 homozygotes. The flushing response was largely absent in ALDH2*1/ALDH2*1 individuals who did tend to consume alcohol and show increased esophageal cancer and acetaldehyde-derived DNA damage [101–103]. Interestingly, individuals who show the alcohol flushing response and still consume alcohol show elevated esophageal cancer rates, whereas those who drink everyday, are heavy drinkers, or are alcoholics show an odds ratios of 12.1, 16.4, and 13.5, respectively, compared to control ALDH2*1 homozygotes [102, 104–106].

*6.4.6 ALDH2*2 Polymorphism Testing*

Many different techniques have been employed in ALDH2 polymorphism testing, including microarray, mass spectrometry, melting curve analysis, sequencing, and conventional fluorescent-based PCR analysis [107, 108]. Itoga et al. [108] compared melting curve analysis, SNaPsnot analysis, denaturing high-performance liquid chromatography (DHPLC), and PCR-SSCP analysis.

Whole blood from 24 healthy men and 74 men with esophageal cancer was collected, and genomic DNA was isolated. The men also answered detailed questionnaires about their alcohol consumption. The portion of the ALDH2 gene surrounding the *2 polymorphism was amplified with the Cy-5 labeled primers Cy5-CAAATTACAGGGTCAACTGCT (forward) and Cy5-CCACACTCACAG TTTTCACTT (reverse) in a 25 μL volume with 100 ng genomic DNA, 0.2 M each primer, 0.2 mM of each dNTP, and 1 U *Taq* polymerase in 10 mM Tris–HCl buffer. The reaction mix was denatured for 3 min at 94 °C and amplified by 30 cycles with

annealing at 58 °C for 30 s, extension at 72 °C for 30 s, and denaturation at 94 °C for 30 s. The resulting amplicons were analyzed as described below.

First, amplicons were subjected to *Fluorescent-Based PCR-SSCP Analysis*. One μL of amplicon was diluted in 10 μL of loading buffer solution that contained 99.5 % formamide and 0.5 % blue dextran. The samples were denatured at 94 °C for 3 min; 2 μL of the diluted mix was run into a 10 % native polyacrylamide gel (99:1, acrylamide:bisactymlamide ratio) in 0.5X Tris-borate buffer. Electrophoresis was carried out at 20 W at 18 °C, with data analyzed via Fragment Manager (Amersham Pharmacia Biotech).

Second, samples were subjected to melting curve analysis. One hundred to 500 ng of genomic DNA was mixed in a 20 μL volume with 11.4 μL distilled H_2O, 1.6 μL 25 mM $MgCl_2$, 1 μM of each primer, 1 μL of anchor probe, and 2 μL LightCycler DNA-Master Hybridization Probes. The PCR was carried out with an initial denaturation of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 11 s. Following amplification, melting curve analysis was performed with denaturation at 95 °C for 0 s, annealing at 40 °C for 120 s, and analysis carried out with a 0.2 °C/s increase to 80 °C. Emitted fluorescence was measured during the heat ramping step, and the melting curve was plotted as melting peaks.

Third, amplicons were subjected to *SNaPshot Analysis*. Four μL of amplicon that had been subjected to a 7 min extension at 72 °C at the end of the last cycle was used. The amplicon was incubated at 37 °C for 1 h with 2 U exonuclease I and 2 U shrimp alkaline phosphatase. The mix was heated to denature the enzyme, and 1 μL of digested PCR product was mixed with 5 μL of SNaPshot Multiplex Ready Reaction Mix with 1 μM minisequencing primer (5′-CCACACTCACAGTTTTCACTT-3′) and amplified via 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60° for 10 s. Upon completion, 0.5 U shrimp alkaline phosphatase was added and the reaction was incubated for 1 h. One μL of the resulting reaction mix was added to 12 μL of HiDi formamide and heated to 95 °C for 5 min. The samples were run in an ABU PRISM 310 Genetic Analyzer, and the results were analyzed with GeneScan 3.0 application software.

6.4.7 Denaturing High-Performance Liquid Chromatography

Denaturing high-performance liquid chromatography (DHPLC) was performed with genomic DNA amplified, prepared as for the SNaPshot procedure. Heteroduplex DNA was produced by mixing ALDH2*1/2*2 by denaturing the PCR products at 94 °C for 3 min and then slowly cooling them. Homoduplex samples were made in the same manner by mixing equal amounts or ALDH2*1 or ALDH2*2 and treating them as per the heteroduplex samples. The DHPLC analysis was carried out using WAVE DNA Fragment Analysis System. The PCR mixture was loaded on a DNASep column and the hetero- and homoduplexes where eluted with a linear

acetonitrile gradient formed by mixing two different elution buffers at a constant flow rate of 0.9 mL/min. DNA elution was detected by measuring absorbance at 260 nm at 58 °C.

Interestingly, the study revealed all four techniques to have 100 % concordance. Melting curve analysis required 4 h to complete, PCR-SSCP took 6 h, DHPLC required 21 h, and SNaPshot analysis required 23 h. The costs of each reaction were $4.16, $2.50, $2.50, and $8.33, respectively. Melting curve analysis had the advantage of not requiring opening of the reaction tube, thus eliminating the possibility of contamination. The authors concluded that, for routine 10–20 samples/day molecular diagnostics, melting curve analysis was the best option and PCR-SSCP fluorescent-based genotyping was best applied to large-scale genotyping for epidemiological studies. Interestingly, the study showed that the highest esophageal cancer rates were seen in habitual drinkers with ALDH2*2 homozygosity.

6.5 CDX2 Protein Expression in Barrett's Esophagitis

The molecular events underlying the transition of normal esophageal epithelium to BE is incompletely understood, but recent studies have demonstrated a role for the Caudal homeobox (Cdx) genes in the morphogenesis of BE. Animal studies have shown that CDX expression plays a role in the morphogenesis of normal intestinal epithelium. For example, in normal murine embryogenesis Cdx1 and −2 expression appears at day 12 and continues throughout life, with Cdx protein expression being required for the transformation of endoderm into large and small intestinal columnar epithelium [109]. Mice genetically engineered to express Cdx1 or −2 in their gastric cells develop metaplastic intestinal-type epithelium with goblet cells, enterocytes, and enteroendocrine cells [110–114]. Additionally, while ablation of global *Cdx2* expression is lethal in embryonic mice, causing death before gastrulation [115, 116], conditional ablation of *Cdx2* in the developing gut results in partial foregut like differentiation of the large and small intestines, loss of intestinal villi, and significantly lowered expression of the pro-intestinal transcription factors *Cdx1, Isx, HNF1α*, and *H*N*F4*α, which together activate the intestinal transcriptome [117].

Cdx1 and −2 is strongly expressed in normal human large and small intestine, but not in the stomach or esophagus [110, 118]. In BE Cdx2 expression is 400 times higher than normal esophageal epithelium and it is 100 % expressed in BE with dysplasia and in EAC [118–120]. Some experiments done on cultured esophageal cells has demonstrated that bile salts and acidic conditions can increase Cdx2 expression, although by itself increased Cdx2 expression is insufficient to cause all the changes seen in BE [121, 122]. Cdx2 is expressed in BE, BE with dysplasia, and EAC, and is a sensitive marker for intestinal dysplasia. However, it cannot differentiate low-grade from high-grade dysplasia, and the expression differences seen in the BE→dysplasia→EAC are not sufficient for immunohistochemistry to be useful [110, 118–124]. Presently the diagnosis of BE, BE with dysplasia, and EAC

presently rests almost solely on the examination of histologic sections combined with clinical findings and patient history. Cdx2 immunohistochemistry may become more important in the future as our understanding of its function and down-steam signaling cascades increases.

6.6 Tylosis

Tylosis is an autosomal dominant syndrome first recognized more than 50 years ago. Its common manifestation is palmoplantar keratoderma, an abnormal thickening and callusing of the palms and soles due to abnormal keratinization. Oral leukoplakia and esophageal hyperkeratosis often accompany the palmoplantar keratoderma. Tylosis has a strong association to ESCC, but not EAC, with the ESCC risk reported as 40–95 % by the age of 70 years [125–130]. Initial linkage studies revealed the heritable Tylosis abnormality to be on chromosome 17, with later studies narrowing the region to a 42.5 kb region at 17q25, designated as the TOC gene. This region has also been implicated in some cases of sporadic ESCC [131–139]. One gene within this region, cytoglobin (*CYGB*), has both alleles ~ 70 % down-regulated in Tylosis, while in sporadic esophageal cancer both alleles appear to be suppressed by an epigenetic mechanism. Interestingly, sequencing of the TOC gene in Tylosis and sporadic esophageal cancer failed to show any heritable or somatic mutations, suggesting epigenetic suppression in both disease processes [138, 140]. Tylosis is rare, with only a few known kindreds having the disease. There are few recommendations on how to clinically manage the disease, although at least yearly upper endoscopic screening with intensive biopsies is recommended. Presently, it is not known if upper endoscopic screening will lower the mortality seen in Tylosis [140].

The diagnosis of Tylosis is based on clinical findings, combined with the personal and family history; molecular diagnostic techniques rarely employed [56]. McRonald et al. [140] collected esophageal biopsies during routine upper endoscopy from 18 affected individuals from a Tylosis family, seven biopsies from individuals with gastro-esophageal reflux, and ten individuals without esophageal cancer, gastro-esophageal reflux, or other esophageal pathology. The tissues were collected into RNAlater and stored at −20 °C until the RNA was isolated using RNeasy. Two micrograms of RNA were reverse transcribed with oligo-dT primers. The resulting cDNA was amplified with 0.2 μM of the primers 5′-CTTCGGGGAAGTTGAGTCAG-3′ (forward) and 5′-CAAGGTGGAACCGGTGTACT-3′ (reverse) for 26 cycles with the *TBP* mRNA employed as a control. Twenty-six cycles were chosen as this was determined to be within the exponential phase of the reaction. The resulting amplicons were identified and quantified by agarose gel electrophoresis with the gels scanned with an ABI GeneScanner. As described above, the *CYGB* was ~ 70 % down-regulated in patients with Tylosis compared to normal esophageal epithelium, with both alleles being equally repressed. The authors concluded that the *CYGB* gene was down-regulated by three possible mechanism: (1) there may be an aberrant anti-sense RNA species produced in Tylosis that impairs the function of the *CYGB* gene;

(2) the transcription of the gene is impaired; or (3) the *CYGB* gene may be down-regulated due to the aberrant expression of another gene. Further research will be required to find the mechanism of transcriptional repression.

6.7 Gastric Cancer

Gastric cancer is the fourth most common cancer worldwide with 934,000 new cases each year. In addition, due to its aggressive clinical course, it is also the second most common cause of cancer deaths worldwide, causing an estimated 700,000 deaths each year. The incidence of the cancer varies widely between different geographical locations. Two-thirds occur in the developing countries, with China accounting for 42 % of all cases. High-risk areas included East Asia, Eastern Europe, and parts of Central and South America. Low-risk areas include Southern Asia, North and East Africa, North America, New Zealand, and Australia [1].

6.8 Environmental Versus Genetic Factors for Gastric Cancer

Studies of migrant populations have clearly demonstrated strong environmental factors in the risk for gastric cancer. In particular, studies of populations moving from high-risk to low-risk areas for gastric have highlighted many environmental risks factors, with some studies indicating that the risk factors exert effects beginning in childhood [1, 141, 142]. Many risk factors have been identified several are discussed below:

6.8.1 Helicobacter pylori *(*H. pylori*)*

H. pylori is listed by the international Agency for Research on Cancer as carcinogenic in humans [143]. More than 50 % of the world's population is infected with *H. pylori*, with infection conferring a two to sixfold increased risk for gastric carcinoma [144, 145]. Not surprisingly curing an *H. pylori* infection significantly reduces the risk for gastric cancer in high-risk population [146, 147]. *H. pylori* is a Gram-negative bacterium that synthesizes numerous virulence factors, including urease, arginase, adhesin, vacuolating toxin, and cytotoxin-associated antigen [148, 149]. Evidence indicates that causes gastric cancer via a three-step mechanism; (1) *H. pylori* infection, (2) the development of precancerous including severe gastric atrophy, intestinal metaplasia, and corpus predominant gastritis, and (3) and carcinogenesis [150]. The mechanisms of *H. pylori*–induced carcinogenesis are incompletely understood, however multiple factors appear to promote cancer including increased oxidative

stress, chronic inflammation, and impaired host antioxidant defenses and ability to repair DNA damage [151–155]. Presently once found *H. pylori* infections are treated with antibiotics to eradicate the infections [146, 147].

6.8.2 Diet

Extensive data have indicated that diet plays a strong role in gastric cancer risk, that is, a diet high in certain foods such as persevered, salted and or smoked foods, especially meats and pickled vegetables with carcinogenic *N*-nitroso compounds. Animal studies have also demonstrated that ingesting relatively high amount of salt induces gastritis and enhances the potency of carcinogens such as N-methyl-N-nitro-N-nitrosoguanidine and 4-nitroquinoline-1-oxide [156, 157]. Additionally, a diet high in fresh fruits and vegetables exerts a protective effect, lowering the risk for gastric cancer, while the polyphenols found in green tea (*Camellia sinensis*) have anti-inflammatory and anti-oxidant effects, and additionally inhibit nitosation – an event implicated in gastric carcinogenesis [158–164].

6.8.3 Tobacco

Not surprisingly, smoking increases the risk for gastric cancer significantly. Interestingly smoking increases the incidence of distal gastric cancer [165, 166].

6.8.4 Obesity

Obesity is a major risk factor for gastric cardia adenocarcinoma as it promotes gastroesophageal reflux, the development of BE and hence increases the risk of adenocarcinoma of the gastroesophageal junction [48]. One Swedish study revealed that the heaviest quartile of the population had a 2.3fold increased risk for gastric cardia adenocarcinoma compared to the light quartile of the population [167]. Thus weight loss, which reduces reflux, also lowers the risk for gastric cardia adenocarcinoma.

6.9 Genetic Risk Factors for Gastric Carcinoma and Molecular-Genetic Testing

About 90 % of gastric malignancies are sporadic, with the remaining 10 % showing a familial clustering, and 1–3 % representing hereditary gastric cancer syndromes [168]. Molecular diagnostic testing is generally not often performed on gastric malignancies, except for hereditary diffuse gastric carcinoma and mucosa-associated lymphoid tissue lymphomas.

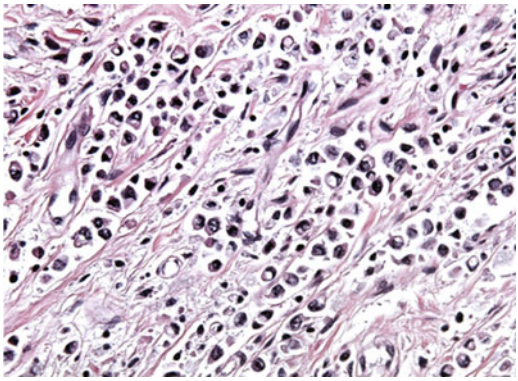

Fig. 6.4 The typical histology of gastric signet ring cell carcinoma (From Ref. [170])

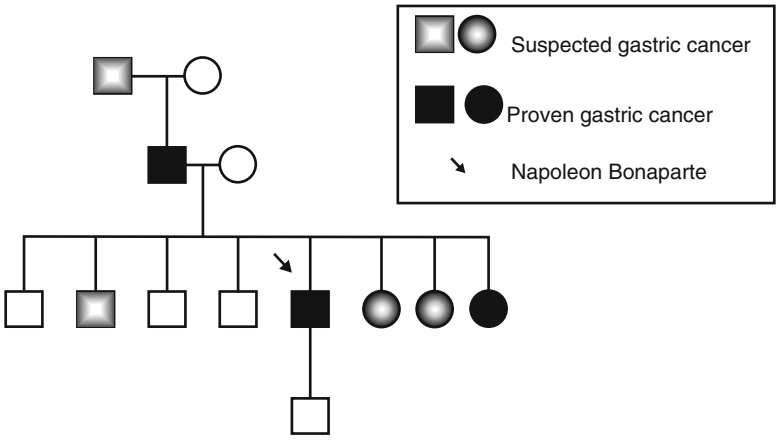

Fig. 6.5 The family pedigree of the Bonaparte family showing a pattern of heredity gastric cancer (From Ref. [56])

6.9.1 Hereditary Diffuse Gastric Carcinoma

Hereditary diffuse gastric carcinoma is an autosomal dominant cancer susceptibility syndrome characterized by an estimated cancer risk of 67 % for men and 83 % for women [169]. Histologically, it is characterized by the development of an aggressive diffusely infiltrating signet ring cell gastric carcinoma that results in linitis plastica once the majority of the stomach is involved (Fig. 6.4). The syndrome was first described in three New Zealand Maori families in 1964, with genetic linkage studies employing microsatellite markers showing that the E-cadherin gene (*CDH1*) at 16q22.1 was involved [171, 172]. Since these early studies, the syndrome has been identified in multiple kindreds in many countries and, interestingly, in the family of Napoleon Bonaparte [56, 173–176] (Fig. 6.5).

Hereditary diffuse gastric carcinoma is defined as follows by the International Gastric Cancer Linkage Consortium [177]:

1. Two or more documented cases of diffuse gastric cancer in first/s degree relatives, with at least one diagnosed before age 50 years; OR
2. Three of more cases of documented diffuse gastric cancer in first/s degree relatives, independently of age of onset.

More recent criteria (also by the International Gastric Cancer Linkage Consortium) are:

1. An individual diagnosed with diffuse gastric cancer before 35 years of age.
2. An individual diagnosed with both diffuse gastric cancer and lobular breast cancer.
3. One family member with diffuse gastric cancer and another with either lobular breast cancer or signet ring colon cancer.

Individuals who meet or exceed these criteria should receive genetic counseling, be tested for germline E-cadherin gene mutations, undergo upper screening endoscopic exams every 6 months with extensive biopsies taken, and undergo prophylatic gastrectomy. The timing of the prophylatic gastrectomy is based on multiple factors, such as patient age, ability to tolerate the operation, and the decision to have children [56, 177].

6.9.2 Molecular-Genetic Testing in Hereditary Diffuse Gastric Carcinoma

Molecular diagnostic testing for hereditary diffuse gastric carcinoma can be performed by several different molecular diagnostic technologies. However, because many of the mutations in this syndrome are missense or inactivating mutations that can occur in different areas of *CDH1,* testing by DNA sequencing of amplified genomic DNA is an efficient method of testing. Suriano et al. [178] employed PCR, DNA sequencing, and DHPLC to examine *CDH1* mutations in 31 previously unreported kindreds with hereditary diffuse gastric carcinoma. Twenty of the families had a positive history of diffuse gastric cancer, ten had early-onset diffuse gastric cancer, and one had lobular breast cancer and diffuse gastric cancer. Genomic DNA was purified from peripheral blood leukocytes using a Puregene DNA Purification Kit. The compete coding sequence of the E-cadherin gene including the splice junctions was PCR amplified using 100 ng genomic template DNA, 0.5 mM/L PCR primers with deoxynucleotide triphosphates, $MgSO_4$, and 1.25 units Optimase polymerase in appropriate buffer conditions. The samples were denatured at 95 °C for 2 min and subjected to 15 touchdown cycles with 0.5 °C annealing temperature decreases at each cycle, and 20–50 s extensions at 72 °C. The end result amplicons were denatured for 4 min at 94 °C and cooled to room temperature at 1 °C/min. Resulting amplicons (3–15 μL) were screened for mutations with high-performance

liquid chromatography. The optimal temperatures for resolution of hetero- and homoduplexes were established by analyzing the melting behavior of PCR fragments in temperature ranges corresponding to that calculated on Wavemaker software. PCR amplicons identified as aberrant by chromatography were directly sequenced in both directions using florescent-labeled dye terminators in an ABI 3,700 capillary sequencer. Samples showing a putative pathogenic mutation were confirmed by a second independent PCR and sequencing reaction.

Of the 20 families with a positive history of gastric cancer, 30 % harbored *CDH1* mutations. 20 % of the families with early-onset gastric cancer were mutation positive, and the case with both lobular breast cancer and gastric cancer was mutation positive. Nine different mutations were identified: four nonsense, two deletion, one splice site substitution, one single nucleotide affecting a start condon, and one misssence mutation. With the exception of one previously identified nonsense mutation, 1003C>T, all where novel mutations. All carriers were heterozygotes for their mutations. Although by this assay some *CDH1* mutations, such as deletions, could have been missed due to assay insensitivity, the authors concluded that many of the families probably harbored mutations in other genes that led to gastric cancer.

6.9.3 Other Familial Gastric Cancer Syndromes

An increased risk for gastric cancer is seen in hereditary nonpolyposis colorectal cancer and Peutz-Jeghers syndromes. These cancer-prone syndromes are covered in Chap. 5, which discusses the molecular diagnostics of colorectal carcinoma.

6.10 Gastrointestinal Stromal Tumors

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasm of the gastrointestinal tract and can occur anywhere from the esophagus to the anus, with approximately two-thirds occurring in the stomach and less than 5 % occurring in the esophagus. GISTs are submucosal lesions that arise from multipotential stem cells [179]. They are rare; having an incidence of 10–20 per one million people diagnosed every year and show considerable histologic variation, showing spindle, epithelioid, and mixed patterns. Immunohistochemical analyses of GISTs are usually limited to CD117 (c-kit), which the majority of GISTs express, and DOG-1, a cell surface protein of unknown function, expressing roughly 98 % of GISTs regardless of c-kit or PDGFRα gene expression status [180].

GISTs also manifest wide variations in their clinical behavior, ranging from benign neoplasms to very aggressive metastasizing malignancies. The prognosis of a GIST is determined by several factors, including factors such as tumor size at presentation, mitotic index, coagulative necrosis, hypercellular sarcomatous histology,

and increased expression of cell cycle progression proteins, such as Ki-67 [181–183]. They typically have activating mutations of either the c-Kit or PDGFRα proto-oncogenes [184]. Most commonly activating c-Kit mutations are found in exon 11 with less common mutations in exons 9, 13, and 17 [185]. The treatment for GISTs, when possibly, is surgical resection. However, for metastatic or recurrent GISTs, imatinib mesylate or other oral tyrosine kinase inhibitors can be employed to slow tumor growth and improve the survival with advanced/aggressive GISTs. The response to tyrosine kinase inhibitors is usually not complete, and most patients eventually develop resistance. Interestingly, the kinase inhibitor response depends on the genomic location of the specific c-Kit mutation [181]. For example, GISTs with c-kit exon 9 mutations show intermediate imatinib sensitivity and better relapse-free survival and overall survival times than those with exon 11 mutations. The exon 11 mutation-bearing GISTs show an increased sensitivity to imatinib and have a worse relapse-free and overall survival time [186].

Molecular diagnostic procedures for the c-Kit mutations found in GISTs have employed FISH, immunohistochemistry, microarrays, and PCR amplification of tumor-derived genomic DNA followed by sequencing [187, 188]. Presently, most c-Kit testing for GISTs involve immunohistochemistry for c-Kit protein expression, as an adjunct to a histology-based diagnoses. Molecular diagnostics is presently rarely preformed to analyze the specific c-Kit mutation [181].

6.11 Conclusion

Presently, molecular diagnostics has limited application in esophageal and gastric pathology, with hereditary diffuse gastric carcinoma testing probably being the best known testing modality. As our knowledge of the growth-promoting signal transduction pathways underlying gastro-esophageal malignancies increases, molecular diagnostics for these organ is likely to become more common and useful in the clinical decision-making and determining treatment options. Additionally, as new molecular diagnostic technologies such as accurate full genome sequencing emerge, our ability to predict an individual's risk for these malignancies may play a role in identifying individuals likely to benefit from periodic screening procedures.

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Chapter 7
Molecular-Genetic Testing in Hepatocellular Carcinoma and Its Premalignant Conditions

Rodney E. Shackelford, Greg Mayhall, Shankar Japa, Aejaz Nasir, Mokenge Malafa, and Domenico Coppola

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R.E. Shackelford, DO, Ph.D. • G. Mayhall, M.D. • S. Japa, Ph.D.
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, SL-79, New Orleans, LA 70112, USA

A. Nasir
Diagnostic & Experimental Pathology & Tailored Therapeutics Program, Eli Lilly & Company, Indianapolis, IN, USA

M. Malafa
Department of Surgical Oncology, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

Department of Oncological Sciences, University of South Florida College of Medicine, Tampa, FL, USA

D. Coppola, M.D. (✉)
Department of Pathology, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

Department of Oncological Sciences, University of South Florida College of Medicine, Tampa, FL, USA
e-mail: Domenico.Coppola@moffitt.org

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Abstract Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third most common cause of malignancy-associated death. Presently there are relatively few molecular genetic testing directly performed on HCC. However, many molecular genetic tests are performed on conditions that increase the risk for HCC or modify drug treatments for conditions that increase HCC risk, including hepatitis B and C, hemochromatosis, and interleukin-28b testing. Here we review the common molecular diagnostic genetic tests associated with HCC diagnosis and treatment.

Keywords Hepatocellular carcinoma (HCC) • Hepatitis B (HBV) • Hepatitis C (HCV) • Hemochromatosis • Interleukin-28b • p53 • MicroRNA

7.1 Introduction

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm worldwide and the third most common cause of malignancy-associated death. In 2008 more than 70,000 cases of HCC where diagnosed worldwide, with ~85 % occurring in the developing countries. The greatest incidence is seen in Eastern Asia and parts of sub-Saharan Africa. HCC has a high fatality rate, as only a minority of afflicted individuals are candidates for potentially curative treatments such as liver transplantation, resection, or complete ablation, making the overall mortality to incidence ratio an abysmal 0.93. The vast majority (70–90 %) of HHCs arise in a background of established chronic liver disease, especially cirrhosis and hepatic steatosis [1, 2].

7.2 Environmental Protective and HCC

The worldwide HCC incidence reflects regional differences in chronic liver disease risk factors, particularly to those causing cirrhosis. In Eastern Asian and sub-Saharan Africa where 80 % of HCCs occur, the main risk factor is chronic hepatitis B (HBV) infection, often combined with other risk factors, such as alcohol and aflatoxin B1 exposure. In Japan, Europe, and North America, the main risk factor is chronic hepatitis C (HCV) infection, often combined with alcohol abuse. Epidemiologically, the HCC incidence has developed in parallel with the spread of HCV in Japan, Europe, and North America. Once HCV induces cirrhosis, the HCC risk is high, with 3–5 % of affected individuals developing HCC/year [1–4]. Not surprisingly, the HCC risk increases with the increased hepatitis viral load and with the duration of infection, and it is likely decreased by anti-HBV vaccination programs and by the effective use of antiviral agents [5–7]. Co-infection with HIV and HBV or HCV causes a more rapid progression to liver disease and an increased risk for HCC once cirrhosis has developed [8].

Other risk factors for HCC include iron overload, either from dietary sources and the genetic disease hereditary hemochromatosis (HH) [9–11]. The mechanisms by which excess iron leads to hepatocarcinogenesis likely involves redox-active labile iron causing the formation of toxic reactive oxygen species via Fenton and Haber-Weiss chemistries, leading to chronic oxidative stress with concomitant lipid peroxide formation, DNA damage, mutagenesis, and eventual hepatocarcinogenesis [9, 10, 12]. A well-known example of dietary iron overload is seen in Southern and Central Africa where large volumes of traditional beer are consumed that has been fermented in cast iron containers. Under the acidic fermentation conditions ionized, highly bioavailable iron leaches from the fermentation vessel. High iron levels accumulate in the hepatocytes, causing portal fibrosis and eventual HCC. For individuals with the highest hepatic iron levels, the relative risk for HCC is 23.5 [9, 13]. Not surprisingly, hepatic iron overload, combined with either chronic HBV or aflatoxin B1 exposure increases an individual's HCC risk [9]. Smoking, heavy alcohol use, and diabetes are also risk factors for HCC, especially when they are combined with concurrent HBV or HCV infections [14, 15]. Interestingly, coffee consumption lowers HCC risk [16]. Less common environmental risk factors for liver cancer includes vinyl chloride exposure that can cause hepatic angiosarcoma and liver flukes, which can increase the risk for development of cholangiosarcoma [17].

7.3 Genetic Factors for HCC Risk

Multiple genetic factors can increase HCC risk, including HH, single nucleotide polymorphisms (SNPs) that modify HBV and HCV related HCC risk, family history, and epidermal growth factor polymorphisms. Many of these genetic factors

interact with and modify environmental risk factors, especially those associated with viral hepatitis (see below).

Individuals with HH have an increased HCC risk, with early studies showing up to a 200-fold increased risk, prior to the use of repeated phlebotomy treatment to lower overall iron load [9, 10, 18–20]. HH is caused by multiple mutations in five different genes, with HFE mutation at 6p21.3 being the most common in Northern European populations [10]. Most HH results from decreased hepcidin synthesis, resulting in increased enteric iron import via ferroportin activity [10]. Given time, iron accumulates causing complications such as fatigue, diabetes mellitus, cardiac arrhythmias, heart failure, hypothyroidism, cirrhosis, and HCC [9–11, 18, 21]. Most data indicates that HCC arises in individuals with HH who have hepatic cirrhosis due to iron overload, as individuals with HH in whom cirrhosis is prevented by repeated phlebotomy have the same long-term survival as individuals without HH [10, 17, 21, 22].

Host genetic factors can also modify environmental risk factors for HCC. For example, genome-wide association studies have indicated that SNPs at 1p36.22 and 8p12 promote HBV-associated HCC [23, 24]. Similar studies have shown that six different SNPs effecting T-cell receptor activity, immune surveillance, and genes associated with antigen presentation and processing correlated strongly with the risk for HBV or HCV-induced HCC versus liver cirrhosis [25]. One SNP (an A → G transition) at 61 in the 5′ untranslated region of the epidermal growth factor gene causes a fourfold increased HCC risk, following adjustment for age, sex, race, etiology, and severity of cirrhosis [26]. Lastly, a family history of a first-degree relative with HCC confers a roughly fourfold greater risk for developing HCC, independently of having viral hepatitis [27]. Undoubtedly, the genetic risk for HCC is polygenetic and many more genetic variations will be found that modify individual HCC risk.

7.4 Hepatocarcinogenesis

Hepatocarcinogenesis is a complex, multistage event that usually progresses from chronic viral hepatitis/chronic liver disease, to cirrhosis, low- and high-grade dysplastic nodule formation, low-grade HCC, and advanced HCC (Fig. 7.1, [28]). While most of these stages are histologically well defined, several can be diagnostically difficult, such as the discrimination between a high-grade dysplastic nodule and low-grade HCC, although in some cases stromal invasion can help in differentiating the two conditions [29]. Recent molecular studies have identified gene expression patterns associated with HCC development, recurrence risk, and prognosis. For example, Hoshida et al. [30] employed genome- wide expression profiling to indentify 186 genes whose expression correlated with HCC recurrence risk and survival prognosis. Similarly, other studies employing serial analysis of gene expression (SAGE) and gene expression and methylation profiling have indentified

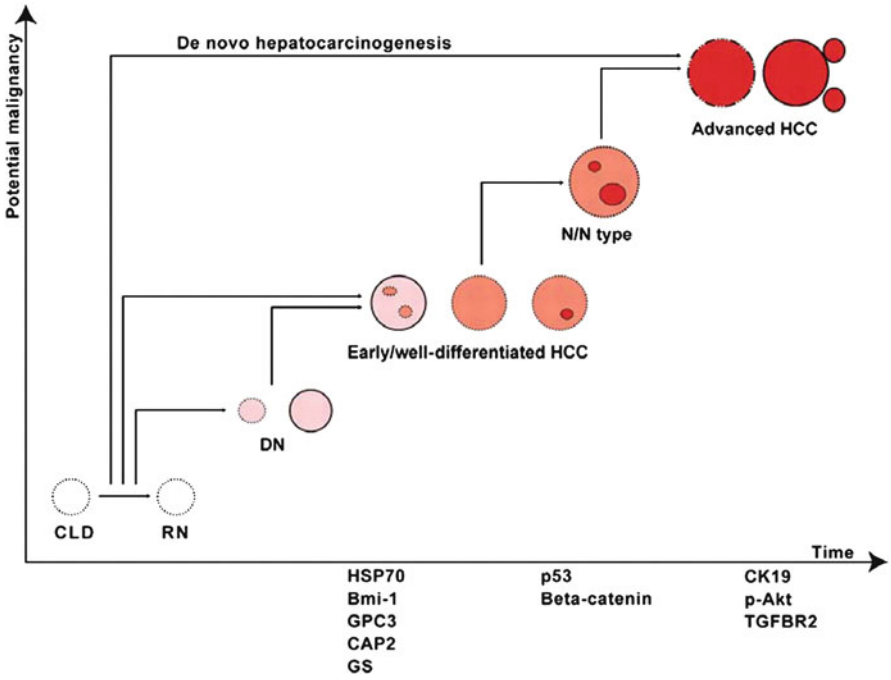

Fig. 7.1 HCC evolution and its related molecular and immunological markers. *CLD* chronic liver disease, *RN* regenerative nodule, *DN* dysplastic nodule, *N/N* nodule-in-nodule [28]

alterations in HCC gene expression and methylation patterns that correlate with hepatocarcinogenesis. Most of these genes are involved in cell proliferation and survival, biosynthesis, signal transduction, and cellular transport [31, 32]. Recently, several molecular signatures have been identified whose immunohistochemical expression correlates with early/well-differentiated HCC and advanced HCC (Fig. 7.1). Although most of these immunohistochemical markers are not completely specific for HCC, they can be useful adjunctive tools in histologically difficult cases. Here several are reviewed:

7.4.1 Heat Shock Protein 70 (HSP70)

HSP70 is a family of 70-kDa chaperone stress proteins that assists in a wide range of protein folding processes, including the folding and assembly of newly synthesized proteins, refolding of misfolded, denatured, or aggregated proteins, and the membranous translocation of proteins [33]. HSP70 is a potent antiapoptotic cellular survival factor, whose overexpression promotes cell cycle progression, and plays a

role in tumorigenesis, with high expression levels associated with adverse outcomes in endometrial, colorectal, breast, prostate cancers and some leukemias [34–39]. Chuma et al. [40] analyzed the expression of ~12,600 genes in seven nodular HCCs and seven corresponding noncancerous hepatic tissue. HSP70 was overexpressed in early HCC compared to most high-grade dysplastic nodules, and was further increased in high-grade HCC. Interestingly, non-malignant/benign nodules, focal nodular hyperplasia, and hepatocellular adenomas showed low HSP70 expression [40, 41]. HSP70 expression is not entirely specific for HCC, as ~5 % of high-grade dysplastic nodules overexpress this protein [41].

7.4.2 Bmi-1

Bmi-1 is identified as a stem cell gene based on the observation that Bmi-1 deficiency compromises adult stem cell function [42]. Bmi-1 encodes a 37 kDa member of the Polycomb gene group which imparts stem cell features to malignant cells, including the induction of telomerase activity and the negative regulation of the p16 and p19 tumor suppressor genes [43, 44]. Bmi-1 overexpression correlates with therapy failure in many different human malignancies [45–47]. Interestingly, down regulation of BMi-1 results in apoptosis and senescence, and in increased cellular sensitivity to cytotoxic agents [48]. Bmi-1 expression is increased in early and well-differentiated HCC usually appearing as small dots within the nucleus. In one study 65 % of well differentiated, 45 % of moderately differentiated, and 50 % of poorly differentiated HCCs expressed Bmi-1 [49].

7.4.3 Glutamine Synthetase (GS)

GS is found predominantly in the brain, kidneys, and liver, where plays a central role in nitrogen metabolism by catalyzing the condensation of ammonia and glutamate to glutamine [50]. Interestingly, glutamine is known to act as an energy source for tumor cells [51]. In normal liver GS expression is found in the hepatocytes surrounding the terminal hepatic venules, while in HCC there is diffuse and extensive accumulation of GS in the malignant hepatocytes [52]. One study demonstrated increased GS expression in 14 % of high-grade dysplastic hepatic nodules as compared to 59 % of well-differentiated HCCs, and 86 % of moderately to poorly differentiated HCCs [41]. GC overexpression is associated with β-catenin mutations or activation of the β-catenin pathway [53, 54]. Interestingly, increasing GS expression appears to promote HCC progression and metastasis [55, 56]. GS is not entirely specific as increased expression is seen in 14 % of high-grade dysplastic hepatic nodules and in the β-catenin-activated hepatocellular adenoma subtype [41, 57].

7.4.4 α-Fetoprotein (AFP)

AFP is a well established serum marker for HCC, with ~50 % of individuals with HCC showing elevated AFP and plasma levels above 400–500 ng/ml AFP being virtually diagnostic of HCC [58]. Unfortunately APF levels are seldom elevated in early HCC and it has low sensitivity as a tissue marker, being expressed in only about 17–68 % of paraffin embedded HCC sections [58, 59]. In addition, AFP levels are usually not elevated in the fibrolamellar subtype of HCC.

7.4.5 Cyclase-Associated Protein 2 (CAP2)

CAP2 is a member of the cyclase-associated protein family and is involved in multicellular organism development, vesicle trafficking, and endocytosis, via regulating adenylyl cyclase [60, 61]. CAPS expression is increased with increasingly higher grades of HCC [62].

7.4.6 Glypican-3 (GPC3)

GPC3 is a cell-surface heperan sulfate proteoglycan normally expressed in fetal liver and placenta, but not in normal adult liver. Recently, GPC3 has been established as a serum and tissue maker for HCC. One recent study demonstrated GPC3 immunoreactivity in 69 % of well differentiated and 81 % of moderately to poorly differentiated HCCs. Only 9 % of high-grade dysplastic nodules and no low-grade nodules were positive [41]. GPC3 immunoreactivity shows 77 % sensitivity and 96 % specificity for small HHC, demonstrating that positivity for this immunostain can support the diagnosis of HCC. GPC3 typically shows a cytoplasmic staining pattern, although canicular or membranous patterns are also seen [41, 63, 64]. GPC3 immunopositivity needs to be interpreted in the correct clinical and histological context, as benign liver lesions with active inflammation and regeneration, and melanocytic lesions can also be GPC3 immunopositive [65, 66].

7.4.7 Immunostain Combinations for HCC

Like for immunohistochemical procedures, various immunostain combinations give better result in the diagnosis of HCC then a single stain. The combination of GPC3, HSP70, and GS allowed 72 % sensitivity and 100 % specificity in well differentiated HCC detection when two immunostains were positive. GPC3 and HSP70 alone gave a 59 % sensitivity and 100 % specificity for well-differentiated HCC. These results are significantly better than those achieved with any single immunostain [41].

7.5 Molecular-Genetic Testing for HCC

Currently, there are relatively few molecular-genetic tests routinely performed for HCC. Presently the Association for Molecular Pathology Solid Tumors Test Directory lists p53 mutational analysis for HCC, offered by two labs and no other tests [67]. However, there are several important molecular tests related to HCC risk and several areas of molecular pathology research that are likely to lead to new molecular diagnostic tests for HCC.

7.6 Hereditary Hemochromatosis

HH is one of the most common genetic diseases, affecting about one in 200–300, with Northern Europeans showing the highest known incidence. HH increased an affected individuals HCC risk, with different HH types often conferring a different risk. Presently there are five different known molecular causes of HH. Since untreated HH can result in cirrhosis and HCC, and can be inherited, molecular testing for HH followed by correct clinical care is necessary [10]. Presently, molecular diagnostic tests are available and routinely preformed in many labs, with HFE gene mutation being the most common.

7.6.1 Indications for HH Testing

The initial diagnosis of HH can be difficult due to the protean manifestations of HH. The symptoms of HH can include heart failure/cardiac arrhythmias, cirrhosis, diabetes, arthritis, joint pain, skin bronzing, fatigue, hypogonadism, hypothyroidism, and HCC [9–11]. It is important to identify individuals with HH as early as possible. While some manifestations of HH can be reversed by therapeutic phlebotomy, such as the liver and skin manifestations, other complications, such as hypogonadism, arthropathy, and diabetes, are irreversible once they have occurred [68]. Should an individual have a family history of HH, molecular testing is warranted to avoid missing HH in its earliest and most treatable form [10, 68]. Evaluation of iron overload should first consider non-genetic causes, many of which are more common than HH.

Iron overload can be measured by several tests, including total iron binding capacity, transferrin saturation, liver biopsy, and serum ferritin (SF). Initial testing is often performed by serum ferritin analysis, which is highly sensitive for iron overload, showing hyperferritinemia. Care must be taken in interpreting the test result, as SF is an acute phase reactant and hyperferritinemia also occurs in inflammatory processes, diabetes, chronic alcohol use, and liver damage [10]. Testing should be performed to exclude other disease processes. Santos et al. [69] proposed the following four-step strategy:

1. *Rule out other causes of hyperferritinemia.* Taking a personal and family history, performing a clinical exam, and performing tests such as iron parameters,

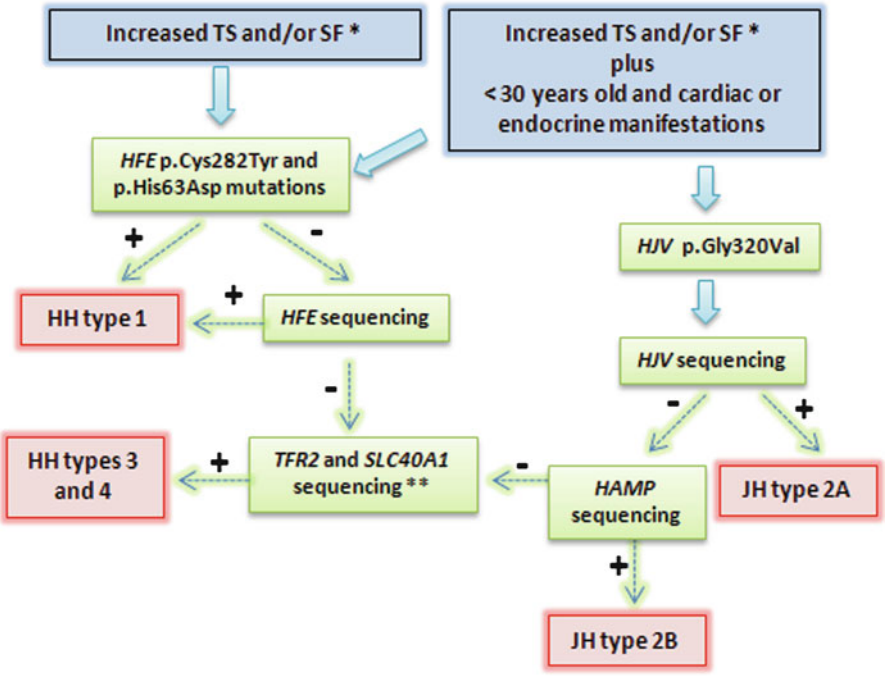

Fig. 7.2 Representation of diagnostic strategy for patients suspected HH. Recommendations report TS > 45 %, SF > 200 μg/L in females and >300 μg/L in males; or in advanced stages: TS > 50 % in females and TS > 60 % in males, in the absence of secondary causes. Some patients with primary iron overload may not present mutation during this genetic approach. Very rare mutations in other genes can be involved. *TS* transferrin saturation, *SF* serum ferritin, *JH* juveline hemochromatosis. + means positive result, and – means negative result; lane 7, heterozygous for C282Y, wild-type for H63D; lane 8, wild-type for both C282Y and H63D [10]

C-reactive protein, hemoglobin, and alanine aminotransferase and aspartate aminotransferase will usually give a diagnosis of acquired hyperferritinemia and avoid unnecessary molecular testing.

2. *Confirm iron overload.* Since SF levels can vary throughout the day, repeating SF can be useful. Along with other iron studies and diagnosis of iron overload can be rendered. Possible causes of iron overload include excessive iron supplementation (oral, parenteral, or transfusions), chronic liver disease (alcoholic, viral or metabolic), end stage liver disease, chronic anemia (thalassemia major, myelodysplastic syndrome, sideroblastic anemia, chronic hemolysis), and porphyria cutanea tarda.
3. *Assess hepatic iron stores.* This can be done via liver biopsy or magnetic resonance imaging. Hepatic iron store analysis may be necessary where other iron studies fail to give definitive results.
4. *Perform molecular genetic testing for HH.* While mutations of the HH gene are most common, other genetic causes of HH must also be considered. An algorithm for molecular genetic testing protocols is given in Fig. 7.2.

7.6.2 HFE Testing

The gene encoding *HFE* is located at 6p21.3 and encodes a seven exon, six protein domains, 47.5 kDa protein similar in structure to the major histocompatibility class I-like proteins. The exoms encode an initial signal sequence, three extracellular domains, a transmembrane region and a short cytoplasmic tail, forming a 4.2 kb long transcript. The seventh exon does not appear to be expressed [70]. HFE is mainly expressed in the liver, duodenum, small intestine, spleen, and heart. Several alternatively spliced variants are expressed at low levels in many human tissues [70–76]. Most cases of HH are caused by two single nucleotide polymorphisms (SNP), Cys282Tyr and His63Asp, making the inheritance autosomal recessive. Some 20 other less common HFE mutations are linked to HH, which contribute to the phenotypic heterogeneity of the disease. Most commonly, individuals with HH are homozygous for Cys282Tyr, or heterozygous for Cys282Tyr/His63Asp [10]. In Northern European populations, roughly 90 % of *HFE* mutations are Cys282Tyr, with Cys282Tyr/Cys282Tyr being the most common HH genotype. Homozygousity for His63Asp does not cause HH, but this SNP contributes to increased hepatic iron levels when the Cys282Tyr SNP is also present [76]. *HFE* mutation causes excess iron accumulation via reducing the amount of hepcidin levels, resulting in increase ferroportin activity and increased enteric iron import [77].

HFE testing for HH is performed employing a variety of different molecular tests. Usually the testing modalities include a combination of PCR amplification, restriction enzyme digest, allele- specific PCR, heteroduplex analysis, sequencing, and single-strand conformation polymorphism testing. Here a few of these techniques are reviewed.

7.6.2.1 Mutliplex PCR with Restriction Endonuclease Digestion

Stott et al. [78] employed a combination of mutliplex PCR with primer mismatches, followed by restriction endonuclease digestion to identify the Cys282Tyr and His63Asp mutations in peripheral blood samples. Five milliliter of blood was taken via venipuncture and the DNA was extracted. The DNA was then PCR amplified with the primers depicted in Fig. 7.3a. Note that the Cys282Tyr and His63Asp each had a different set of mismatched primers that introduced a *Bbr*PI restriction site into the wild type, but not in either mutant amplicon. The amplification was carried out under standard conditions with a 2 min denaturation at 96 °C, followed by an initial annealing step of 30 s at 55 °C, with 30 cycles each of 30 s at 72 °C, 30 s at 94 °C, and 15 s at 55 °C. A final extension period of 10 min at 72 °C completed the PCR.

The entire PCR reactions were then subjected to *Bbr*PI restriction digestion for 4 h using five Units of enzyme. The restriction fragments were separated by gel electrophoresis using 3 % Nusieve:Agarose (3:1) gels for 50–60 min at 100 V. Products were visualized by staining in 1 mg/L ethidium bromide followed by transillumination at 302 nm. Each possible combination of the two SNPs and wild type sequence are represented in Fig. 7.3b. Each combination gave a specific and reproducible pattern that allowed identification of the two common *HFE* SNPs.

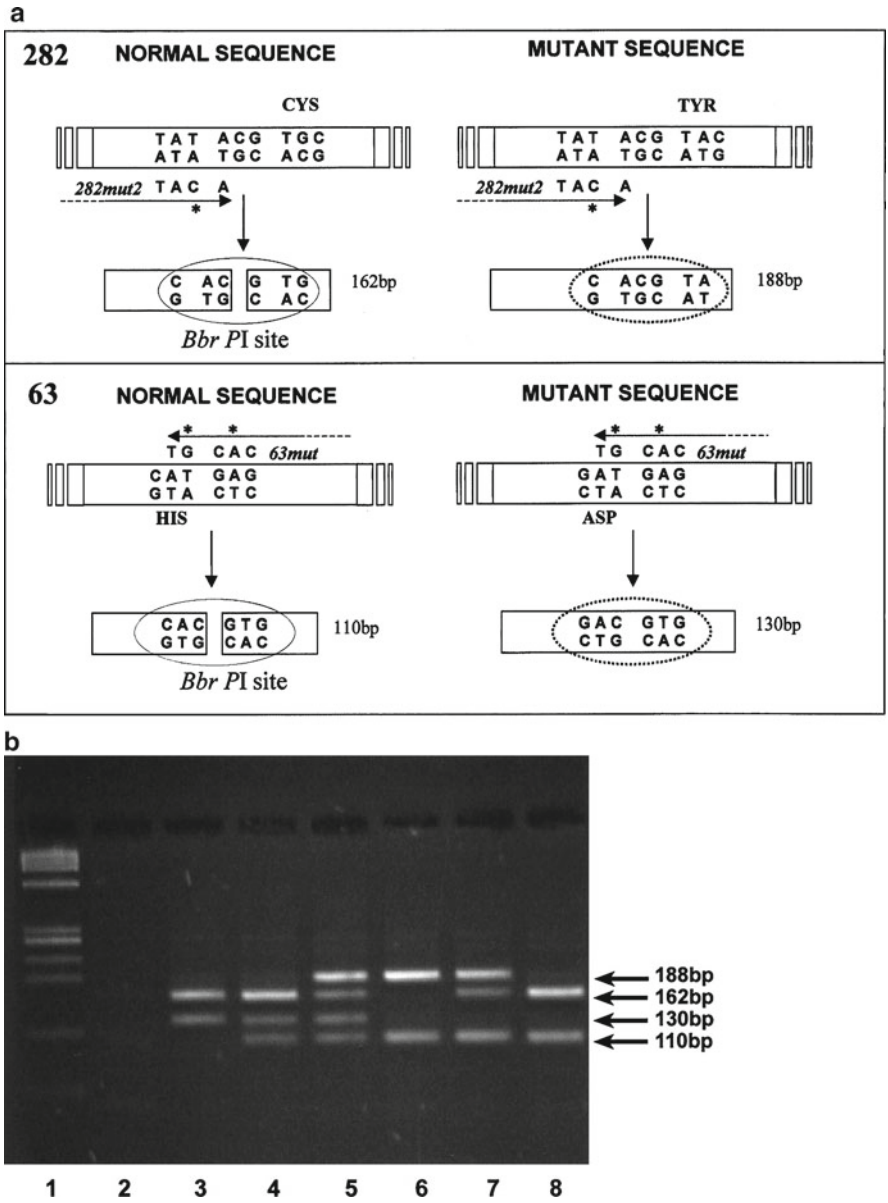

Fig. 7.3 (**a**) Schematic outline of PCR and restriction digestion used to determine the presence of HFE gene mutations and detection of both hemochromatosis mutations, C282Y and H63D, by PCR and restriction digestion (**b**). The site of mutation and primer mismatches are shown for the 282 locus (*top*) and for the 63 locus (*bottom*). **(b)** Restriction fragments were separated on 3 % Nusieve:Agarose. Results representative of all six possible genotypes are shown. Lane 1, PHI-X174-HaeIII molecular mass marker; lane 2, negative control; lane 3, wild-type for C282Y, homozygous for H63D; lane 4, wild-type for C282Y, heterozygous for H63D; lane 5, heterozygous for C282Y, heterozygous for H63D (compound heterozygote); lane 6, homozygous for C282Y, wild-type for H63D [79]

7.6.2.2 Allele-Specific PCR (ARMS)

ARMS takes advantage of the 10^{-3} to 10^{-6} lower amplification efficiency of the *Taq* DNA polymerase initiating from mismatched template-primer 3′-termini [79]. Through the use of primers specific for either a wild type or SNP sequence, it is possible to design primers that will preferentially amplify the sequence they share 100 % homology with [80]. In some systems ARMS technology can detect one mutant allele in a background of 104 wild-type alleles [81]. Sánchez et al. [81] used ARMS to analyze the blood of 5,370 Spanish blood donors for the Cys282Tyr and His63Asp *HFE* mutations. DNA was isolated via cell lysis, high-speed centrifugation, multiple washing steps, and protein removal by proteinase K digestion [82, 83]. The DNA was subjected to PCR amplification with the following probes:

Cys282Tyr	His63Asp
C282Y-R1: CCACTGATGACTCCAATGACTA	63Fw: AGCTGT TCGTGTTCTATGATC
C282Y-Rm: CCTGGGTGCTCCACCTGGT	HFE187Gas: CTCCACACGGCGACTCTCATC
C282Y-Fw: GGGAAGAGCAGAGATATACGTG	HFEEx2U: GCTCCCCTCCTACTACACAT
C282Y-F: TGGCAAGGGTAAACAGATCC	HFEEx2L: GGTCCCTATTTCCACCATCC

The allele-specific PCR was preformed under standard conditions for 35 cycles and the resulting amplicons were analyzed. Of the 5,370 blood samples analyzed, 8 C282Y homozygotes (0.15 %) and 74 compound C282Y/H63D heterozygotes (1.38 %) were identified, giving a C282Y allele frequency in the Spanish population of 3.16+/–0.46 %, or 1 homozygote in every 1,004 individuals. The H63D homozygote frequency was 20.8 %, or 1 in every 23 individuals. The allelic frequencies were not significantly different between men and women. To confirm that the above assay worked, the researchers employed the assay by Stott et al. [78] and found a 100 % results concordance. Four of the 8 C282Y homozygotes, all men, had high serum ferritin and transferring iron saturation, while only 1 of the 74 C282Y/H63D heterozygotes had the same serum results, demonstrating that the compound heterozygotes were less likely to develop HH.

7.6.2.3 DNA Sequencing

AHH is characterized by a number of common SNPs and in most cases analysis of these SNPs by techniques, such as ARMS, will locate specific *HFE* gene mutations. However, since HH can be caused by loss of *HFE* expression, resulting in increased ferroportin activity [10], other less common mutations that result in the loss of *HFE* protein expression can also cause HH. Sequencing is the ideal technique to locate these HFE gene variants, as it gives the specific nucleotide type at each point interrogated by the molecular assay. Cukjati et al. [83] analyzed a 47-year-old man, with no family history of HH, who had repeated high serum iron levels without any history of chronic anemia, infectious liver disease, alcohol

abuse, or oral or parenteral iron administration. The patient's blood and the blood from 22 Slovenian patients with HH was collected and the DNA was extracted using a QIamp DNA Blood Mini Isolation Kit. RT-PCR analysis for the common HFE mutations (C282Y, H63D, and S65C) was performed on these blood samples. Of the 22 Slovenian patients with HH, four did not carry any of the common *HFE* gene SNPs tested for above. Additionally, the patient and his son were negative for all three common *HFE* gene SNPs.

To further analyze this set of patients, exons 1→6 with PCR amplified and subjected to Sanger sequencing using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and analyzed on an ABI PRISM 310 Gene Analyzer. In three patients with HH, no mutations were identified by this analysis method, while the 47-year-old had a frameshift homozygous mutation of the A nucleotide in exon 3, at position 471 (c.471del, p.A158fs), which resulted in a prematurely truncated protein. The patient was also homozygous at both HLA-A and HLA-B, suggesting that loss of heterozygosity might have occurred during mitotic recombination. While rare, unusual *HFE* gene mutations require testing by sequencing or other molecular techniques capable of interrogating every nucleotide within the DNA sequence being analyzed. Although not performed often, HFE gene mutations have also been analyzed by DNA pyrosequencing with good results [84].

7.6.2.4 Genotyping HFE Gene Mutations by Melting Point Analysis

DNA melting point analysis typically involves PCR amplification followed by allele-specific fluorescent probe melting profile analysis. This technique is commonly employed to locate changes in nucleic acid sequences, although by itself it does identify specific nucleotide changes. It has the advantage of allowing melting analysis to be performed on the closed PCR reaction without the need of opening the reaction tube once amplicon amplification is complete. This significantly lowers the chances of sample contamination.

Bach et al. [85] used this technique to analyze genomic DNA from over 3,500 donor samples to look for the presence of the C282Y (845G>A), H63D (187C>G), and S65C (193A>T) *HFE* gene mutations. The mutations were amplified with the following primers:

C282Y	H63D and S65C
F5′-TGGCAAGGGTAAACAGATCC-3′	F5′-GCTCTGTCTCCAGGTTCACACTC-3′
R5′-CTCAGGCACTCCTCTCAACC-3′	R5′-CCCTCTCCACATACCCTTGC-3′

The amplification was performed as follows: denaturation 1 cycle 95 °C 10 min, followed by 45 cycles of 95 °C 10 s, 55 °C 10 s, and 72 °C 10 s, with a temperature transition rate of 20 °C/s. The melting curve was performed at 95 °C for 5 s and 45 °C for 15 s with a temperature transition rate of 20 °C/s ramping to 85 °C at 0.1 °C/s. The C282Y/845A>T transition was measured with a 3′-fluorescein-labeled sensor (5′-GAG ATA TAC GTG CCA GGT GGA GCA C-3′) and a

5′-LC-Red640-labeled anchor probe (5′-AGG CCT GGA TCA GCC CCT CAT TGT-3′). The H63D/187C>G was monitored with a 5′-LC-Red705-labeled anchor (5′-CTG GTA TCC ACG TAG CCC AAA GCT TCA A-3′) and a 3′-fluorescein-labeled sensor (5′-GG CGA CTC TCA TGA TCA TAG AAC ACG AAC A-3′) probe. This second probe- anchor combination was also used to monitor the S65C/193A>T mutation as the SNP was located only six nucleotides away from the H63D/187C>G mutation. Abnormal melting curves were analyzed by Sanger sequencing on an ABI PRISM 310 Gene Analyzer. By this method two new *HFE* gene substitutions were identified and one melting curve typical of the C282Y/845A>T transition was actually G>A transition located two nucleotides away from the C282Y/845A>T transition, giving the same melting curve. The authors concluded that this analysis method works well, but in some cases results should be further analyzed by DNA sequencing or restriction endonuclease digest.

7.6.3 Juvenile Hemochromatosis (JH)

JH or Type 2 HH is a rare autosomal recessive disorder that leads to significant iron overload before the age of 30 and is characterized by cardiomyopathy, liver damage, endocrine dysfunction, hypogonadotrophic hypogonadism. JH typically has a rapidly progressive clinical course, making the rapid initiation of therapy vital for patient care and also allowing the clinical differentiation from HFE mutation-induced HH (see Fig. 7.2). Molecular analyses have demonstrated that two genes are mutated in JH; *HJV* and *HAMP*, leading to JH types 2A and 2B, respectively. The *HJV* gene consists of four exons, encoding the hemojuvelin protein Individuals with *HJV* gene mutations and *HJV* knockout mice have low hepcidin, suggesting that hemojuvelin is involved in regulating hepcidin expression. The *HAMP* gene encodes three exons and encodes hepcidin, a peptide hormone produced in hepatocytes that plays a role in iron absorption and ferroportin degradation in intestinal enterocytes [10].

7.6.3.1 HJV Gene Analysis

The *HJV* gene has a large number of mutations that lead to JH 2A, with the most common one being a Gly320Val change [86–89]. Due to the rarity of *HVJ* gene mutations and the relatively large number of mutations that can cause type 2A JH, sequencing has often been chosen to analyze *JHV* gene mutations. Lee et al. [89] employed these primers to amplify the HJC gene exons in unrelated kinships in the Southeastern US in which HJ had been identified:

HJV	Primer sequence	Fragment size in BP
HJ Ex 1 F	GTACTCTGGCCAGCCATATACT	286
HJ Ex 1 R	CGAGAGACATCCAAGTAGGTGT	

(continued)

(continued)

HJV	Primer sequence	Fragment size in BP
HJ Ex 2 F	ATCTCCCCAAATTCCAGTCTG	359
HJ Ex 2 R	ACATAGCAGCCTACCCTCTAG	
HJ Ex 3 F	GCAAACTACACTCCGATAGAG	669
HJ Ex 3 R	GAATCTCATGAGGTGGATCGG	
HJ Ex 4A F	TAGTCCTGCATCTCTACTTGG	394
HJEx4AR	TGCAGGTCCTGTTCAGCTG	
HJ Ex 4B F	ATGGAGGTGACCGACCTGG	374
HJEx4BR	AGCTGCCACGGTAAAGTTGG	
HJ Ex 4C F	GCTCTCCTTCTCCATCAAGG	430
HJEx4CR	AAACTAGTAATGGGACTGATGG	
HJ Ex 4D F	TGTGGGCTCTTTGTTCTGTG	407
HJEx4DR	GTCTTCTGCTTTCAGCTCTTG	
HJ Ex 4E F	ATAAGTTTAGAGGTCATGAAGG	404
HJEx4ER	GCCCTCTTTCAGTGGAGTG	

The amplicons were purified using a Qiaquick PCR purification kit and the genotypes were determined by direct sequencing with an ABI 3100 DNA sequencer. By this method first group had two novel *HVJ* gene mutations, while to second group had two previously identified mutations.

7.6.3.2 HAMP Gene Analysis

HAMP gene mutations in HJ are very rare [10, 90]. *HAMP* gene mutations are usually identified by PCR amplification of the three gene exons, followed by DNA sequencing [90, 91].

7.6.4 Transferrin 2 Gene Mutation (TFR2) *and Hemochromatosis*

TFR2 gene mutations cause Type 3 HH, an HH type with similar clinical manifestations the HFE mutation-induced HH [10, 92]. The *TFR2* gene encodes an 18 exon, 801 amino acid protein that shares 45 % identity with TFRC. The protein mediates cellular iron import and has two predominant transcripts (alpha and beta), with the alpha form expressed mainly in the liver, spleen, lung, muscle, prostate, and peripheral blood mononuclear cells, and the beta form expressed in all tissue examined [92, 93]. Once identified, Type 3 HH is treated with periodic phlebotomies, like most other HH types [10].

TFR2 genes mutations leading to HH are relatively rare [10]. Roetto et al. [94] examined four different families with HH unrelated to *HFE* gene mutations. DNA was prepared from peripheral blood by standard phenol-chloroform

extraction. The DNA was PCR amplified in a Thermal Cycler using 10 pmol of primers specific to all 18 *TFR2* exons. A total of 32 PCR cycles (denaturation, 94 °C for 30 s; annealing, 56 °C for 45 s; extension, 72 °C for 45 s) were performed. The amplicons were then transcribed into RNA with 10 units T7 RNA polymerase in an appropriate buffer with ^{35}S-UTP and subjected to gel etectrophoresis, drying, and autoradiography. Bands showing altered electrophoretic mobility were directly sequenced. Direct sequencing was performed on the purified PCR amplicons using a Thermo Squenase Cy5.5 dye terminator cycle sequencing kit. After removal of the unincorporated dye, the sequence was identified via electrophoresis on an automatic sequencer (model 373A; Applied Biosystem). The *Mae*I enzyme was used to detect the previously described Y250X mutant within the amplicon for exon 6.

Of the patients with altered *TFR2* gene exon electrophoretic mobility two patients had a previously described Y250X mutation in exon 6. In one large inbred family a frameshift mutation (84–88 insC; E60X) in exon 2 was identified which formed a premature stop codon. A single patient with nonfamilial HH carried a T → A transversion (T515A). All patients with a non-protein expressing phenotype showed severe clinical complications.

In another study, Biasiotto et al. [92] used denaturing high-performance liquid chromatography to analyze TFR2 gene mutations using blood samples from 657 individuals from Northern Italy who either had a diagnosis of HH or serum ferritin concentrations greater than 400 μg/L or a transferrin saturation above 50 %. The *HFE* gene C282Y and H63D mutations were identified by endonuclease digestion before this study was carried out. Genomic DNA was isolated from 0.2 ml of blood with a QIAamp DNA Blood Mini Kit and portions of the *hepcidin*, *HFE*, and *TFR2* genes were PCR amplified with appropriate primers. Specifically, exons 2, 4, and 6 of the *TFR2* gene were amplified. These exons were chosen for analysis as previous studies had shown mutations in these exons [92–94]. The resulting amplicons were heated to 94 °C for 10 min cooled at 56 °C for 60 min to obtain heteroduplexes. Mutations were analyzed by denaturing high-performance liquid chromatography on a Transgenomic WAVE® System with a preheated C_{18} reversed-phase column. Eight μL of the PCR mixture were loaded into column duplexes were eluted at a flow rate of 0.9 μL/min. DNA was detected by monitoring the absorbance at 260 nm and DNA fragment melting characteristics were predicted by Wavemaker™ software. The samples with altered elution profiles were subjected to DNA sequencing. As this technique does not detect homozygous mutations, the amplicons were also mixed with equal amounts of wild-type DNA, denatured, reannealed, and analyzed for heteroduplexes. All identified C282Y and H63D *HFE* gene mutations were confirmed by enzymatic digestion. No discrepancies between endonuclease assay- indentified C282Y and H63D *HFE* gene mutations and denaturing high-performance liquid chromatography *HFE* gene mutation results were detected, indicating that the chromatographic assay worked well and had 100 % sensitivity, at least for these two mutations.

Out of the 657 individuals tested, three new intronic variations, a previously identified IVS5-9T > A transition, and a new Val22 → ILE *TFR2* gene mutation

where found. Only one individual (72 years old and hepatitis B surface antigen positive) with *TFR2* gene mutations and no concomitant *HFE* gene mutations had a high serum ferritin value, possibly due to viral hepatitis. Based on these results, the authors concluded that *HFE* and *TFR1* gene mutations are often associated in HH and that HH due solely to the rare *TFR2* mutations is uncommon.

7.6.5 SLC40A1 *Gene Mutation and Type 4 HH*

The *SLC40A1* gene mutations cause a rare autosomal dominant HH, showing unusual clinical features including high serum ferritin levels with low or normal transferrin saturation until the end stage of the disease. Additionally, urinary hepcidin is usually reduced. Strangely, it may initially present as mild iron-deficient anemia with a reduced tolerance to therapeutic phlebotomy. *SLC40A1* consists of eight exons and encodes the ferroportin protein – a multipass transmembrane protein located on the basolateral membrane of enterocytes that regulates iron export into the circulation [10, 95]. Ferroportin also regulates the efflux of iron from macrophages of the spleen, liver, and bone marrow, and Type 4 HH is unique in that it often shows increased Kupffer cell iron ([10, 95, 96], Fig. 7.3a, b). *SLC40A1* gene mutations lead to increased iron loading, often due to ferroportin losing its responsiveness to hepcidin [97].

Like molecular testing for HH types 2 and 3, *SLC40A1* gene mutation analysis is not commonly performed, due to the rarity of the disease. Most molecular tests involve sequencing the entire coding region of the gene. Montosi et al. [98] examined genomic DNA isolated from peripheral blood from an Italian pedigree that included 15 individuals with iron overload, resulting in hepatic fibrosis, diabetes, impotence, and cardiac arrhythmias. None of these 15 individuals carried the C282Y *HFE* gene mutation [99]. The ferroportin exons were PCR amplified, sequenced, and compared to control sequences. Individuals with iron overload, but not those with normal iron levels, showed an exon 3 mutation that converted an alanine at residue 77 to an aspartic acid (GCC → GAC). The authors hypothesized that this mutations resulted in a gain of protein function that resulted in increased dietary iron accumulation. At least 26 other ferroptorin mutations have been identified by various molecular tests [10].

7.6.6 Genetic Testing for HH

While most cases of HH are due to *HFE* gene mutations in European populations, a subset of HH in this population and non-European populations can carry different gene mutations. Santos et al. [10] recommended the testing algorithm shown in Table 7.1.

Table 7.1 Common serological patterns associated with HBV infection (105–107)

HBsAg	Negative	
Anti-HBsAg	Negative	Susceptible to Infection
Anti-HBcAg	Negative	
HBsAg	Negative	Immune due to Natural Infection
Anti-HBsAg	Positive	
Anti-HBcAg	Positive	
HBsAg	Negative	Immune due to HVB Vaccination
Anti-HBsAg	Positive	
Anti-HBcAg	Negative	
HBsAg	Positive	
Anti-HBcAg	Positive	Acute infection
IgM anti-HBcAg	Positive	
Anti-HBsAg	Negative	
HBsAg	Positive	
Anti-HBcAg	Positive	Chronically Infected
IgM anti-HBcAg	Negative	
Anti-HBsAg	Negative	
HBsAg	Negative	Several Possibilities:
Anti-HBsAg	Negative	1. Resolved HVB Infection
Anti-HBcAg	Positive	2. False-Positive anti-HBcAg
		3. Low-Level Chronic Infection
		4. Resolving Acute Infection

7.7 Molecular Diagnostic Testing for Hepatitis B and C

Chronic HBV, HCV, and the satellite virus of HBV, hepatitis D (HDV), are major risk factors for HCC, with approximately 500 million people infected with viral hepatitis worldwide [1–4]. Proper medical management of chronic viral hepatitis requires timely and accurate diagnosis, staging, and disease monitoring. In general serological markers are commonly used for viral hepatitis diagnosis and staging, while molecular diagnostic tools are used for the identification of specific viral strains and for assessing drug responses. The end-point of such treatment is obtaining a sustained virological response, preferably with undetectable serum viral RNA, 6 months post-therapy completion [1–4, 99, 100]. Here we will review the serological and molecular testing for HBV, HCV, and HDV, with emphasis on molecular diagnostic techniques.

7.7.1 HBV

The WHO estimates that over two billion people have been infected with HBV and currently some 350 million people are chronically infected worldwide, with about 75 % living in the Asia Pacific region. Chronic HBV confers an increased risk for

hepatic decompensation, cirrhosis, and HHC, and it is estimated that the worldwide mortality from this disease is 0.5–1.2 million deaths per year. In adults, most HBV infections are eventually cleared with only about 5 % resulting in chronic infections. In neonates, however, over 90 % fail to clear the virus and become chronically infected. HBV is spread parenterally by percutaneous or permucosal exposure to infected body fluids, especially blood. Risk factors for HBV infection include reusing syringes in between intravenous drug users, transfusions with untested blood, tattooing with unsterilized instruments, sexual promiscuity, working in a healthcare setting, and living in a correctional facility [101, 102].

HBV is a double-stranded (ds), circular, enveloped 3,200 base pair DNA virus of the hepadenaviridae family. HBV has at least eight major genotypes (A through H), which shows a greater than 8 % divergence in viral nucleic acid sequence. Following entrance of the HBV into the hepatocyte, the viral genome enters the nucleus and integrates into the host genome, where the viral DNA functions as a transcriptional template making viral RNA. Unfortunately, because HBV integrates into the host genome and remains as an intercellular replicative intermediary (covalently closed circular DNA) complete elimination of HBV from an infected individual is not usually possible. Some of the viral RNA intermediates are reversed transcribed into DNA. The transcription products are used to make viral proteins, including the HBV surface and e antigens. Although HBV has a strong preference for the liver, viral DNA can be found in the kidneys, pancreas, and mononuclear cells of infected individuals. In acute infections the HBV infection becomes apparent roughly 45–180 days post initial infection, with the first 6 months of infections defined as acute phase of infection. Typically clearance of the HBV infection begins several weeks prior to the onset of clinical symptoms, and it is caused by antiviral cytokines produced by the adaptive and innate immune responses. Later, a cytologic immune response causes hepatocyte necrosis and apoptosis, responsible for the concomitant onset of clinical symptoms, and a rise in serum alanine aminotransferase (ALT). Individuals with a strong and specific CD4/CD8 immune response will usually clear the HBV, while a weaker response is associated with chronic HBV [101, 102].

7.7.1.1 HBV Serologic and Histopathologic Testing

Liver damage and function are typically analyzed serum ALT, bilirubin, and albumin levels, and the prothrombin time. Liver biopsy is used to quantify the grade of inflammation and the stage of fibrosis. Non-invasive methods such as ultrasound or rising alpha-fetoprotein levels can, on the other hand, often detect cirrhosis and HCC. Several serologic tests are employed in HBV infection and serum analysis based assays are presently the mainstay testing modality. Detectable HBV surface antigen (HBsAg) is a marker of chronic infection, while viral core antigen (HBcAg) indicates high hepatic viral replication. Loss of serum HBcAg with concomitant raise in anti-HBcAg antibodies (termed seroconversion) is associated with clinical improvement and reduced serum ALT, hepatitis, and reduced HBV DNA levels

[101–103]. Serum analysis of HBV-related antigens and antibodies identify the different phases of the infection and are summarized in Table 7.1 [101–103].

7.7.1.2 HBV Molecular Genetic Testing

Molecular HBV testing is usually employed in the diagnosis and management of HBV infection; quantification of viral load, genotyping, drug resistance, mutation analysis, and core promoter/precore mutation assays. Of these assays, the quantification of viral load is the most commonly used test [103].

7.7.1.2.1 HBV Viral Load Quantification

HBV viral load quantification is usually performed on plasma or serum. It is used to measure the viral load prior to the initiation of therapy, to monitor the effectiveness of anti-viral therapy, and to identify "occult" HBV viral infections, where the serologic tests can fail to detect HBV infections. It is also important in liver transplantation to avoid unnecessary HBV transmission [101–103]. In general, individuals with chronic HBV that are HBsAg positive, HBeAg negative, anti-HBeAg positive and serum HBV DNA levels below 10^5 copies/ml (>20,000 IU/ml) show histologically mild HVB-induced hepatic lesion as graded according to Ishak et al. [104, 105].

7.7.1.2.2 Nucleic Acid Hybridization Assays for HBV Quantification

The first molecular assay employed to quantify HBV viral loads were hybridization assays. Although these assays worked well, they were less sensitive and more cumbersome to perform that most PCR-based assays and are now seldom performed [104]. The degree of HBV probe hybridization does directly quantify the amount of serum HBV DNA present and correlates closely with infectivity [106]. Guo et al. [107] employed digoxigenin-labeled probes to detect HBV DNA in serum. Full-length HBV was inserted into a pBR322 plasmid at a *PstI* site, grown up in bacteria, subjected to restriction endonuclease digestion and isolated by gel electrophoresis. The probed DNA was purified by ion-exchange chromatography and either labeled with [α-^{32}P]dATP with a random labeling heximer or with a digoxigenin-11-dUTP labeling according to Boehringer Mannheim kit protocol. HBV DNA was isolated from human sera by two means: phenol-chloroform extraction followed by ethanol precipitation, or by alkaline denaturation in 1 M NaOH, followed by neutralization. Both samples were applied to nitrocellulose prewetted with appropriate buffers, baked to attach the HBV DNA to the nitrocellulose, pre-hybridized, hybridized with the HBV probes, washed, dried, and exposed to X-ray film. For the digoxigenin-labeled probes, the labeled DNA was detected immunologically using an antibody-enzyme conjugate, anti-digoxigenin-alkaline phosphatase with an enzyme-linked

color reaction. Both assays included a standard curve within the same blot. Both assays yielded a lower detection limit of ~0.25 pg, corresponding to 7.0×10^4 genome copies. Since this number is close to the 10^5/ml copy number that defines viral loads that are not usually damaging to the liver. The sensitivity of this hybridization assay is adequate, but the sensitivity of the PCR assay is greater. Additionally, the digoxigenin-based assay had a longer shelf life and is safer to work with than the ^{32}P-based assay.

7.7.1.2.3 Branched DNA Technology in HBV Detection

Branched chain DNA (bDNA) is a sensitive, specific, and reliable tool for the diagnosis of viral infections and in monitoring drug response and/or disease progression. It has been successfully on multiple microorganisms, including T. *bricei,* cytomegalovirus, human papilloma virus, HIV, HCV, and HBV. bDNA does not depend on nucleic amplification, but instead uses a bDNA probe that hybridizes to the target sequence(s), resulting in a complex of probes and target sequences that can be quantified with great accuracy.

To perform this assay HBV is isolated from patient serum and treated to insure that the DNA can bind the assay "extender probe". The assay is usually performed on a multi-well plate, such as a 96 well plate. Attached to the walls and floor of the plate are many "capture probes". Extender probes are added under the correct buffer conditions to allow the capture and extender probes to hybridize. Part of the extender probe binds to the capture probe while the other, non-bound, single-stranded portions hybridizes to the target HBV DNA. A "label extender" is then added which binds to contiguous regions of the bound target HBV DNA. A "preamplifer oligonucleotide" is added which hybridizes to the label extender. This oligonucleotide partially hybridizes to the label extender and also usually has multiple branched oligonucleotides that hybridize to an amplifier oligonucleotide having alkaline phosphatase attached to it. After the required multiple washing steps, the amount of HBV target DNA present in the serum sample will be directly related to the signal created by the reporter molecule (alkaline phosphatase). A standard curve with positive and negative controls is used to estimate the number of HBV genomes in the serum sample. Essentially this assay depends on the HBV DNA linking the fixed capture probe to the amplifier/reporter, allowing the amount of HBV DNA to be quantified [108, 109]. The procedure may be summarized as follows:

Well → Capture Probe → Label Extender → HBV Target DNA → Label Extender → Preamplifier Oligonucleotide → Amplifier Oligonucleotide-Alkaline Phosphatase Conjugate

The advantages of bDNA technology include the fact that the technique lacks any amplification steps (therefore lowering the chances of cross contamination), that it is easy to automate, and is not labor intensive. Its sensitivity is usually less than that of PCR-based assays, but its reproducibility and specificity are excellent. Thus this assay would work well in patient populations that typically have higher HBV titers [109].

7.7.1.2.4 HVB Quantification by PCR

PCR has the advantages of high sensitivity and specificity and has largely replaced hybridization assays for serum HBV DNA quantification. While the sensitivity of hybridization assays is limited to about 70,000 viral genomes/ml, many PCR assays can reliably detect serum viral genomes over a 10^1 and 10^8/ml range [107, 110]. Abe et al. [110] developed an early RT-PCR assay for quantifying serum HBV. To begin this work, nucleotides 1–2,182 of the HBV genome were inserted into a BlueScript II SK(+) plasmid (Stratagen), grown in bacteria, and the recombinant plasmid was purified and then quantified by the optical density at 260 nm. Real-time detection PCR was performed on the HBV DNA with three sets of PCR primers and a probe; two primer sets would amplify the HBV surface (S) antigen gene and one the viral X gene. The amplified HBV viral gene segments were chosen based on being highly conserved between different viral strains, on having high sensitivity/specificity, and being placed relatively widely apart within the viral genome.

To quantify the HBV load, DNA was extracted from 100 μl of plasma and place into 20 μl of water. 10 μl of this DNA was amplified in a 50 μl volume with one of the primer-probe sets, *Taq* polymerase, appropriate buffer conditions, and nucleotide triphosphates. The mix was subjected to amplification cycles of 95 °C for 20 s and 60 °C for 1 min. The amount of target DNA was quantified by measuring the increase in probe fluorescence, following probe degradation with concomitant probe-quencher disassociation, normalized to an unchanging passive reference signal. The amount of target DNA amplified was thus the change in probe fluorescence minus the reference signal measured at the beginning of the PCR reaction. The threshold Ct value was calculated on the mean baseline of the first 15 amplification rounds and set at 10 standard deviations above this mean. The amount of HBV target DNA was measured against a standard HBV DNA curve ranging over 10^1 to 10^8 copies/reaction. A linear relationship was obtained between the Ct and the number of HBV viral copies. In parallel experiments employing HBV DNA quantification by bDNA technology, the detection limit was found to be 7×10^5 viral copies/ml. When applied to the sera of HBV infected and viral-free individuals, this PCR protocol was able to detect HBV viral DNA over a life range (10^1–10^8 copies/reaction) and have similar sensitivity of bDNA technology. This assay was also able to detect HBV DNA in patients who had undetectable HBV level by the bDNA testing method. The authors of this early study conclude that this assay was superior to other methods for HBV viral DNA quantification in its sensitivity, specificity, simplicity, and reproducibility.

Since this study, RT-PCR has become the gold standard for HBV DNA detection in patient sera and this technique is routinely employed in the automated detection of serum HBV [111–113]. However, RT-PCR still has limitations in detecting very low HBV loads. Wang et al. [114] used a locked nucleic acid PCR (LNA-PCR) probe to quantify serum HBV loads compared to standard TaqMan RT-PCR probe and achieved a significant increase in the viral detection limit. LNAs carry 2-methyele units between the O_2 and C_4 of ribose, locking the ribose in the 3′-endo (North) conformation, often found in A DNA. This locked conformation enhances

base stacking and increases the melting temperature 2–8 °C compared to unmodified RNA or DNA oligonucleotides, increasing mismatch discrimination [115]. Wang et al. [114] used an LNA or an unmodified oligonucleotide probe designed to match the HBV X gene and produce a 114 base pair amplicon. The same oligonucleotide primers were used in both reactions:

Primer 1	GACCACCAAATGCCCCTAT
Primer 2	CCRAGAYYGAGATCTTCTGCGAC
LNA probe	FAM-TCGTCTAACAACAGT-BHQ1 (underlines denote methylene bridge)
TaqMan probe	FAM-TCGTCTAACAACAGT(TAMMRA)AGTTTCCGGAAGTGT-P

Serum samples were taken from 39 individuals, previously demonstrated by have chronic HBV by ELISA. These samples were divided into three groups: (1) 15 cases that were HBsAg positive only, (2) 10 cases that were HBsAg and HBeAg positive, and (3) 14 cases that were HBsAg, HBeAg, and HBc positive. Additionally, 19 cases of normal serum were taken from volunteers. The HBV DNA was isolated from 100 µl serum and 2 µl of purified DNA were used per reaction out of a final 25 µl volume. The amplification reaction consisted of 40 µl of 200 nM each primer, 75 nM LNA probe, dGTP, dATP, dCTP, and dUTP, 3.5 mM $MgCl_2$, 2 U HotSartTaq DNA polymerase, 0.5 U uracil DNA glycosylase and, 2 µl of purified DNA. The amplification was carried out in a iCycler iQ5 (Bio-Rad) consisting of an initial activation of UDG at 37 °C for 5 min, activation of HotStarTaq DNA polymerase and template denaturation at 95 °C for 3 min, and 40 cycles in two steps: 95 °C for 5 s, 60 °C for 30 s. Standard curves were created in the range of $40–4 \times 10^7$ IU/ml by 1:10 serial dilutions of an HBV standard. All samples were run in duplicate.

A linear relationship was obtained between the Ct values and the log10 concentrations of HBV DNA. Over a serial dilution of $40–4 \times 10^7$ IU/ml the maximum sensitivity of the standard oligonucleotide primers was 80 IU/ml, while the maximum sensitivity for the LNA probe was 40 IU/ml. Thus the LNS probe roughly doubled detection sensitivity compared to the standard oligonucleotide probe. When applied to patient samples, both probe types were able to detect HBV DNA in patient samples. Interestingly, in the 15 HBsAg positive only samples with low HBV DNA, the LNA probes were able to detect HBV DNA in four samples determined to be HBV DNA negative by standard probes. The authors concluded that LNA probe has higher precision, an improved signal to noise ration, and a better detection limit in clinical assays compared to standard probes.

7.7.1.2.5 HBV Molecular Genotyping Assays

HBV has at least eight different genotypes, A–H, which show greater than 8 % genomic variation between the viral types and numerous subtypes which show at least 4 % genomic variation. Most likely these variations have arisen due to the relatively high rate of nucleotide replication substitution ($1.4–4.57 \times 10^{-5}$ per base pair/year)

seen in HBV. Phylogenetic evidence suggests that human HBV is a relatively recent viral pathogen, that first appeared about 3,000 years ago [116, 117]. Many of the HBV genotypes show significant differences in: (1) geographic distribution, (2) clinical course, (3) treatment responses, and (4) modes of transmission. For example, HBV genotype A is more common in Northwest Europe and the USA, while HBV genotype C is more common in Asia and the Pacific region. The HBV genotype C induces a more severe disease, characterized by higher fibrosis scores and a more frequent progression to cirrhosis than does HBV genotype B, while the D genotype appears to cause a higher incidence of HCC [118–122]. Additionally, while all HBV genotypes appear to respond equally well to nucleoside/nucleotide analogue treatment, genotypes A and B respond better to pegylated interferon-α than do genotypes C and D following 52 weeks of treatment [123, 124]. Last, different HBV genotypes are preferentially spread by different modes of transmission, although all genotypes can be spread by the common transmission modes. For example, HBV genotype A has been associated with sexual transmission while the D genotype is often transmitted by blood transfusions and IV drug abuse. Interestingly, genotype A appears to also be preferentially transmitted by homosexual or bisexual contact [125, 126].

Presently there are roughly ten different HBV genotyping techniques that entirely or partially use molecular technology (for review, [127]). Here these techniques will be briefly reviewed and the advantages and disadvantages of each technique discussed.

7.7.1.2.6 Direct HBV Sequencing

Sequencing remains the gold standard for HBV genotyping and presently commercial kits are available for this purpose, such as HBV sequencing test kit offered by Abbott Molecular (Illinois, USA). Sequencing has the advantage of directly interrogating every nucleotide within the viral genome or viral gene being sequenced, allowing the identification of new sequence variations, and it can be a relatively sensitive test. Since all nucleotides are interrogated, sequencing works equally well for genotyping, identifying drug resistant HBV clones, and other mutation testing. The drawback of this technique is that it can be expensive, time-consuming, and can easily miss less commonly represented HBV genotypes within a genotype mix [127].

Okamoto et al. [117] developed an early method for Sanger sequencing of the HBV genome. HBV DNA was isolated from 20 ml of serum taken from individuals demonstrated to have HBV by solid phase immunoassay for HBsAg and by dot blot hybridization for HBV DNA. The gapped region of the HBV genome was repaired with an endogenous DNA polymerase and the HBV DNA was cloned into a pSP65 plasmid *Bam*HI, grown in bacteria, and subcloned into different phage vectors. The HBV genomic fragments were removed with the appropriate restriction endonuclease and subjected to standard Sanger sequencing. A total of 18 HBV genomes were sequenced, five of which comprised newly identified viral subtypes. Although an

early study, this work did demonstrate that Sanger sequencing is an excellent, although slow way to analyze HBV viral sequence variations.

Since the eight different HBV genotypes show significant sequence variation, efficient and accurate genotyping methods employing Sanger sequencing combined with genotype-specific PCR primers have been developed. The commercial TRUGENE HBV Genotyping commercial kit (Siemens Medical Solutions Diagnostics, NY, USA, [128]) uses these techniques. First, DNA is extracted from serum and the HBsAg gene is amplified via PCR. The resulting amplicons are then bidirectionally Sanger sequenced and compared to the known A through H HBV genotypes via a bioinformatics program. The bidirectional Sanger sequencing improves accuracy, as each sequence is effectively read twice. The detection limit of this assay is approximately 2.0×10^3 HBV genome copies/ml. Although less commonly used, pyrosequencing has worked well for some HBV sequencing applications, such as the analysis of specific nucleotide substitutions associated with dug resistance [129].

7.7.1.2.7 Multiplex Polymerase Chain Reaction

Multiplex PCR has been used to analyze HBV genotypes A through H in an accurate and cost-effective manner. Liu et al. [130] employed this technique to analyze the HBV genotypes in the sera of 441 HBV DNA-positive patients and compare it to restriction fragment length polymorphism analysis. The primers employed in this study were designed by analyzing 369 HBV full-length nucleotide sequences in GenBank which had been classified into eight different genotypes (A–H) using the Clustal X1.81, GenDoc2.6.002 and Mega2 software. From this database, genotype-specific primers were designed that were specific to at least 90 % of the viral isolates corresponding to the genotype.

Following patient DNA isolation, the HBV DNA was amplified by multiplex PCR performed with a GeneAmp PCY System 9600 in a 50 ml volume. The amplification protocol incubated the samples for 5 min at 94°, followed by 40 cycles consisting of 94° for 1 min, 59.5° for 1 min, and 72° for 2 min. The amplicons were visualized by electrophoresis in a 4 % agarose gel, stained with ethidium bromide, and evaluated under UV light. Each primer set gave an amplicon with a different molecular weight, allowing identification of the HBV genotype. HBV infections containing different genotypes gave multiple bands of different sizes. In order to be detected, a co-infecting HBV genotype had to represent at least 10 % of the total HBV infection load. When the results of the multiplex PCR were compared to genotyping results obtained by restriction fragment length polymorphism (RFLP) analysis a 93 % concordance was achieved, indicating that genotyping with multiplex PCR is relatively accurate. The authors concluded that this genotyping method could be applied only to areas where HBV genotypes A–G are prevalent. It could also provide an efficient alternative for clinical diagnosis and large-scale studies due to its low cost and fast turnaround time. PCR has also been combined with melting curve analysis to identify different HBV genotypes. This technique allowed as little as 100 HBV genome copies/ml sera and was more accurate than RFLP analysis [131].

7.7.1.2.8 Restriction Fragment Length Polymorphism (RFLP) Analysis

RFLP analysis interrogates the sequence difference between to homologous DNA sequences by using restriction endonucleases to cut the DNA into fragments of different lengths based on the sequences having sites cut by different restriction endonucleases. The resulting DNA fragments are often resolved by gel electrophoresis. Lindh et al. [132] used RFLP to genotype HBV. Seventy-three HBV sequences were obtained through the Entrez database of the National Center for Biotechnology Information (NCBI, Bethesda, MD) and analyzed with MacVector DNA analysis software (International Biotechnologies, New Haven, CT). A restriction endonuclease fingerprint pattern was predicted following treatment with the *Tsp509*I, *Hinf*I, and *Bsr*I endonucleases. To test the predicted fingerprint pattern, 187 HBeAg-positive chronic HBV carriers who resided in western Sweden were then analyzed by a commercial HBV genotyping serology kit (Abbott, Abbott Park, IL), or by RFLP. To perform the RFLP DNA was prepared from 30 μl of patient serum and the segment between nucleotides 256 and 796 were PCR amplified by 40 PCR cycles. Three minutes denaturation at 94 °C, 40 cycles of amplification, including denaturation for 45 s at 94 °C, annealing for 60 s at 53 °C, and extension for 90 s at 72 °C (prolonged by 3 s/cycle), were done and followed by a final 7 min extension at 72 °C.

The resulting amplicon was incubated for 3 h with either the *Tsp509*I or the *Hinf*I restriction endonucleases. The cut amplicons were then run into a composite gel containing 2 % NuSieve agarose and 1 % standard agarose, with uncut amplicon as a negative control. In some cases the cut amplicons were cut further with the *Bsr*I endonuclease. The DNA fragments were identified with ethidium bromide staining and the restriction fragment pattern read visually. One hundred and sixty-six of the samples fell into one of ten major patterns, each corresponding to specific HBV genotype. Seven additional patterns appeared to represent rare HBV genotypes. Thus roughly 92 % of the HBV genotypes were easily identified. Of the others, 14 samples were incubated with *Bsr*I, allowing seven additional cases to be genotyped. Comparison of this method to serological testing showed a 100 % genotyping correlation for those samples that could be RFLP analyzed.

Lee et al. [133] used RFLP combined with matrix-assisted laser desorption/ionization-time- of-flight mass spectrometry to indentify the tyrosine-methionine-aspartate motif associated with lamivudine drug resistance. This detection technique, although expensive, had a lower detection limit of 100 copies/ml. than that of gel-based methods.

7.7.1.2.9 Oligonucleotide Microchip Arrays

Many different molecular assays employ specific gene sequences attached to a solid matrix, combined with washing steps and a hybridization detection reporter, to identify specific nucleic acid sequences within a complex mix. Often these methods show very high sensitivity and specificity [134–137]. Gauthier et al. [138] employed

the Affymetrix in situ synthesis (Affymetrix) to design an HBV microarray consisting of 520,327 different 20-mer oligonucleotide probes allocated to detect HBV polymorphisms/mutations. The array was designed to detect 994 mutants located at 298 genomic positions in the HJBV genome, including positions in the S, C, P, and X viral genes. The fluorescence hybridization patterns were detected via DNAEM automated software (bioM erieux, Marcy-l'Etoile, France) and the fluorescence intensities were translated into sequence information by selecting the most probable nucleotide at each interrogated position.

A total of 253 patient sera that had been previously analyzed by Sanger sequencing were used in this study. DNA was extracted from the seras and amplified in a duplex PCR reaction as previously described [139]. Those amplifications that failed to give an expected 1.5 and 1.7 kb pair fragments were subjected to an alternative nested-PCR amplification procedure to produce an analyzable amplicon. The amplicons where cleaved and fluorescently labeled by successively adding 5 μl RNase-free water, 5 μl of PCR product, 5 μl of M bis-biotin-phenylmethyl-diazomethyl, and 5 μl of 20 mM HCl and incubating for 25 min at 95 °C. The labeled fragments were purified, hybridized on the microarray, and stained. The microarrays were immediately scanned at 570 nm. Analysis of the genotyping performance revealed that samples containing less than 400 HBV genome copies/ml could be successfully analyzed by this technique, demonstrating a high sensitivity. The results were 94 % concordant with Sanger sequencing. The technique also worked well for indentifying HBV sequences associated with adefovir and entecavir drug resistance, and with precore mutations. The authors concluded that this microarray technology could have clinical, epidemiological, and research applications for HBV analysis. Although relatively time-consuming and expensive, this technique is superior to the Sanger sequencing as it gives exhaustive information at multiple positions over the entire 3.2 kb HBV genome in a single reaction.

7.7.1.2.10 INNO-LiPA HBV Assay

The INNO-LiPA HBV assay is a commercially available reverse hybridization line probe assay (Innogenetics, Belgium) that is easy to performed and capable of identifying mixed HBV genotypic infections. In this assay HBV DNA is PCR amplified with biotinylated primers complementary to the conserved S/pre-S ORF. The amplicons are then hybridized to probes immobilized on a strip that has targets corresponding to HBV genotypes A though H. Following washing steps, alkaline phosphatase-labeled streptavidin added, followed by another washing step. The position of the hybridized biotin-labeled amplicon is visualized by the deposition of an insoluble purple/brown precipitate created by alkaline phosphatase catalysis acting on a soluble substrate. The INNO-LiPA HBV assay identifies mixed HBV infections.

Ali et al. [140] compared the INNO-LiPA HBV assay, direct sequencing, and subtractive PCR-RFLP for HBV genotyping. All three assays were performed on 80 consecutive HBV DNA positive serum samples obtained from individuals with

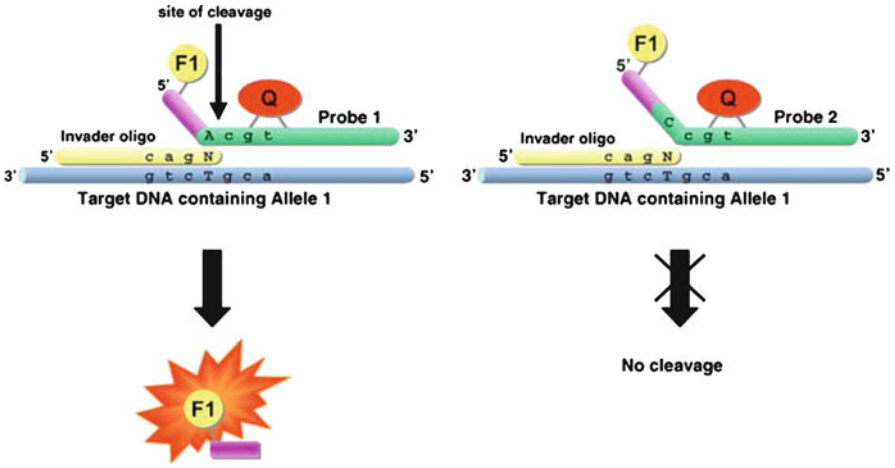

Fig. 7.4 The Invader assay from Ref. [146]

chromic HBV. The DNA was isolated with a High Pure Template Preparation Kit (Roche Applied Science, Germany). Ten µl out of a final 100 µl volume was used as a template for the HBV DNA amplification. The HBV DNA was amplified by nested PCR and the amplicons were hybridized to genotype-specific probes impregnated on membrane strips, and analyzed as described above. The INNO-LiPA HBV assay and direct sequencing both identified 63, 4, and 13 of the HBV strains as genotype D, genotype A, and mixed genotypes A and D, respectively. The PCR-RFLP-based method was less accurate, identifying all four A genotypes, but only 53 of the 63 D genotypes. In fact, seven HBV strains, previously categorized as genotype D, were identified as indeterminate. The authors concluded that the PCR-RFLP-based method failed to identify some genotype D HBV strains and it was therefore a less useful genotyping method as compared to the other assays. Additionally, since the INNO-LiPA HBV assay is available as a largely complete kit and does not require automated sequencing, it lends itself to use by less sophisticated laboratories.

7.7.1.2.11 PCR Invader Assay

HBV has been successfully genotyped by PCR invader assay. The advantage of this assay is that, when compared to the commonly used HBV genotyping methods (direct sequencing and PCR-RFLP analysis), it is less expensive and less difficult to perform for most routine clinical applications. In this assay the basic mechanism involves two specific oligonucleotide probes that bind to target DNA, forming a tripartite structure that can be recognized and cut by a thermostable structure-specific flap endonuclease or "DNA cleavase" (Fig. 7.4) [141]. The first probe, called the "Invader", is complementary to the 3′ end of the target DNA, but the last

base is a mismatch with the target DNA. The second probe in this assay, the "allele-specific probe", contains the base complementary to the mismatched Invader probe and extends 5′ of this nucleotide with additional non-complementary nucleotides. Once the two probes anneal to the target DNA, they form a three-dimensional structure over the mismatched single nucleotide that can be cut by the DNA cleavase. Commonly the Invader probe is designed to permanently anneal to the target DNA at the assay temperature. The allele-specific probe is designed to have a melting temperature close to the assay temperature and therefore constantly anneal and detach. This allows the DNA cleavase to cut multiple allele-specific probes in succession and up to 3,000 allele-specific probe molecules can be cut/target over a 90-min period [141]. Additionally, a fluorophore is often added to the 5′ end of the allele-specific probe and a quencher placed at the 3′ end to the fluorophore such that DNA cleavase activity separates the two allowing the fluorophore signal to be measured. In tripartite structures where the allele-specific probe does not match the base mismatched by the Invader probe, the DNA cleavase does not cut the tripartite structure. Since the DNA cleavase is highly specific in its activity, doubly mismatched tripartite structures are cut at a very low rate and the Invader assay in highly specific for matched target DNA-allele-specific probe tripartite structures.

Tadokoro et al. [142] used the Invader assay to genotype HBV DNA taken from 505 HBsAg positive serum samples obtained from Toranoman Hospital, Japan. Initially the HBV genotypes were determined by two assays; (1) ELISA assay using an HBV genotyping EIA, and (2) a genotype- specific probe assay using seven oligonucleotides corresponding to sequences in the preS1 region. The viral strains were classified as genotypes A–F by ELISA and genotypes A–G by genotype-specific probe assay.

The genotype-specific Invader probes were designed by analyzing 491 individual complete HBV genomes from the National Center for Biotechnology Information (NCBI) database. The Invader Technology Creator (TWT, Madison WI) was used to design the specific probe/invasive oligonucleotides. The oligonucleotide probes were either labeled with FAM (carboxyfluorescein) or RED (REDmond RED). The HBV DNA for the Invader assay was extracted from 100 μl of serum and the HBV S and core gene sequences were amplified by PCR:

S gene	Sense: 5′-GGTCACYATATTCTTGGGAACAAGAKCTA-3′
	Anti-sense: 5′-CTGACTGCCGATTGGTRGAGGCAG-3′
Core gene	Sense: 5′-TATAAAGAATTTGGAGCTWCTGTGGAGTT-3′
	Anti-sense: 5′-GCGGCGATTGAGAYCTKCKT-3′

The Invader reactions were performed in 384-well plates with a Cleavage XI Invader core reagent kit containing 2 μl purified HBV amplicon. The plates were incubated at 65 °C for 15 min in a DNA thermocycler and the fluorescent intensities were measured from zero time to 15 min. Second fluorescent intensities were measured continuously on a Cytoflour 4000 fluorescence plate reader (Applied Biosystems) for FAM (carboxyfluorescein) (wave-length/bandwidth: excitation, 485/20 nm; emission, 530/25 nm) and RED (REDmond RED) (excitation, 560/20 nm;

emission, 620/40 nm) dyes. The genotype was identified by calculation of fold-over-zero (FOZ) values. Twenty-one sera without HBV-DNA were used to determine the cut-off value of FOZ. The mean plus 5 S.D. of the FOZ value of these sera in each well was calculated to be 1.21–1.51 (L1–12) of FAM-FOZ and 1.20–1.64 (L1–12) of RED-FOZ. Therefore, the cut-off level was set at 2.00. Four HBV genotypes and two subgenotypes (Ae, Ba, Bj, C, D) were also amplified, cloned, and sequenced for use in detection sensitivity assays for single and mixed genotype preparations.

The detection threshold for the PCR Invader assay was about ten HBV copies/reaction. Mixtures of two genotypes could be detected even where the ratio of the two genotypes was 1,000:10. All eight genotypes and four subgenotypes were identified by this assay. Thus the assay had a high sensitivity and specificity. In 168 serum samples with HBV, the ELISA and Invader assays gave identical results in 165 (98.2 %) of the samples, with three of the samples (1.8 %) not agreeing. When the three discordant samples were sequenced, the genotypes were found to be those determined by the Invader assay. Comparison of the genotyping results for the Invader and genotype-specific probe assays were in agreement, with 333 of 343 (97.1 %) samples having identical genotypes. Ten samples (2.9 %) were discrepant and could not be genotyped by the genotype-specific probe assay, but were genotyped by the Invader assay. The authors concluded that this assay is sensitive, accurate, relatively low in cost compared to other genotyping methods, and has a short, 7-hour turn-around time. Thus the Invader assay would be very useful in both the clinical and research settings.

7.7.1.2.12 Serological HBV Genotype Testing

HBV is commonly genotyped by serological methods. Typically antibodies directed towards genotype-specific HBsAg epitopes are employed, with antibodies to one constant determinant and two or more antibodies directed to mutually exclusive HBV HBsAg epitopes. Serological HBV genotyping is often used and it is cost-effective, has a high throughput, and it is easy to perform. Serological assays are also superior to molecular genotyping methods in typing HBV-PCR-negative carriers [127].

7.7.1.2.13 HBV Drug Resistance Assays and Promoter/Precore Mutation Testing

Molecular genetic testing is often performed on HBV to detect the development or presence of drug resistance and promoter/precore mutations. Most of the assays described above have been employed in this type of testing, especially sequencing, which interrogates all the nucleotides examined. In addition, other assays have been developed specifically for these purposes. For example, Innogenetics has developed a commercial INNO-LiPA assay that detects lamivudine, telbivudine, emtricitabine, adefovir, and entecavir resistance-associated HBV mutations [143]. Additionally,

Quest Diagnostics offers both HBV drug resistance and precore mutation testing performed by PCR amplification, followed by sequencing at specific codons [144].

7.7.2 HCV

The WHO estimates that three to four million people are newly infected with HCV every year and about 170 million people, or ~3 % of the world's population, are chronically infected and are therefore at risk for HCV-related cirrhosis and HCC. The highest HCV infections prevalence (>3.5 %) is found in Central and East Asia, North Africa, and the Middle East. Southern Asia, sub-Saharan Africa, and most of South and Central America have a moderate HCV infection prevalence (1.5–3.5 %), while the Asian Pacific and North America have a low prevalence (<1.5 %, [145]). Like HBV, HCV is a significant risk factor for cirrhosis and HCC and globally, it is the leading cause of liver transplantation. In the US HCV is the most common cause of liver disease related death (1–4, 145, 146). HCV is an enveloped, single-stranded positive-sense RNA virus of the *Flaviviridae* family consisting of approximately 9,600 nucleotides with two short untranslated regions at each end and a single open reading frame (ORF) of about 9,000 nucleotides. The HCV ORF, known as the polyprotein, encodes three structural proteins known as core, E1, and E2, and seven non-structural proteins; P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The ORF is initially translated into a large polyprotein that is later cut by cellular and viral proteases into smaller proteins. The virus replicates in hepatocytes and peripheral blood mononuclear cells, by the action of an RNA-dependent RNA polymerase and a negative RNA strand intermediate [147]. HCV has at least six major genotypes that show 30–35 % nucleotide divergence [147–150]. Not surprisingly, each HCV genotype exhibits a unique geographical distribution and response to different HCV directed therapies [150]. Thus optimal HCV treatment regimes requires knowledge of the HCV genotype, as well as of the serial viral titers. It also requires evaluation of livers biopsies and analysis of liver enzymes. In fact multiple patient parameters can effect the clinical course of HCV, including the viral genotype and load, hepatic iron load, gender, alcohol use, ethnicity, length of infection, patient adherence to medication regimens, obesity, and co-infection with HIV [151]. Numerous molecular genetic tests are performed on HCV, including the quantification of viral load and genotyping [152]. Additionally, IL-28B molecular testing is a powerful predictor of a patient's chance of achieving a sustained virologic response following interferon-based HCV therapy [153].

7.7.2.1 HCV Viral Load Quantification

Like HBV load quantification, there are many different molecular genetic tests employed in HCV load quantification. The main uses of HCV load quantification are to establish a prognosis, to evaluate treatment options, to guide possible changes

in treatment, and to consider further treatment(s) [154, 155]. Here we will review the most commonly used molecular techniques. Most of them are similar to those used for HBV load tests, with the important difference that the nucleic acid analyzed in HCV testing is RNA, while for HBV the main nucleic acid tested is DNA [101, 102, 147].

7.7.2.1.1 Reverse Transcriptase PCR for HCV (RT-PCR)

RT-PCR is often used to quantify the HCV serum load and is currently the main HCV load quantification testing method [152]. Kleiber et al. [156] used RT-PCR to quantify serum HCV levels and were able to distinguish therapy responders from nonresponders and responder-relapsers. In their study three HCV RNA-positive samples with viral titers ranging from 4,000 to 1,600,000 IU/ml were used the analyze test precision. The specificity was determined by analyzing a set of 100 HCV negative plasma samples. The ability of the assay to monitor changes in viral load was analyzed by taking serial samples from three individuals being treated with five million units of interferon-α 3 times/week. Different HCV genotypes were generated by transcribing cloned HCV plasmid DNA genotypes 1a, 1b, 2a, 2b, 3a, 4, and 5 into RNA, isolating and quantifying the RNA, and diluting the samples to 100,000 copies/ml. These were serially diluted in HCV negative human plasma.

HCV RNA was isolated from 200 μl serum or plasma. A 250 base pair target sequence located in the highly conserved 5′ untranslated HCV genomic region was amplified. The reverse transcription and amplification steps were performed with a modified version of the *Thermus* species ZO5 DNA polymerase, which under appropriate buffer conditions has both reverse transcriptase and DNA polymerase activities occurring within the same reaction. The reaction was performed with HCV-specific oligonucleotides and fluorescently labeled probe oligonucleotides. The thermocycling parameters were 50 °C for 4 min, 30 min at 61 °C, two PCR cycles of 15 s at 95 °C and 50 s at 58 °C, and 48 PCR cycles of 15 s at 91 °C and 50 s at 58 °C. An ABI Prism 7700 Sequence Detector System measured fluorescent emissions, which increased in direct proportion to the increase of amplified product during the PCR amplification. The amount of HCV RNA was quantified by running a set of five external HCV RNA standards covering a four log concentration range, that were reverse transcribed and amplified in separate reaction run in parallel. The limit of detection for this assay proved to be 100 viral copies/ml. The different viral genotypes tested were all amplified with comparable efficiency with only small variations in amplification efficiency. The addition of significant amounts of nucleic acids from adenovirus types 2, 3, and 7, *Chlamydia trachomatis, coxsackievirus* B1, cytomegalovirus, echovirus 1, hepatitis A, hepatitis B, herpes simplex types 1 and 2, human herpesvirus, HIV-1, Group M subtypes A–E and G and Group O, *P. acnes*, *S. aureus*, *S. epidermis,* and varicella zoster did not result in the assay showing HCV positivity. Last, of the 100 negative plasma samples 96 were negative for HCV and four gave invalid results. Thus this assay showed high specificity. The linear range of the assay was determined to be over a braid 64 and 4,180,000 IU/ml range. Last,

the assay was able to detect changes in HCV load during interferon therapy which paralleled changes in serum ALT, demonstrating that this assay could have value in monitoring patient response to therapy. Last, the test showed high precision as the assay highly repeatable results over a 10-day period where the assay was repeated 180 times. The authors concluded that this test has better sensitivity, a grater dynamic range, and a higher throughput than many other current HCV tests.

Currently many companies offer real-time PCR testing on serum from HCV infected individuals. For example, two real-time PCR tests offered by Roche and Abbott were analyzed by Halfon et al. [157] who concluded that both assays had high sensitivities and large dynamic ranges that were fully adequate for the clinical and therapeutic management of HCV. Other less often used methods of HCV load quantification included branched chain amplification and transcription mediated amplification assays [152].

7.7.2.2 HCV Genotyping Assays

Like HBV, different HCV genotypes typically have different clinical responses to treatment. For example, individuals with HCV genotypes 2 or 3 are three to six times more likely to achieve a sustained virological response to pegylated interferon than is genotype 1 [158, 159]. Unfortunately, data on how to treat HCV genotypes 4, 5, and 6 is scarce, but currently these genotypes are given the same treatment regime as HCV genotype 1 [158–162]. Thus HCV genotype testing plays an important role in predicting the clinical response to therapy. Similar to HBV infections, a sustained virological response for HCV is defined as a 6-month period negative HCV testing following the cessation of anti-viral therapy.

7.7.2.2.1 HCV Genotyping Strip Assays

As for other molecular applications, strip assays typically show high sensitivity and accuracy, and can be performed at a relatively low cost, usually from a commercial kit. Innogenetics provides a commercial kit for HCV genotyping (VERSANT® HCV Genotype 2.0 Products). The strip assay based technology can process up to 48 assays simultaneously and identify HCV genotypes 1–6. For most viral types, subtype information is also obtained. Verbeeck et al. [163] evaluated this assay by analyzing 326 HCV-positive clinical specimens consisting of either serum or EDTA plasma samples with viral loads about 2,000 IU/ml. RNA was collected from these samples using a QIAamp DSP virus kit in combination with a QIAvac 24 Plus vacuum system (Qiagen GmbH, Germany). Each extraction included positive and negative controls from the kit. The 5′ UTR region (NS5b) and the core region of the HCV genome were reverse transcribed and amplified using the kit. This was performed in two steps, with the first step involving reverse transcription and the second PCR activated by high heat with simultaneously denaturation of the reverse transcriptase and activation of the DNA polymerase. The primer sets were biotinylated,

yielding two biotinylated amplicons of 240 and 270 base pairs, corresponding to the 5′ UTR and core HCV viral regions, respectively. The amplicons were hybridized to oligonucleotide probes immobilized on nitrocellulose. Following washing steps the strip are incubated with alkaline phosphatase labeled strepavidin and the hybridized bands are visualized with BCIP/NBT chromogen. The Versant HCV genotype (LiPA) 1.0 assay was used in this study as a reference assay.

Specific genotypes were identified by comparison to the patterns produced by known positive controls. Of the 326 samples analyzed, 93.3 % (304/326) gave interpretable genotyping results, with 2.1 % (7/326) failing to amplify and 4.6 % (15/326) amplifying with uninterpretable results. Of the 313 samples that did yield interpretable results, 99/4 % (311/313) agreed with the reference assay method. The method also showed very high reproducibility. The authors concluded that this genotyping method is accurate, rapid, and reliable, out performing the previous Versant HCV genotype (LiPA) 1.0 assay, and recommended it for large-scale genotyping.

7.7.2.2.2 HCV Sequencing Assays

HCV Genotyping by sequencing is commonly performed and considered the Gold Standard in genotyping assays. Both Sanger and pyrosequencing methods are commonly used [164, 165]. Most studies have demonstrated that sequencing works well and gives close or does give 100 % concordant results with assays such as the LiPA assay above [165]. The main difference between HBC and HCV genotyping by sequencing is that HCV has an RNA genome, usually requiring a reverse transcription step to convert the RNA genome into DNA prior to Sanger or pyrosequencing [164, 165].

7.7.2.2.3 Other HCV Genotyping Methods

HCV has been successfully genotyped with Invader technology, genotype-specific primers in reverse transcription PCR technology, and with the assays described above. Most of them are commercially available [158–166]. Since HCV genotyping methods are very similar to those employed for HBV genotyping, they will not be extensively reviewed here.

IL-28B Testing for HCV

As of 2011, it is estimated that 130–170 million people worldwide are chronically infected with HCV. HCV complications include increased risk of cirrhosis, HCC, and liver failure. PEG-INF-α plus Ribavirin therapy remains a mainstay HCV treatment, despite the fact that fewer than half of patients are able to achieve sustained viral response with this therapy. Moreover, treatment response and HCV clearing capability seem to differ among different ethnic groups infected with the

same HCV genotype, suggesting a genetic predisposition, for some patients, to be more responsive to therapy than others [146–150]. Recent studies have shown that single nucleotide polymorphisms (SNPs) within the IL-28B gene locus are found in HCV infected patients. Patients with a common variant of the gene (rs12979860 CC) are significantly more likely to respond to PEG-IFN-α plus Ribavirin treatment [158–162]. These results have led to a more personalized approach to HCV management, with many medical centers now offering IL-28B testing to predict therapy response.

IL-28B is a member of the type III IFN-gamma cytokines and it is located on chromosome 19. Just like IFN-gamma, IL-28B and other INFs can be triggered by viral infection, inducing antiviral activity through both cellular and humoral immune pathways. IL-28B acts through pathways such as JAK-STAT and NF-kappaB to activate IFN stimulated genes (ISGs) in a viral infection. Successful induction of ISGs in response to IFN treatment is associated with favorable SNPs within the IL-28B gene. The independent effect of different SNPs in IL-28B suggests a major role in the regulation of antiviral activity against HCV [167, 168]. Ge et al. [169] first showed an association between SNPs and sustained virological response (SVR); a variable region of IL-28B (rs12979860) in a non-coding region 4 kb upstream the gene. In a multi-ethnic study of patients affected with HCV genotype 1, patient homozygous for the major allele of rs12979860 (CC) of IL-28B were twice as likely to achieve SVR as patients with even one copy of the minor allele (TT or CC) following combination therapy. Later McCarthy et al. [170] confirmed these findings in another multi-ethnic study involving patients infected with HCC genotypes 1, 2 and 3. Thus, the SNP status of this gene has remained the single strongest predictive factor for SVR.

SVR is affected in several ways by the favorable CC genotype. For instance, these patients show a higher likelihood of clearing the virus spontaneously even without treatment [170]. Differences in the antiviral cytokine response conveyed by IL-28B polymorphisms can also lead to different inflammatory states with the unfavorable genotype leading to higher frequencies of hepatic steatosis and fibrosis [146–150]. Due to its predictive value, IL-28B genotyping is now frequently used as a molecular test for HCV. Methods used to determine SNPs include RFLP, direct sequencing, high-resolution melting, sequencing, denaturing high-performance liquid chromatography, and allele- specific PCR. In most cases these assays have a near 100 % concordance [171–174].

The role of IL-28B in chronic HCV infection is an example of how personalized genotyping can be incorporated into the clinical arena affecting therapeutic decisions. Even with the recent introduction of new direct acting antiviral agents, the test is still useful in predicting treatment outcomes. It's useful in helping predict or control re-infection following liver transplantation [175]. In addition, extensive studies of IL-28B have yielded novel insights into the antiviral response to HCV. For example, the therapeutic role of INF-α is being studied as a less toxic alternative to INF-α therapy. Future studies may provide additional insights into ISG response that may even extend beyond the scope of HCV.

7.8 Direct HCC Molecular Tests

Presently there are relatively few molecular diagnostics tests that are performed directly on HCC and most molecular testing is directed towards conditions such as HH, hepatitis B and C, and IL-28B testing which modify an individual's risk for HCC and other liver diseases. The main importance of these tests, as related to HCC, is that the vast majority of HCC arises in the context of chronic liver disease and cirrhosis, which HH, and hepatitis B and C cause. While several direct HCC molecular tests show promise, a few are presently done and the main diagnostic tool for HCC remains a combination of imaging and histological analysis supplemented with immunohistochemistry [28, 175]. Here we will briefly review these tests.

7.8.1 Gene Expression Profiling in HCC

A major cause of morbidity and mortality for individuals with HCCs small enough to be enough to be surgically respected is eventual recurrence, which happens in most patients [176, 177]. Histological parameters are useful in predicting recurrence, including tumor multinodularity and vascular invasion [176]. Hoshida et al. [30] used gene-expression profiling to identify gene expression patterns associated with HCC recurrence. Initially, tissue samples from 106 patients with primary HCC were collected and the patients' clinical outcomes were followed over period of 7.8 years. Later a "validation set" of 234 HCCs from different patients were also obtained. The formalin-fixed, paraffin-embedded tumor samples were cut into 10 μM thick sections, after being macrodissected to isolate the HCC and adjacent benign liver, and were subjected to RNA extraction. The gene profiling was done by DNA-mediated annealing, extension, and ligation assay hybridized to a 6,100 transcriptionally informative gene array. Genes whose expression was associated with disease-specific survival and recurrence time were selected via Cox score analysis. One hundred and eighty six genes were identified that correlated with recurrence. Genes associated with a better prognosis were usually related to normal liver function and to drug metabolism, while those associated with a poor prognosis were involved in inflammation, interferon signaling, NF-κB, and TNF-α. The downstream targets of IL-6 were especially associated with a poor prognosis. These results were applied to 225 HCC tumor samples from the US and Europe and, despite a modest follow-up period (2.2 years), a significant difference in survival was seen that strongly correlated with the expression pattern of the 186 previously identified genes. Although this assay would be difficult to apply to a clinical setting, in the future simpler strip assays may be a useful prognostic test for HCC recurrence.

Similarly, several different studies have used gene expression profiling and similar techniques to demonstrate differences between hepatic dysplastic nodules and early HCC [40, 178–180]. Llovet et al. [180] identified 12 genes that were significantly differently expressed between hepatic dysplastic nodules and early HCC.

These genes included TETm GPC3, gankyrin, survivin, TOP2A, LYVE1, E-cadherin, IGFBP3, PDGFRA, TGFA, cyclin D1, and HGF. Analysis of these genes allowed a 94 % discriminative accuracy. Interestingly, GPC3 immunostaining was found to be 100 % expressed in HHC and not at all in dysplastic nodules.

7.8.2 MicroRNA Testing in Hepatocellular Carcinoma

miRNAs are major regulators of gene expression and are associated with HCC and other liver diseases. They are evolutionarily conserved, endogenously expressed non-coding ssRNA sequences usually 20–24 nucleotides long. They regulate the integrity and translational stability of up to 500 different mRNAs, enabling them to often control entire transcriptional programs that determine cellular properties and behavior. Interestingly, it has been estimated that 20–80 % of transcribed human genes are in part miRNA regulated. The dysregulated expression level of specific miRNAs has been shown to influence liver disease from fibrosis to HCC development, progression, and metastasis [181].

Recently, several studies have shown dysregulated miRNA expression in HCC compared to benign liver, with some of these miRNAs found to be aberrantly expressed in more than one study. Among the upregulated miRNAs are miR-18, miR-21, miR-221, miR-222, and miR-224. Among the downregulated miRNAs are miR-122, miR-125, miR-130a, miR-150, miR-199 and miR-200 [182]. For example, the liver-specific tumor suppressor miR-122 is significantly downregulated in HCC tissues. It functions by suppressing Cyclin G1 protein expression; a cyclin that promotes cell cycle progression. ADAM17 (a disintegrin and metalloprotease family 17) is involved in metastasis and is also suppressed by miR-122. There are also many mechanisms by which tumor-promoting miRNAs affect target genes. For example, miR-221 suppresses the cyclin-dependent kinase inhibitors CDKN1B/p27 and CDKN1C/p57, shifting hepatocytes from G_1 to S phase and facilitating cell cycle progression [181, 182]. The amount of data gathered on miRNAs and HCC is impressive and specific miRNAs have been associated with HCC differentiation, metastatic potential, recurrence, and patient survival. Interestingly miRNAs are also able to differentiate between HBV and HCV infections [183–188]. Quantitative real-time reverse transcriptase PCR is most commonly used to identify and quantify specific miRNAs in HCC [189, 190]. Other researchers have successfully used microarray analysis to analyze the expression patterns of several dozen miRNAs simultaneously, in HCC [191].

7.8.3 Promoter Methylation in HCC

Like most tumors examined, HCC shows epigenetic changes often involving specific CpG island promoter cytosine hypermethylation, often resulting in lowered expression of tumor suppressor genes [192–196]. Yang et al. [32] examined the

tissue from 62 patients who had undergone resection for HCC with curative intent. Following surgery the patients were seen every 3–6 months and assessed for tumor recurrence by such parameters as serum α-fetoprotein and high-resolution, high-detection CT. Gene expression and methylation profiling were performed on the 62 HCCs and 139 adjacent benign liver samples by initial RNA isolation followed by amplification, labeling with strepavidin-Cy3, and hybridized to an Illumina Human-6 BeadChip (Illumina Inc., CA, USA). The arrays were scanned with an Illumina BeadArray Reader confocal scanner (BeadStation 500GXDW, Illumina, Inc). Gene expression profiles of 48,803 probes from the benign liver and HCC tissue were obtained. DNA was also extracted with the QIAamp DNA Mini Kit and checked for quality by gel electrophoresis. The EZ DNA methylation kit (Zymo Research, Orange, CA, USA) was used for the bisulfite conversion of 2 μg of genomic DNA. Following this treatment and further standard treatments, the DNA was analyzed with the Infinium HumanMethylation27 V1.o BeadChip array from Illumina to genotype bisulfite-converted and unconverted CpG sites of over 14,000 genes. The DNA was biotinylated and assay oligonucleotides were added and hybridized to resuspended DNA. Allele-specific extension and ligation of the hybridized oligonucleotides was performed; fluor-labeled strands were then hybridized to the Illumina HumanMethylation27 BeadChip. Arrays were scanned with an Illumina BeadArray Reader confocal scanner. The chip interrogates 27,578 CpG sites of over 14,000 genes. Within the HCC tissue samples, 12 genes were identified showing Cog site hypermethylation with concomitant lowered mRNA expression and a significant association with HCC recurrence. An additional 25 genes showed CpG site hypomethylation, increased concomitant mRNA expression, and a significant association with HCC recurrence. While encouraging, the author state that their results need further validation, but may eventually have prognostic and diagnostic value.

7.8.4 Specific Genes Dysregulated/Mutated in HCC

Several gene have been identified which are often mutated in HCCs. Some of these gene alterations can be tested in HCC, but only a few are routinely done presently.

p 53 Testing p53 is mutated in over 50 % of human malignancies [197]. p53 is the most commonly mutated tumor suppressor gene in HCC, with 27 % showing p53 mutations (range 0–67 %), and is correlated with tumor grade, progression, survival, and recurrence [198–203]. p53 is analyzed by many different methods, although immunohistochemistry and Sanger sequencing have been the most commonly used [203, 204].

β-catenin β-catenin (CTNNB1) is mutated in approximately 30 %, making it the commonly mutated oncogene in HCC [205]. HCCs with β-catenin mutations tend to show increased chromosomal instability, a well-differentiated histology, and cholestasis [206]. Molecular testing for β-catenin mutations is usually done via DNA isolation followed by PCR amplification of specific exons, followed by sequencing

[205]. β-catenin mutation testing in HCC is predominately performed for research purposes and has few clinical applications.

CK19 CK19 is not normally expressed by benign hepatocytes, although normal bile duct cells express it. CK19 expression is significantly higher in HCC lymph node metastases and its expression is a predictor of early postoperative recurrence due to increased invasiveness. CK19 testing is almost always performed by immunohistochemistry and molecular techniques are seldom used in its analysis [207].

H-Tert H-Tert is widely over expressed in many human malignancies [208]. Yang et al. [209] used real-time quantitative fluorescent PCR to quantify H-Tert in the Plasma samples were collected from 60 HCC patients, 21 patients with hepatitis B virus (HBV) and 29 healthy controls. H-Tert DNA was significantly higher in the plasma of individuals with HCC compared to those with HBV and normal controls. Thus plasma H-Tert DNA may have some value in screening for and detecting HCC.

Metastatic Liver Disease The majority of hepatic malignancies are metastatic to the liver with HCC far less common [210]. Molecular diagnostics performed on malignancies metastatic to the liver are those done for the primary tumor, such as KRAS testing performed on colorectal carcinoma metastatic to the liver [211].

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Chapter 8
Molecular Diagnostics of Pancreatic Cancer

Edward J. Richards, William Kong, Mokenge Malafa, Jin Q. Cheng, and Domenico Coppola

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E.J. Richards • W. Kong • J.Q. Cheng
Department of Molecular Oncology, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

M. Malafa, M.D.
Department of Surgical Oncology, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

Department of Oncological Sciences, University of South Florida College of Medicine, Tampa, USA

D. Coppola, M.D. (✉)
Department of Anatomic Pathology, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

Department of Oncological Sciences, University of South Florida College of Medicine, Tampa, USA

Department of Pathology and Cell Biology, H. Lee Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: Domenico.coppola@moffitt.org

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Abstract Pancreatic cancer is a malignant disease relating to uncontrollable cell growth in the tissues of the pancreas. The vast majority of pancreatic cancers are ductal adenocarcinomas, originating in the epithelial layer of the exocrine pancreas, however some pancreatic cancers do originate in the endocrine compartment. Ductal adenocarcinomas are very aggressive cancers, associating with poor prognosis and late staging upon discovery. One reason for this is that many patients do not present symptoms until late onset of disease. Numerous research efforts have been put forth to earlier diagnose pancreatic cancer. Understanding of pancreatic cancer etiology and identification of associated tumor markers has progressed, however there is a clear need for more specific and sensitive technologies. This chapter aims to address current status of molecular diagnostics in pancreatic cancer.

Keywords Pancreatic cancer • Molecular diagnostics • Tumor markers • KRAS • Micro RNA

Abbreviations

CA-19-19	Carbohydrate antigen 19 9
CA-242	Carbohydrate antigen 242
CEA	Carcinoembryonic antigen
CK7/17/20	Cytokeratin 7/17/20
CTCs	Circulating tumor cells
GWAS	Genome wide association study
IPMNs	Intraductal papillary mucinous neoplasms

MCNs	Mucinous cystic neoplasms
MIC-1	Macrophage inhibitory cytokine 1
miRNA	MicroRNA
MSLN	Mesothelin
mtDNA	mitochondrial DNA
MUC4	Mucin 4
OPN	Osteopontin
PanINs	Pancreatic intraepithelial neoplasias
PDAC	Pancreatic ductal adenocarcinoma
SELDI	Surface enhanced laser desorption and ionization
SNP	Single nucleotide polymorphism
TPS	Tissue polypeptide specific antigen

8.1 Introduction

Pancreatic cancer ranks fourth among cancer related death in the United States. It is estimated that in 2012 there will be 43,920 new pancreatic cancer cases and that 37,390 patients will die of this disease [1]. Pancreatic cancer has a very poor prognosis with a 5-years survival of 6 % when considering tumors of all stages [2]. The majority of pancreatic cancers are ductal adenocarcinomas originating in the epithelial tissue of the exocrine pancreas, responsible for almost 95 % of cases. Though, a small percentage of pancreatic cancers can initiate in the endocrine component of the pancreas. Exocrine pancreatic cancers have a poorer prognosis compared to that of endocrine pancreatic cancers, and 5-year survival is 1 % and 15 % respectively in stage 4 presentation of disease [2]. There are three well characterized precursor lesions believed to progress to pancreatic cancer, and it is well established that such progression occurs through the accumulation of genetic mutations [3].

Pancreatic ductal adenocarcinoma is commonly diagnosed as a high stage tumor and absolute removal by surgery is not always a therapeutic option. In these cases, chemo- or radiation therapy has shown limited benefit [4]. Timely detection and diagnosis is critical to increase survival rate of this disease.

This chapter aims to address pancreatic cancer development and the current state of molecular diagnostics. The understanding in both is vital for appropriately diagnosing a patient at the earliest stage of disease. Numerous serum, pancreatic juice, and tissue markers have been evaluated for their diagnostic utility. Of greatest importance, discussed will be the recent advances in technology that have provided more specific and sensitive assays in molecular diagnosis of pancreatic cancer.

8.2 Precursor Lesions of the Pancreas

Ideally, detection of pancreatic cancer would occur prior to malignant presentation of the disease. Three well defined precursor lesions have been associated with pre-invasive disease with the potential to progress to pancreatic cancer.

8.2.1 Mucinous Cystic Neoplasms (MCNs)

MCNs occur almost exclusively in women and arise in the mucin-producing epithelial layer of cells. MCNs typically occur in the head or tail region of the pancreas and do not directly communicate with the pancreatic ductal system [5, 6]. Their defining diagnostic and histological feature is that the mucinous cystic neoplasm is supported by "ovarian-type" stroma [7]. Patients presenting with these lesions typically are on average 40–50 years of age and 5–10 years younger than those presenting invasive disease [8]. MCNs are graded as low, moderate, or high grade based upon the degree of dysplasia and architectural atypia present. One third of these lesions progress into invasive carcinoma [5, 6].

8.2.2 Intraductal Papillary Mucinous Neoplasms (IPMNs)

IPMNs arise within the epithelium of the main pancreatic duct or corresponding branch. They are mucin producing, and papillary in structure [9]. IPMNs are the most common pancreatic cystic lesion and likely to occur in the head of the pancreas in males between 60 and 70 years of age [10, 11]. It is possible to identify IPMNs using the current imaging technology available because of their large size [12]. Upon diagnosis, IMPNs are subcategorized according to the same metrics used for MCNs, and about 40 % of them progress to invasive neoplasms [13]. Both MCNs and IPMNs, if non-invasive, can be completely cured by surgical resection [14, 15].

8.2.3 Pancreatic Intraepithelial Neoplasias (PanINs)

PanINs are microscopic (<5 mm) lesions that arise in the epithelium of the pancreatic ducts, with prevalence commonly increasing with age [16]. PanINs are columnar to cuboidal cells containing mucin and displaying different degrees of architectural atypia [17]. Classified into grades based on histological analysis, these lesions are pathologically characterized as PanIN-1, PanIN-2, and PanIN-3 [16]. At the molecular level, many of the same genetic alterations seen in invasive carcinoma are also found in PanINs. For this reason, PanINs are closely associated with the genetic progression model towards low-grade neoplasia, high-grade PanIN, and eventually invasive carcinoma [18].

8.3 Molecular Pathogenesis of Pancreatic Cancer

In recent years, the molecular pathways associated with pancreatic cancer tumorigenesis have become more evident through exhaustive research efforts. Four central genetic alterations are commonly associated with malignant disease, yet these alterations can be present at the premalignant stage. Precursor lesions presenting any of these alterations should be considered to have a high risk of progression to cancer.

8.3.1 KRAS2

A member of the GTPase superfamily of proteins responsible for a range of biological functions, KRAS2 is locates at chromosome 12p12.1 and if is frequently mutated in pancreatic cancer. It is estimated that more than 90 % of pancreatic ductal adenocarcinomas harbor an activating point mutation in KRAS2, with the majority of these mutations causing an amino acid substitution at codon 12 or 13 [19, 20]. Constitutive activation derives from this mutation and results in the locking of KRAS2 in a GTP-bound active conformation. In this state KRAS2 generates aberrant signaling to downstream members of the phosphoinositide-3-kinase (PI3K) and RAF-mitogen-activated protein kinase (MAPK) pathways [21]. A small subset of pancreatic cancer without a point mutation in the KRAS2 oncogene can alternatively present with a gain-of-function point mutation in BRAF, a serine/threonine kinase downstream of KRAS2 [22].

8.3.2 p16/CDKN2A

Loss of function of the 16/CDKN2A gene (INK4A) is the most frequently observed tumor suppressor deficiency occurring in almost all pancreatic cancers [23]. Interestingly, selective inactivation of INK4A occurs through a variety of mechanisms: homozygous deletion (40 %), intragenic loss-of-function mutation of one allele and loss of second allele (40 %), and hypermethylation of the gene promoter (20 %) [23, 24]. This gene critically functions as a cyclin-dependent-kinase (CDK) inhibitor at the G1/S checkpoint of cell cycle and division therefore preventing Rb phosphorylation by CDKs and cyclin-CDK complexes [25, 26].

8.3.3 TP53

Loss of TP53 activity is the most common abnormality in human cancer and occurs in more than 50 % of pancreatic cancers. Inactivation of the TP53 tumor suppressor predominantly arises through intragenic loss-of-function mutation of one allele and

loss of second allele [27]. TP53 plays a critical role in cell cycle arrest and the DNA damage response. Importantly, the p53 transcriptional targets 14-3-3 and p21 can no longer be induced for expression by a mutated p53 and fail to function in mediating these biological processes [28, 29].

8.3.4 DPC4/SMAD4

The tumor suppressor deleted-in-pancreatic-carcinoma-4 gene (DPC4 or SMAD4) loses function through homozygous deletion (30 %) or intragenic loss-of-function mutation of one allele and loss of second allele (25 %). This alteration is present in 55 % of pancreatic cancers [30]. SMAD4 signaling occurs through the transforming growth factor-beta (TGF-B) pathway with activation resulting in phosphorylation and nuclear trafficking of SMAD transcription factors and subsequent growth inhibitory effects [31, 32]. Loss of SMAD4 can be found in PanIN-3 lesions, but not in IPMNs and MCNs, and it is almost exclusive to pancreatic ductal adenocarcinoma [33, 34].

8.4 Pancreatic Tumor Markers

Numerous markers are under evaluation for their diagnostic utility in pancreatic cancer. A pancreatic tumor marker is defined as any molecule elevated to a significant degree in the diseased state that can be present in the bodily fluids and tissue. Serum carbohydrate antigen 19 9 (CA-19-9) is currently the only marker accepted for clinical use, and there is a clear need for further candidates.

8.4.1 Serum Markers

8.4.1.1 Carbohydrate Antigen 19–9 (CA-19-9)

CA-19-9 is the sialylated Lewis-a blood group oligosaccharide tumor associated antigen first identified through hybridoma antibodies generated from colorectal carcinoma [35]. It is still accepted as a clinically useful diagnostic marker for pancreatic cancer with some reservations. In 2006 the American Society of Clinical Oncology (ASCO) claimed that CA-19-9 is inadequate for reliable diagnosis when used alone, yet monitoring its levels can be of use in combination with other markers [36]. The National Academy of Clinical Biochemistry (NACB) guidelines reflect similar conclusions for its use in diagnostics, asserting that CA-19-9 can be useful in conjunction with imaging tests, such as computed tomography (CT) or endoscopic ultrasound (EUS) [37].

Nevertheless, CA-19-9 remains the standard to which performance of other candidate serum markers are compared. Two major reviews compiling 46 different studies evaluating CA-19-9 (cutoff 37 U/mL in the majority of studies) in comparison to controls groups have found the overall mean sensitivity to be 81 % and specificity to be 68 %, and the overall median sensitivity to be 79 % and specificity to be 82 % [38, 39].

There are several limitations in using CA-19-9 as a single diagnostic tool in pancreatic cancer. Namely, CA-19-9 has been shown to be elevated in other cancer types and numerous benign conditions such as acute and chronic pancreatitis, cholangitis, hepatic cirrhosis, obstructive jaundice, and choleocystitis [38–41]. Also, it is estimated that 5–10 % of Caucasian population lack the Lewis antigen, a condition in which CA19-9 cannot be detected [40, 42, 43].

8.4.1.2 Carbohydrate Antigen 242 (CA-242)

CA-242 marker was first isolated through immunization of mice with COLO 205, a human colon adenocarcinoma cell line [44]. The suggested antigenic determinant of CA-242 is sialylated carbohydrate structure related to type I chain, a similar but distinct epitope from CA-19-9 [45].

Numerous studies have been performed comparing CA-242 to other pancreatic cancer diagnostic markers including CA-19-9, carbohydrate antigen 50 (CA-50), and carcinoembryonic antigen (CEA) [46–50].

The general consensus remains that CA-242 is comparable in diagnostic performance to CA-19-9, and yet it has not proven to be superior [37]. The diagnostic utility and clinical usefulness of CA-242 is the fact that, in contrast to CA19.9, its expression is independent of Lewis-a secretor status [41].

8.4.1.3 CAM17.1

CAM17.1 antibody is a mucin based marker that detects an epitope of a sialylated blood group antigen, likely type I antigen related [51]. CAM17.1 was first assessed in pancreatic cancer through the development of an enzyme-linked antibody sandwich assay (CAM17.1/WGA) in combination with CA-19-9 assay with satisfactory results. CAM17.1 and CA-19-9 tested independently had comparable sensitivities, 78–77 %, yet CA-19-9 achieved greater specificity (88 % vs. 76 %). Combining the two assays increased sensitivity to 85 %, yet did not increase the specificity originally achieved with CAM17.1 alone [52].

Interestingly, in one study CAM17.1 significantly outscored CA-19-9 in specificity when comparing pancreatic cancer to chronic pancreatitis (90 % vs. 78 %) [53]. Equally remarkable is a large patient cohort comparing 250 patients with (n = 89) and without (n = 165) jaundice where CAM17.1 served to be a better marker in patients without jaundice. In this cohort both sensitivity and specificity increased (85–89 % and 81–94 % respectively) [54].

8.4.1.4 Tissue Polypeptide Specific Antigen (TPS)

TPS is an epitope derived from multiple fragments of soluble cytokeratin 18 and has shown to reflect active tumor growth rather than simple tumor burden providing diagnostic utility [55, 56]. The potential effectiveness of TPS as a useful marker in pancreatic cancer has been debatable and while outperforming CA-19-9 in monitoring palliative treatment response [57], it has not yet reached levels comparable to other diagnostic serum markers [58–60]. However, it is possible that these differences may likely be due to differential cutoffs and non-uniform study design.

More recently it has been shown that increasing the cutoff value of TPS could prove useful in discriminating pancreatic cancer versus chronic pancreatitis. When TPS (>100 U/L) and CA-19-9 (>37 U/mL) were assessed in the preoperative serum of pancreatic carcinoma patients, 100 % (46/46) patients had elevated levels of TPS compared to 70 % CA-19-9. In the same study, TPS and CA-19-9 were elevated in 22 % and 19 % of patients with chronic pancreatitis respectively. Further increasing the cutoff to 200 U/L significantly raised both sensitivity and specificity, facilitating almost complete discrimination between patient groups [53, 61].

8.4.1.5 Tissue Inhibitor of Matrix Metalloproteinase 1 (TIMP-1)

TIMP-1 gene is located at chromosome Xp11.3–p11.23 and encodes a protein capable of inhibiting the matrix metalloproteinase (MMP) family of enzymes that degrade the extracellular matrix. TIMP-1 RNA expression levels are elevated in both pancreatic tumor tissue and serum of pancreatic cancer patients [62].

In one study, TIMP-1 was significantly higher in pancreatic cancer patient sera compared to normal controls, with no correlation established between resectable versus unresectable groups. This may suggest elevated serum TIMP-1 as an early event in pancreatic tumor progression, and thus may carry diagnostic potential. When used as a single diagnostic marker, TIMP-1 did not meet the same standard as CA-19-9 in specificity, yet by combining measurements of TIMP-1, CEA, and CA-19-9, 100 % specificity was achieved [62].

8.4.1.6 Osteopontin (OPN)

OPN locates at chromosome 4q22.1 and encodes for an adhesive glycoprotein of ~44 kDa identified as a sialoprotein in bone [63]. OPN is secreted into most of the bodily fluids and is produced in osteoblasts, macrophages, smooth muscle, and other epithelia [64]. It has become clear that OPN aids in tumor progression and increases metastatic potential specifically through facilitating anchorage independent growth in transformed cells, increasing migration and invasion potential, and activating matrix metalloproteinases [65–67].

Numerous malignancies have demonstrated serum levels of OPN to be elevated, including colon, prostate, breast, and ovarian cancer, implicating its potential use as

a biomarker in cancer [68, 69]. Global gene expression profiling technology first identified elevated levels of OPN in pancreatic cancer in 2002 [70]. Through the development of an ELISA based assay in initial studies, OPN demonstrated better performance in sensitivity (80 %) and specificity (97 %) as compared to CA-19-9 (sensitivity 62 % and specificity 79 %) [71].

8.4.1.7 Macrophage Inhibitory Cytokine 1 (MIC-1)

MIC-1 locates at 19p13.11 and is a divergent member of the transforming growth factor-beta (TGF-B) superfamily identified for its role inhibiting macrophage activation [72]. MIC-1 goes by several other names including growth differentiation factor 15 (GDF15), placental bone morphogenic protein (PLAB), placental transforming growth factor (PTGF-B), prostate derived factor (PDF), and non-steroidal anti-inflammatory drug-activated protein 1 (NAG-1). The MIC-1 gene product encodes a precursor peptide that is cleaved into a ~12 kDa mature form containing 7 conserved cysteine residues at the carboxyl-terminal and is secreted as a 25 kDa homodimer [72].

It has been shown that MIC-1 can perform adequately in comparison to CA-19-9 when used as a single marker in distinguishing pancreatic and periampullary adenocarcinomas from precursor lesions of the periampullary region. When combined, even greater diagnostic accuracy was achieved [73]. This same study demonstrated through a variety of molecular techniques that MIC-1 is significantly overexpressed in the cytoplasm of pancreatic tumor cells. MIC-1 levels in the serum were much lower for IPMNs and noncancerous neoplastic pancreatic cysts compared to invasive adenocarcinomas. This findings suggest that MIC-1 could be a useful marker to discriminate between these entities [73]. However, MIC-1 had limitations in differentiating between chronic pancreatitis and pancreatic adenocarcinoma and CA-19-9 still serves as a more specific marker in this regard [74]. Also, MIC-1 has been shown to be elevated in several other diseased states [75–77].

8.4.2 Tissue Markers

8.4.2.1 Cytokeratins (CK7, CK17, and CK20)

Cytokeratins, as the name implies, are cytoplasmic keratin polypeptides of intermediate size that function to provide scaffolding and structural support in epithelial cells [78]. Previously, epithelial carcinomas of unknown origin have been differentially diagnosed through the use of differential expression patterns of CK7 and CK20 [79, 80].

Two independent reports have shown similar differential CK7/CK20 expression profiles in assessing pancreatic adenocarcinomas. The results indicate that pancreatic adenocarcinomas are positive for both (CK7+/CK20+) in 65–62 % of cases.

Positivity for only CK7 (CK7+/CK20-)was seen between 26 % and 30 % of cases. The other cytokeratin profiles (CK7-/CK20+ and CK7-/CK20-), in both studies, were seen in less than 10 % of all cases [81, 82].

A later study comparing pancreatic carcinomas with nearby ampulla of Vater carcinomas demonstrated that through the use of another marker, CK17, adenocarcinomas of pancreatic origin can more readily be distinguished [83].

8.4.2.2 Mucins (MUC4)

Mucins are densely glycosylated proteins of the epithelia. These high molecular weight molecules are secreted in a gel-like form, which among other functions, facilitates their function as a protective barrier in the mucosal system. Two initial investigations of mucin expression, one using in situ hybridization and the another using immunohistochemistry, were consistent in reporting that mucin-1 (MUC1) was the predominant apomucin expressed in the normal pancreas [84, 85].

In comparing normal pancreas to pancreatic cancer using more sensitive techniques, like Northern blotting and RT-PCR, it was found that MUC4 was expressed in 75 % of pancreatic tumor tissue and 73 % of tumor cell lines, yet it was undetectable in normal pancreas and chronic pancreatitis [86]. Immunohistochemical analysis demonstrated up-regulation of MUC4 throughout pancreatic intraepithelial neoplasia progression (PanIN-1 [17 %], PanIN-2 [36 %], PanIN-3 [85 %]) that continued to elevate into invasive adenocarcinoma (89 %) [87]. This report recapitulates a progressive model for pancreatic cancer development.

8.4.2.3 Carcinoembryonic Antigen (CEA)

CEA is an anchored cell surface glycoprotein produced and utilized for cell adhesion in the developing fetus. Its expression is halted prior to birth. The fact of not being normally present in tissue and blood of healthy adults gives CEA diagnostic potential. Immunohistochemical analysis of tissue sections using dual antibody interrogation in one study demonstrated that pancreatic adenocarcinoma stained positive for CEA in 70–77 % of cases. Within this group, moderately and well differentiated tumors had a significantly greater proportion of positive cells. Normal pancreas was negative for detection using either antibody [88].

A later study using an indirect immunoperoxidase technique detected CEA in 20 of 22 pancreatic ductal adenocarcinomas. In this case, CEA detection was specific for the luminal membrane or cytoplasm of the malignant ducts, yet the surrounding normal pancreas was negative [89].

The clinical usefulness of CEA as a serum marker in pancreatic cancer diagnosis has been debatable. Studies have shown that CEA in combination with other serum markers gives limited benefit, and has proved inferior to CA-19-9 when used alone [59, 90].

8.4.2.4 Mesothelin (MSLN)

MSLN, located on chromosome 16p13.3, encodes a 69 kDa precursor that is proteolytically processed into two distinct protein products, megakaryocyte potentiating factor (MPF) and MSLN [91, 92]. MSLN results from the 40 kDa c-terminal fragment that potentially functions as a cell adhesion molecule via glycosylphosphotidylinositol (GPI) anchoring. Constitutive expression of MSLN has been confined to mesothelial cells in normal tissue of the pleura, peritoneum, and pericardium, with the potential to be overexpressed in solid tumor malignancy [91, 93].

Serial analysis of gene expression (SAGE) identified the MSLN transcript to be over-expressed in pancreatic adenocarcinoma in comparison to normal pancreas [94]. More in depth analysis combining RTqPCR, in situ hybridization, and immunohistochemistry techniques supported that MSLN is over-expressed in the vast majority of pancreatic adenocarcinomas at both RNA and protein levels [95]. Further analysis has revealed MSLN as a reliable discriminatory marker to differentiate pancreatic adenocarcinomas from both normal pancreas and chronic pancreatitis [96].

8.4.3 Pancreatic Juice Markers

8.4.3.1 DNA Methylation Alterations

It is common observation in human cancer to detect hypermethylation of CpG islands within particular gene promoters through methylation specific PCR analysis (MSP) [97]. In one study, pancreatic juice from 155 patients suspected to have pancreatic disease was collected surgically or endoscopically and analyzed using conventional MSP. Methylation in promoter regions was detected in 71.3 % of the 13-gene panel in patients confirmed to have pancreatic cancer, in comparison to chronic pancreatitis (26.7 %) and normal controls (10.9 %). Using the six most promising markers within this panel (Cyclin D2, FOXE1, NPTX2, ppENK, p16, and TFP12), quantitative MSP was able to detect pancreatic cancer with 82 % sensitivity and 100 % specificity [98].

In another study, global gene expression analysis was performed following treatment with a DNA demethylating agent to determine candidate genes that became preferentially upregulated. Three candidate genes (NPTX2, SARP2, and CLDN5) were selected and assessed for methylation status in pancreatic cancer and pancreatic juice in comparison to normal epithelia. Of these three genes, aberrant methylation of at least one was detected in 100 % of primary pancreatic carcinoma and in 75 % of pancreatic juice in known cases of cancer [99]. The results suggest DNA methylation signatures can help discriminate in the molecular diagnosis of pancreatic cancer.

8.4.3.2 KRAS Mutations in DNA

It has been well documented that KRAS point mutations occur as an early event in pancreatic tumorigenesis including precursory benign states, and therefore its identification could be of significant value in prevention and diagnostics [100–102]. A study including three groups, no pancreatic disease, benign pancreatic disease, and pancreatic cancer, assessed the presence of KRAS mutations in the pancreatic juice. Analysis using PCR mediated restriction length polymorphism (PCR/RFLP) demonstrated 0 % KRAS mutations in the groups with no pancreatic disease and benign pancreatic conditions. Yet, KRAS mutations were detected in 77 % of patients known to have malignant pancreatic cancer [103].

Another similar study found KRAS mutant alleles in the pancreatic juice via PCR/RFLP at a rate of 87.8 % of pancreatic cancer patients in comparison to 23.9 % of benign pancreatic disease. This study also compared diagnostic usefulness of detecting mutant KRAS to mutant p53 in the pancreatic juice, with KRAS significantly outperforming the latter [104].

Recently, newer technology has been developed to detect and quantify single nucleotide differences in a more sensitive manner. The LigAmp Assay, which utilizes a two-oligonucleotide interrogation system and qPCR step, has been applied to identify KRAS2 mutations in the pancreatic juice. Follow-up studies are needed, however the assay has shown to accurately quantify levels of KRAS2 mutant DNA which may prove effective in aiding early diagnosis given the fact that progressive mutation events are associated with disease progression [105].

8.5 Advances in Molecular Diagnostics of Pancreatic Cancer

In the past decade many resources and efforts have been put forth to developing better molecular based assays to detect cancer at earlier stages through more sensitive means. Despite the fact that no kits are currently commercially available for the molecular diagnosis of pancreatic cancer, the rate at which this research progresses gives a clear hint that this trend will end soon. Below is a summary of the current assays being developed likely to be soon available for use in the clinical arena in the near future.

8.5.1 MicroRNA (miRNA) Expression Profiling

MicroRNAs (miRNAs) are evolutionarily conserved short (~22 nt) non-coding RNA molecules that directly affect biological function through inhibition of target messenger RNA (mRNA) [106]. Cleavage of a precursor and trafficking of miRNA from the nucleus to the cytoplasm has previously been described in detail [107–109].

After processing, a mature form, capable of perfect or imperfect base pairing at the seed sites on mRNA, results in degradation of the transcript via the RNA induced silencing complex (RISC) or inhibition of translation through interaction with the 3′ untranslated region (3′ UTR) [110, 111]. A major attraction to miRNAs is that they are stable and can be quantified by means of real-time PCR using limited starting material required.

Initial investigations of miRNA expression profiles (miRNome) compared normal pancreas (n = 5), chronic pancreatitis (n = 6), pancreatic ductal adenocarinoma (n = 8), and pancreatic cancer cell lines (n = 6), to a reference set of 33 human tissues. Pancreatic tissue was characterized by miR-216 and miR-217 expression, and lacking miR133a expression. Twenty-six miRNAs were identified as being dysregulated in pancreatic cancer, with miR-217 and miR-196a levels sufficient to discriminate between normal pancreas, chronic pancreatitis, and other cancer tissues [112].

A similar study comparing ductal adenocarcinoma (n = 65) and adjacent normal pancreas (n = 65) identified 21 miRNAs up-regulated and 4 miRNAs down-regulated in pancreatic cancer. When comparing the same adenocarcinoma samples to chronic pancreatitis (n = 42), 15 miRNAs were found to be overexpressed while 8 miRNAs were down regulated. Notably, miR-21 and miR-155 were preferentially elevated and able to distinguish pancreatic ductal adenocarcinoma from normal pancreas and chronic pancreatitis [113]. However, further studies in pancreatic cancer miR expression profiling are needed, before specific miRNAs can be used as potential diagnostic markers in the clinic.

8.5.2 Cancer Genome Sequencing

Cancer can be classified as disease that manifests itself at the genetic level. Inherently, whole genome analysis and sequencing is a critical step in understanding the pathogenesis and progression of disease. Recent advances in massively parallel sequencing have made it possible to not only sequence the cancer genome for novel mutations [114], but also consider somatically acquired rearrangements in the context of cancer genomic instability [115]. The challenge that now faces researchers is making sense of this large amount of data, and will require efforts across multiple disciplines.

The pancreatic cancer genome was sequenced in 2008 and was analyzed for novel somatic mutations and deletions. In this study, a pattern emerged in which 12 major signaling pathways were consistently altered (ranging from 67 % to 100 % in frequency) in a given pancreatic tumor. The pathway playing the major role in driving tumorigenesis and tumor progression varied from case to case. This study has provided tremendous insight into our understanding of driver and passenger mutations in pancreatic tumorigenesis and cancer progression, and has contributed to a new perspective of personalized medicine and cancer prevention [116].

8.5.3 Mitochondrial Genome Analysis (mtDNA)

The human mitochondrial genome (mtDNA) is ~16.5 kb that encode the proteins and RNA elements essential for the organelle's processes and function. Due to its limited size, identifying mutations and copy number variation in mtDNA could prove valuable diagnostically because of the quick turn around in mitochondrial genome sequencing.

In one study, 15 pancreatic cancer cell lines (10) and xenografts (5) were analyzed for somatic mutation and copy number variation. Among the cell lines, 49 homoplasmic variations were identified that had not seen previously reported. Southern blot analysis demonstrated that mtDNA was amplified 6–7.7-fold in comparison to normal duodenal tissue in two patients [117].

It has been shown that mtDNA can be mutated and contain amplified gene copy number in response to damage through oxidative stress caused by smoking [118]. A recent study has linked higher mtDNA copy gene number and a significantly increased risk of developing pancreatic cancer in a cohort of male smokers [119]. These results suggest that mtDNA has potential for diagnostic utility for pancreatic cancer in certain populations.

8.5.4 Genome-Wide Association Study (GWAS) and Single Nucleotide Polymorphisms (SNPs)

In addition to the familial and heritable risk factors described above, newer techniques and analyses are critical for identifying a high risk population to target for the prevention and early detection of pancreatic cancer. GWASs and the identification of particular SNPs have demonstrated to be useful in regards to pancreatic cancer susceptibility.

In a two-stage GWAS initially comparing individuals with pancreatic cancer (n = 1,896) to healthy controls (n = 1,939) adjusted for age, sex, ancestry, and five principle components of population stratification, 558,542 SNPs were genotyped and analyzed. SNPs from three distinct genomic regions were fast tracked for replication, resulting in a strong association for locus rs505922 on chromosome 9q34 and pancreatic cancer [120]. This SNP, residing in intron 1 of the ABO blood group gene, is consistent with previous findings that A and B blood types are at higher risk for developing gastric and pancreatic cancer than blood type O [121, 122].

A complementary GWAS study was performed adding approximately 2,000 cases per affected and control cohorts above in order to identify additional susceptibility SNPs. Eight novel SNPs were identified within three genomic loci (13q22.1, 1q32.1, and 5p15.33) to be associated with common susceptibility in the development of pancreatic cancer [123].

8.5.5 *Microsatellite Marker Loss of Heterozygosity and K-Ras Mutation*

Current diagnostic modalities for pancreaticobiliary cancer (cholangiocarcinoma), or cancer located in the bile duct, requires endoscopic retrograde cholangiography to obtain brushings of cells for pathological evaluation. The process yields a very low diagnostic sensitivity (<60 %) due to the fact that determining malignancy is objective and based on cellularity and morphology [124–127].

As in most pancreatic cancers, numerous tumor suppressor genes are frequently deleted in pancreaticobiliary cancer. In one study, loss of heterozygosity (LOH) of a panel of 12 microsatellite markers linked to six common tumor suppressor genes (RIZ, VHL, APC, p16/CDKN2A, PTEN, TP53) was assessed via PCR in combination with KRAS point mutation at codon 12 for diagnostic utility in brush cytology specimens. Through traditional cytomorphological analysis in 17 patients comparing diseased tissue to a normal adjacent control, 8 were deemed malignant, 10 were indeterminate, and 8 were benign. Tissue categorized as malignant harbored numerous LOH with varying heterogeneity, likely due to intratumoral clonal expansion. All of these samples also contained KRAS mutation, with adjacent normal tissue and benign samples having no LOH. This study, although limited in sample size, proved his technique to have 100 % sensitivity, specificity, and accuracy [128].

A similar methodology was also applied for more accurate diagnosis when using routine endoscopic ultrasound guided fine needle aspiration (EUS-FNA). This procedure sometimes results in unnecessary surgery for patients with benign pancreatic conditions due to inconclusive cytology assessment. In comparing six known cases of benign disease to 15 cases of malignancy, a panel of 16 LOH microsatellite markers (located at 1p, 3p, 5q, 9p, 9q, 10q, 17p, 17q, 21q, and 22q) combined with presence of KRAS point mutation was able to reliably discriminate between masses using EUS-FNA samples [129].

RedPath Integrated Pathology, Inc., a leader in molecular diagnostics, presented results of a multi-institutional prospective study from 492 patient outcomes on integrated molecular pathology (IMP) at the recently held 2013 ACG Annual Meeting in San Diego, CA. Final data results validate the company's *PathFinderTG*® pancreas platform's high negative predictive value (NPV = 97 %) for correctly identifying patients at very low risk of developing pancreatic cancer. Patients with low risk *PathFinderTG* diagnoses had a 97 % probability of follow up without malignancy. These low risk patients could be statistically differentiated from those with much higher risk of malignancy. Patients in the 'statistically higher risk' category have a 31-fold increased risk of cancer and those in the 'aggressive' category have a 76-fold increased risk of cancer, added Dr. Smith Redpath also offers similar tests for predicting esophageal cancer in background of Barrett's. Both tests are medicare approved [130].

8.5.6 Surface Enhanced Laser Desorption and Ionization (SELDI)

SELDI ProteinChip Arrays (Ciphergen Biosystems Inc., Fremont, CA) is a novel approach for more sensitive detection and differential profiling of serum proteins. The technique utilizes an energy absorbing matrix in combination with laser to ionize proteins of a given biological sample enabling molecular weight quantification via time-of-flight mass spectrometry [131]. SELDI offers a more high-throughput diagnostic assay for proteomic analysis and offers potential in developing predictive models in cancer. Initial studies have already demonstrated the potential success of SELDI in differentiating ovarian cancer [132, 133], prostate cancer [134–137], breast cancer [138], renal cell carcinoma [139], hepatocellular carcinoma [140] and bladder cancer [141].

Initial reports have identified two discriminatory peaks that could differentiate pancreatic cancer from healthy controls with a sensitivity of 78 % and a specificity of 97 %, proven to be significantly superior to using the CA-19-9 serum marker alone. Combining these two markers with CA-19-9 further improved diagnostic accuracy. SELDI profiling was again superior to CA-19-9 when differentiating pancreatic cancer from pancreatitis [142].

Another study combined SELDI with an artificial intelligence classification algorithm for serum screening. By using a panel of six peaks, this classification and algorithm was able to discriminate pancreatic cancer from controls with 88.9 % sensitivity and 74.1 % specificity. This methodology was challenged in a double blind test, results showed a sensitivity of 80 % and specificity of 84.6 % in differentiating pancreatic cancer from controls. Impressively, in 39 of the 47 of these cases, this technique was successful in determining patients with surgically resectable disease [143].

8.5.7 Circulating Tumor Cells (CTCs)

CTCs are malignant cells found in the peripheral blood that have detached from primary tumor site and have metastatic potential. Detecting CTCs in the context of prognosis has already proven useful, yet little is known about its potential in diagnostics [144]. Initial studies in pancreatic cancer patients found 11 of 26 (42 %) patients to be positive for CTC (1–105 cells/7.5 ml). None of the patients with chronic pancreatitis and healthy volunteers (100 %) had CTCs using CellSearch (Veridex, LLC, Raritan, NJ, USA). The presence of CTCs was significantly linked to poorer prognosis [145].

Efforts have been put forth to apply CTC detection for use in diagnostics as potential biomarkers. Two platforms, CellSearch (mentioned above) and isolation by size of epithelial tumor cells (ISET) (RareCell Diagnostics, Paris, FR), were used to compare blood samples from 54 patients. A major difference between the assays is that CellSearch captures cells expressing epithelial markers like EpCAM, and ISET is marker-independent and based on cell size and morphology. Similarly to the report above, CellSearch was able to detect CTCs in 40 % of patients, but was outperformed by ISET which detected CTCs in 93 % of cases. It is possible to

account for this dramatic difference by the level of heterogeneity in epithelial markers expressed, as well as not detecting cells which may have undergone the epithelial to mesenchymal transition (EMT) [146].

It is evident that additional evaluation is needed before considering CTCs as a diagnostic tool, yet clear potential exists.

8.5.8 DNA Methylation Profiling

Epigenetic alterations and aberrant methylation of cytosine at CpG motifs are partly responsible for differential gene expression in cancer, and can be exploited in tumor profiling [147–149]. These modifications are critical events in tumorigenesis in the context of proto-oncogene and tumor suppressor gene promoters [150].

Global profiling of normal pancreas, pancreatic carcinoma, and pancreatic cancer cell lines was assessed for 807 gene promoters. The status of 289 markers were shown to be altered, and novel candidate genes for hypo- (23) and hypermethylation (35) were identified as potent oncogenes and tumor suppressors [151]. Notably, previously published markers specific to pancreatic cancer such as CCND2 and 14-3-3 also displayed an irregular methylation status in this study [152, 99].

8.6 Conclusions

Pancreatic cancer has a very poor survival due to diagnosis corresponding with later onset of disease. Clearly defined precursor lesions and the progressive molecular pathogenesis associated with pancreatic disease has provided a window of opportunity to detect pancreatic cancer prior to its development or at the earliest stage of malignancy. Over the past decade, there have been significant advancements in tumor marker identification and evaluation, yet it is clear that the majority of these markers have limitations. Recent advancements in technology and molecular diagnostics have put forth some promising alternatives with higher sensitivities and specificities. Despite the fact that commercial availability of specific diagnostic assays has yet to be approved for use in the clinic, it is evident that they will be available soon.

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Chapter 9
Molecular Pathology and Diagnostics of Prostate Cancer

Rodney E. Shackelford, Janese Trimaldi, Philippe E. Spiess, Wade J. Sexton, Maisoun Qassim Abdelbaqi, and Shang-Tian Chuang

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R.E. Shackelford
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, SL-79, New Orleans, LA 70112, USA

J. Trimaldi
Department of Pathology, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

P.E. Spiess • W.J. Sexton
Department of Genitourinary Oncology, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

M.Q. Abdelbaqi
Department of Pathology, LSU Health Sciences Center Shreveport, 1501 Kings Highway, Shreveport, LA 71130, USA

S.-T. Chuang (✉)
Pathology, East Los Angeles Doctors Hospital, 4060 Whittier Blvd, Los Angeles, CA 90023, USA

4060 Whittier Blvd., Los Angeles, CA 90023-2523, USA
e-mail: tiansmail@yahoo.com

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Abstract Prostate cancer (PCa) is the most common malignancy among men and is the second leading cause of cancer-related deaths in the United States and Europe. The molecular alterations underlying PCa have recently been shown to be quite complex, involving many different genes, microRNA species, epigenetic and mitochondrial changes, and multiple gene translocations. Because of the highly complex molecular changes underlying this malignancy, PCa molecular diagnostics are exceptionally complex and have only recently led to useful molecular tests. Here, we will review several promising areas in the molecular diagnostics of PCa and try to indicate which ones may have clinical utility in the near future.

Keywords Prostate cancer • Translocation • PSA • Molecular diagnostics • Mitochondria • Prostatic intraepithelial neoplasia • Benign prostatic hypertrophy • Environmental risk factors • Genetic risk factors

Abbreviations

AMACR	Alpha-methylacyl-CoA racemase
BPH	Benign prostatic hypertrophy
BPSA	Benign prostate-specific antigen

COPA	Cancer outlier profile analysis
CTC	Circulating tumor cells
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
ETS	Erythroblast transformation-specific
FISH	Fluorescent in situ hybridization
fPSA	Free PSA
GOLPH2	Golgi Phosphoprotein 2
GST	Glutathione-S-transferase
GSTP1	Glutathione-S-transferase P1
hK2	Human glandular kallikrein 2
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MSMB	Microdeminoprotein-ß
PBS	Phosphate-buffered saline
PCa	Prostate cancer
PCA3	Prostate cancer gene 3
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate-specific antigen
RT-PCR	Reverse transcription-polymerase chain reaction
SNP	Single nucleotide polymorphism
uPA	Urokinase plasminogen activator
uPAR	Urokinase plasminogen activator receptor

9.1 Introduction

Prostate cancer (PCa) is the most common cancer in men within the United States (US) and is the second leading cause of cancer deaths in the US and Europe. One in six US men develops PCa at some point in their lives. Each year, 186,320 US men and 345,900 European men are diagnosed with PCa; of these men, 28,660 US and 87,400 European men will die of the disease. Since the incidence of PCa increases with age, the number of men with newly diagnosed PCa will probably continue to rise as life expectancy of the general population increases [1, 2]. The incidence of PCa is much lower in other parts of the world, with Eastern Europe, Asia, and Africa having a significantly lower incidence [3]. Within the US, the age-adjusted incidence of PCa-related mortality peaked in the early 1990s and declined by 4.17 % per year from 1994 to 2004, possibly due to early prostate-specific antigen (PSA) testing and more aggressive treatment of asymptomatic disease [4].

9.2 Environmental Versus Genetic Factors for Prostate Cancer Risk

Multiple environmental and genetic risk factors have been identified that play a role in determining PCa risk. Several studies have implicated diet, chronic inflammation, and obesity in the risk of developing PCa. Obesity confers an increased risk for aggressive PCa. Although poorly understood, the underlying causes may in part relate to the lower testosterone levels and higher levels of estrogens seen in obese men, which might facilitate tumor growth. Additionally, there may be bias against screening obese men for PCa, potentially leading to a delay in diagnosing early-stage disease [5]. A diet high in meat, particularly well-done red meat and highly processed meat, is associated with an increased risk of PCa. Interestingly, the increased risk is likely due to the mutagenic effects of heterocyclic amines, particularly rich in well-done/overcooked red meat, and not due to the effects of high levels of saturated fats found in red meat [6]. Additionally, a diet consisting of less than 35 g of protein/day, eating fish three or more times/week, and high dietary levels of genistein, curcumin, folate, and retinoic acid lowers the risk for PCa [7, 8]. Interestingly, agents such as genistein and curcumin may lower the risk for PCa by altering microRNA expression patterns [8]. Lastly, chronic inflammation plays a role in PCa development, as many factors that increase chronic inflammation/oxidative stress, such as mitochondrial dysfunction, loss of antioxidant defenses, and chronic prostatitis, stimulate cell proliferation in a background of increased genomic instability [9].

Studies of twins and families with strong histories of PCa, genetic studies of single nucleotide polymorphisms (SNPs), and genome-wide linkage studies have demonstrated a genetic component in approximately 42 % of all PCas [10, 11]. Several gene mutations have been implicated in either hereditary or sporadic PCa, including BRCA1 and 2, AR, PALB2, ELAC2, CHEK2, CYP17, CYP1B1, CYP3A4, GSTM1, GSTP1, GSTT1, PON1, SRD5A2, VDR ANXA7, KLF5, NKX3-1, and CDKN1B [12–15]. The relative contribution of each of these genes to PCa varies enormously. For example, BRCA mutations that play an important role in hereditary breast and ovarian cancers only account for 1–2 % of PCas [12, 15]. Similarly, a large number of SNPs have been implicated in PCa risk/susceptibility [14]. Presently, however, there is little molecular diagnostic testing for PCa directed at SNPs or gene mutations associated with increased cancer risk.

9.2.1 PCa Risk and Race

African American men present with PCa at a younger age, have a higher PCa incidence, often present with a more advanced stage, and have a worse clinical course than men of other races. In fact, African American ancestry is a well-recognized PCa risk factor, with African American men often having higher serum PSA and Gleason scores at diagnosis than Caucasian men [13]. Although some of these

differences are due to socioeconomic and lifestyle factors, a significant component of the PCa racial disparity appears to be molecular-genetic factors found in African American men. Many of these factors have been indentified and include higher mean serum testosterone levels (a PCa risk factor), increased androgen receptors in benign prostatic tissue and PCa, variants of 5α-reductase that may convert testosterone to the more active dihydrotestosterone, less active cytochrome P450c17 (an enzyme that inactivates testosterone), increased epidermal growth factor expression, increased *Bcl-2* expression (which exerts anti-apoptosis effects), increased *MDM2* (which suppresses p53 expression promoting genomic instability), and patterns of microRNAs (which may promote PCa) (for review, see [14]).

9.3 PSA Testing

The US Food and Drug Administration approved the serum PSA test in 1986 for monitoring men with PCa. In 1994 the test was approved in conjunction with the digital rectal examination for the detection of PCa [2, 11]. PSA is a member of the Kallikrein gene family, which consists of at least 15 different proteins, all of which are found at 19q13.41. PSA has five exons, is regulated by steroid hormones, and functions as a serine protease [16–18]. PSA testing has been very successful and has led to a dramatic reduction in the incidence of advanced-stage disease, that is, those typically difficult to treat and carrying a poor prognosis [4, 19].

Serum PSA is elevated in benign conditions such as benign prostatic hypertrophy (BPH) and prostatitis, giving the test low specificity, especially in the "gray zone" of a total serum PSA between 4 and 10 ng/mL. In this PSA range, only 25 % of men will have detectable PCa upon biopsy [20]. Additionally, men with a total serum PSA below 4 ng/mL have a PCa risk similar to men with total serum PSA levels between 4 and 10 ng/mL, making clinical decision-making based on PSA values alone often difficult. Attempts to improve the positive predictive value of PSA for PCa detection have included lowering the PSA level threshold for recommending biopsies. The lowering of the PSA threshold has resulted in an increased number of positive PSA screening tests and an increased number of men undergoing prostate biopsies with no definite improvement in the detection of clinically significant PCas [21]. Thus, the sensitivity and specificity of PSA levels in PCa detection are not satisfactory, and others factors, including family history, body mass, race, and age, are usually included in the clinical decision making [20, 21]. Lastly, negative biopsies do not completely rule out the presence of PCa; conversely, many biopsies are performed on patients without PCa or on patients with clinically insignificant indolent tumors that would be better treated with active surveillance and less frequent biopsies. Thus, a major challenge in PCa testing, not met by the PSA test, is the stratification of men into those who need biopsy, those who do not, and those with indolent, clinically insignificant PCa who do not require aggressive local therapy (i.e., radical prostatectomy or radiotherapy) [20–22]. Several variations on serum PSA testing have been developed, which have led to greater clinical utility for PSA testing. Some of these include those listed below.

9.3.1 PSA Density

PSA Density compares the serum PSA value to the size of the prostate. A higher PSA value with a greatly enlarged prostate is far less concerning than an increased PSA with a normal to small sized prostate. One drawback with this test is that a man may have an enlarged prostate that harbors PCa [22].

9.3.2 PSA Velocity

PSA Velocity measures the changes in serum PSA over time. A relatively sudden increase in serum PSA levels may indicate the presence of PCa or a previously indolent PCa becoming more aggressive [22]. For example, Carter et al. [23] demonstrated that a serum PSA increase of over 0.35 ng/mL/year increased the relative risk of death from PCa compared to men with a velocity below 0.35 ng/mL/year. PSA velocity has been demonstrated to be a more accurate way to detect adverse clinical/pathological features rather than measuring the PSA doubling time [24].

9.3.3 Free Versus Bound PSA

This test measures two separate serum PSA forms, one free within the serum and the other attached to a carrier protein, such as serine protease inhibitor 1-antichymotrypsin. The free PSA (fPSA) assay is best utilized in men with serum PSA between 4 and 10 ng/mL. In PCa, a higher percentage of serum PSA is protein-bound compared to that shown with benign prostatic conditions. For instance, men without PCa have a higher likelihood of having a fPSA above 25 %. fPSA values between 10 % and 25 % are not predictive of PCa risk. Finally, men with fPSA values below 10 % have the highest possibility of having a PCa when their serum PSA is within the 4–10 ng/mL range [22, 25, 26].

9.3.4 Benign Prostate-Specific Antigen

Another form of PSA, called benign prostate-specific antigen (BPSA), is derived from the cleavage of fPSA between Lys182 and Ser183. The resulting peptide has a unique, measurable epitope that is found in the prostatic tissue, blood, and seminal fluid. BPSA is localized to the prostatic transitional zone of men with BPH, almost undetectable in healthy men, and increased in men with BPH. Serum BPSA levels correlate strongly with nodular hyperplasia of the prostate, especially with increased transition zone volume. BPSA is a better predictor of prostatic enlargement than serum PSA or free serum PSA and may have use in differentiating BPH from PCa [27–29].

9.4 Molecular-Genetic Testing for Prostate Cancer

Since the introduction of PSA testing in 1986, several other potential biomarkers of PCa have been examined in hopes of increasing PCa detection sensitivity and specificity. Among these markers, only a few have demonstrated clinical value, while others show some promise following further analysis. These include PSA derivatives, microdeminoprotein-β (MSMB), PCa gene 3 (PCA3), *TMPRSS2:ETS* fusions, *SPINK1* overexpression, the t(4;6)(q22;q15) chromosomal translocation, somatic mitochondrial DNA mutation, *C-MYC* amplification, *Bcl-2* overexpression, Kallikrein serum levels, Urokinase Plasminogen Activator activity, *GSTP-1* hypermethylation, alpha-methylacyl-CoA racemase (AMACR) levels, PCa microRNA expression patterns, and SNPs. Here, we will review several of them.

9.4.1 Microdeminoprotein-ß Urine Testing

PSA, prostate acid phosphatase, and microdeminoprotein-ß (MSMB) are the three most abundant proteins found in human semen [25]. MSMB, also known as PSP94 or β-inhibin, is found at 10q11.2 and encodes a cysteine-rich 16-kDa, 94 amino acid protein [30, 31]. MSMB is a strong inhibitor of follicle stimulating hormone. MSMB peptide fragments exert pro-apoptotic effects, inhibit matrix metalloproteinase-9 (MMP-9) secretion, antagonize tumor neovascularization, and inhibit tyrosine kinase pathways downstream of VEGF and PDGF, thus preventing intracellular signaling through the MAPK cascade [32–35]. MSMB is expressed in multiple tissues, including tracheobronchial tract and the cervix, although at levels much lower than are found in the prostate [36, 37].

MSMB expression is low to absent in PCa, but highly expressed in benign prostatic tissue and BPH [38–42]. MSMB loss may be an early event in the PCa development as its expression is lost in prostatic intraepithelial neoplasia (PIN), although some studies refute this finding [40, 42]. Additionally, in pubertal and adult normal prostates MSMB expression is most intense in the review of the peripheral zone, less intense in the transitional zone, and shows variable staining in the central zone. Thus, higher MSMB expression is found at prostatic anatomic sites most likely to develop PCa. Interestingly, no zonal differential expression was identified for PAP or PSA [40–42]. Lastly, MSMB is bound to proteins that in part regulate its bioavailability. One of these proteins, cysteine-rich secretory protein-3, is relatively low in benign prostatic tissue and elevated in PCa, and its expression is an independent predictor of disease recurrence following radical prostatectomy [42]. Taken together, the above data indicate that MSMB exerts tumor suppressor effects on PCa and its loss may have potential as a biomarker for this malignancy.

Two genome-wide association studies have identified a small nucleotide polymorphism, rs10993994 at 10q11.2, that is strongly associated with increased PCa risk ($p = 8.8 \times 10^{-18}$) [43–45]. rs10993994 is 57-bp centromeric of the first MSMB gene exon and has several 2-bp variants. The CT or CC variants express high levels

of MSMB/reporter gene mRNA, while the TT variant expresses only about 13 % of the CT/CC variants, probably due to low CREB transcription factor binding to TT variant promoters [45, 46]. The TT variant occurs in about 30–40 % of European men and 70–80 % of men with African ancestry [47]. The low MSMB expression in the TT variant could lead to less suppression of prostatic epithelial cell growth and increased risk for malignant transformation [45].

9.4.2 Immunohistochemical Analysis of Urinary MSMB for PCa Molecular Diagnostic Testing

Until recently, MSMB has rarely been used in clinical testing, although low to absent levels in PCa have been detected by a variety of techniques, including immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR), and enzyme-linked immunosorbent assay (ELISA) assays [38–42]. Recently, several studies have demonstrated that MSMB testing may have value in analyzing PCa risk, diagnosis, and disease monitoring. For example, Whitaker et al. [48] employed immunohistochemical (sandwich ELISA) analysis of urinary MSMB to detect PCa. The urine of 215 men with no history of PCa and 89 men with PCa was examined. Urine was collected prior to a digital rectal exam, aliquoted, and immediately frozen at −80 °C until needed. Plates for urine MSMB quantification were prepared by incubating 96-well plates overnight at 4 °C with mouse anti-PSP94 antibody at a 1:1,000 dilution in phosphate-buffered saline (PBS). The wells were washed three times between all steps with 0.05 % PBS-Tween. The wells were blocked with 1 % bovine serum albumen for 1 h and 50 μL of sample or standard was added to each well in duplicate and incubated at room temperature for 2 h. Serial dilutions of recombinant MSMB were used as a control and standard curve for MSMB quantification. Goat anti-MSMB was added to each well for 1 h and further incubated with anti-goat horseradish peroxidase-conjugated antibody for an additional 1 h. The amount of bound MSMB was quantified by the addition of 100 μL TMB for 15 min. The reaction was then stopped by acidification, and the absorbance was measured at 450 nm using a Lucy II spectrophotometer. Urinary and serum PSAs were quantified with a commercial kit.

Men with PCa showed a consistent and significant decrease in urinary MSMB compared to men without cancer, which correlated strongly with the rs10993994 TT variant. Urinary MSMB significantly improved upon urinary PSA for PCa diagnosis specificity and sensitivity in this cohort, particularly when the PCa was stratified into low (six and seven) and high (eight and nine) Gleason scores. It did not improve upon serum PSA for specificity and sensitivity. No correlation between urinary MSMB levels and BRCA1 and BRCA2 status was identified. As previously found, MSMB staining was lost in PIN and PCa and was significantly lower in men with the rs10993994 TT variant versus those with the CC variant. MSMB urine protein testing has the following advantages: (1) MSMB urine protein testing does not require digital rectal massage, (2) MSMB expression is not affected by

hormonal status or BPH, and (3) urine MSMB has improved specificity and sensitivity over urine PSA. The authors conclude that urine MSMB may be a useful biomarker for screening for both PCa development and risk, although further studies are required to validate the assay.

9.4.3 Prostate Cancer Gene 3 (PCA3)

Prostate cancer gene 3 (PCA3), also known as "differential display 3," is found at 9q21-22 within intron 6 of the *BMCC1* gene, has four exons, and is transcribed into multiple RNA splice and polyadenylation variants. The PCA3 RNA carries many stop codons and hence appears to produce no detectable protein(s), and thus functions as a non-coding RNA. Interestingly, however, a recent detailed analysis revealed a novel PCA3 isoform with four putative open reading frames from a single ATG initiation site. Although no protein(s) has been found to be coded for by PCA3, some may exist [49–51]. PCA3 was discovered by differential display analysis of normal versus PCa, where it was highly overexpressed in 53 of 56 PCas compared to the same prostate samples taken from non-neoplastic areas. PCA3 expression is prostate-specific and was not detected in normal human artery, brain breast, bladder, colon, heart, duodenum, liver, lung, ovary, pancreas, placenta, seminal vesicles, skeletal muscle, spinal cord, or testis [49]. Although originally thought to be expressed only in PCa, PCA3 is expressed in benign prostate and is on average 66-fold up-regulated in 95 % of PCas (median 158.4×10^5 copies/μg tissue RNA in PCa compared to 2.5×10^5 copies/μg tissue RNA) [52].

9.4.3.1 PCa Molecular Diagnostics and PCA3

Many different molecular diagnostic protocols have been developed for PCA3 analysis. Most employ variations on RT-PCR with simultaneous amplification of control gene(s) products to normalized PCA3 transcript levels and confirm a yield of prostate-specific RNA. PSA is often employed as a normalization control as it is relatively prostate-specific and unlike PCA3 its mRNA levels are not increased in malignant prostatic tissue [51, 52]. Detection of PCA3 in urine is attractive as urine collection is noninvasive and since PCA3 RNA is prostate-specific [49–54], cells and urinary sediments from non-prostatic sources do not interfere with test specificity.

9.4.3.2 PCA3 Quantification in Urine

Hessels et al. [52] employed quantitative RT-PCR to analyze PCA3 in the urine of 108 men with serum PSA above 3 ng/mL. The urine was collected following a thorough rectal examination, cooled on ice to 4 °C, centrifuged at 700 g for 10 min, washed twice with ice-cold PBS, snap frozen in liquid nitrogen, and stored at −70 °C.

Total RNA was later extracted using TRIzol® reagent (Invitrogen). A cDNA was made by heating the purified RNA for 10 min at 68 °C and reverse transcribing the RNA at 37 °C for 1 h with an olido-d$(T)_{18}$ primer. The resulting PCA3 cDNA was PCR amplified with these PCA3-specific primers: forward 5′-TGGGAAGGA CCTGATGATACA-3′ and reverse 5′-CCCAGGGATCTCTGTGCTT-3′. After 35 amplification rounds, the products were quantified by time-resolved fluorescence-based hybridization assay on strepavidin-coated microtitration wells. Target-specific detection was achieved with a PCA3-detection probe labeled with Eu^{3+}. PSA RNA was amplified in a similar manner and detected with a Tb^{3+} labeled detection probe. The amount of PCA3 RNA in the sample was calculated by comparing detected PCA3 to a standard curve, to the PCA3 RNA found in normal prostate, and to the urine PSA RNA levels. Of the 108 men analyzed, 24 had PCa by prostate biopsy, with 84 being negative. Of the men with PCa, 16 were positive by PCA3 testing, giving a sensitivity of 67 % and a negative predictor value of 90 %. The authors concluded that PCA3 testing could have value in urine analysis for PCa and could reduce the number of unnecessary biopsies. However, a larger multi-center study should be performed to further analyze the usefulness of urine PCA3 in detecting PCa.

9.4.3.3 Transcription-Mediated Amplification Platform for Urine PCA3 Analysis

One major drawback of urine based RT-PCR for PCA3 analysis is that this method is too time-consuming and insensitive to lend itself to routine clinical molecular diagnostic testing. Up to ~20 % of all clinical specimens contained insufficient prostate cells for testing, thus giving an unacceptably low informative rate of ~80 % [52]. Groskope et al. [55] employed a transcription-mediated amplification (TMA) system combined with mRNA capture to achieve an informative rate of 98.2 %. Two hundred μL of urine and urine transport medium were initially used to facilitate RNA capture and increase RNA stability. The specimens were vortexed and incubated at 62 °C with magnetic particles with attached PSA or PCA3 RNA target-specific oligonucleotides. After 30 min, the magnetic particles with attached RNAs were placed on a Gen-Probe Target Capture System for 10 min to allow the magnetic particles to migrate to the sides of the tubes. The fluid was removed, the tubes were washed with 1 mL buffer, and the process was repeated once more for complete sample purification. Seventy-five μL of buffer and primers were added to the beads, and the samples were heated to 62 °C for 10 min. Twenty-five μL of enzyme reagents were added to each tube, and the TMA reaction was allowed to proceed for 1 h at 42 °C.

TMA amplifies nucleic acids isothermally and employs two primers and two enzymes: RNA polymerase and reverse transcriptase. One of the primers contains a promoter for RNA polymerase. Amplification is initiated when this primer hybridizes to the purified RNA at a defined site and the reverse transcriptase creates an dsRNA:DNA hybrid, which is rapidly degraded to ssDNA by the RNase activity of

the reverse transcriptase. The second primer then binds the ssDNA, and the reverse transcriptase polymerizes a dsDNA. The RNA polymerase attaches to the DNA promoter sequence and initiates many rounds of transcription. The resulting RNA amplicons reenter the TMA cycle for new replication rounds. Up to one billion copies of the original RNA can be produced within 1 h. The amplicons were detected with specific gene probes in a hybridization protection assay employing a chemiluminescence detection format. All samples were run in triplicate.

To examine the clinical utility of this test, three groups were tested: (1) 70 men with serum PSAs ≥2.5 ng/mL and/or abnormal digital rectal exams, (2) 52 men under 45 years who had no PCa risk factors, and (3) 21 men who had undergone previous prostatectomies at least 3 months before their urine was analyzed. Additionally, the urine of six women was used as a negative control. Of the 70 men with elevated serum PSA, 68 yielded informative specimens. Of these, 16 were PCa positive by biopsy; of the remaining 52, 40 had BPH and/or inflammation. PCA3 analysis of this group gave a specificity of 67 % and a sensitivity of 79 %, while serum PSA yielded a specificity of 28 % and sensitivity of 81 %. The calculated media ratios ($PCA3/PSA \times 10^{-3}$) for healthy, biopsy negative, and biopsy positive groups were 4.5, 27.0, and 81.8, respectively, with the mean PCA3/PCA ratios of the three groups significantly different ($p < 0.01$). The advantages of this system are as follows: (1) urine testing is noninvasive, (2) it has a high informative rate, (3) it has a higher specificity than serum PSA (69 % vs. 28 %), (4) it uses whole urine and not urine sediments, which simplifies specimen-processing, and (5) the assay can be completed in 6 h, giving a relatively short turn-around time.

9.4.3.4 PCA3 Detection by Analysis of Circulating Tumor Cells

Circulating tumor cells (CTCs) are found in 50–70 % of individuals with metastatic breast, colon, and PCas, and their appearance in advanced PCa correlates with a poor prognosis and treatment failure [56–59]. There are many different approaches for isolating and analyzing CTCs, each having different efficiencies and value for molecular analyses [60]. Jost et al. [61] developed an efficient immunomagnetic system for the capture and molecular analysis of circulating PCa cells. Magnetic particles were employed, which either had attached anti-epithelial cell adhesion molecule (EpCAM) or anti-EpCam combined with anti-prostate specific membrane antigen antibodies attached. To initially test this system, 10 mL of normal donor blood harvested by venipuncture into EDTA-containing Vacutainer tubes and 5 mL of the blood was spiked with five C4-2 prostate cell line cancer cells; 100 µL of antibody-coated magnetic particles were added, and the mix was incubated at room temperature for 30 min. The magnetic-bound fraction was subjected to three PBS washings and subsequently treated with Gen-Probe lysis buffer. The immunomagnetically-enriched fractions were analyzed for PSA mRNA expression via a TMA-based system similar to that employed by Groskope et al. [55]. Five C4-2 cells yielded slightly over 1,000 PSA mRNA copies/reaction. Similar reactions carried out without magnetic beads and/or attached anti-EpCAM antibodies failed

to yield any PSA mRNA amplicons, demonstrating system component specificity. The system was also tested with LNCaP prostatic cell lines expressing green fluorescent protein, as measured by phase-contrast and fluorescent microscopy. The immunomagnetic particles bound only the transformed prostatic cells.

To analyze this system with patient samples, 5 mL of EDTA-containing blood from the following groups were examined: (1) 35 men diagnosed with advanced-stage PCa diagnosed by bone scan, (2) 29 men diagnosed with early-stage PCa diagnosed by biopsy, digital rectal exam, and serum PSA measurements, (3) five men diagnosed with BPH, and (4) five healthy men without prostate disease as controls. The CTCs were isolated as above, and PSA, PCA3, and the *TMPRSS2:ERG* gene fusion RNA levels were analyzed by TMA. The *TMPRSS2:ERG* gene fusion occurs in 15–50 % of PCas and confers a more aggressive PCa course in a transgenic murine model, is preferentially detected in metastatic PCa, and is linked with a more aggressive PCa phenotype in humans [62–67] (see below). mRNA for PSA was not detectable in any of the samples from men with BPH or men with early-stage (i.e., confined to the prostrate) PCa. Of the men with advanced-stage PCa, none with androgen-dependent PCa were PCA3 positive, while 31 % of those with androgen-independent PCa were PCA3 positive. Additionally, more of the patients with androgen-independent PCa were *TMPRSS2:ERG* gene fusion positive, compared to that shown in androgen-dependent PCa patients. The addition of anti-prostate specific membrane antigen to the magnetic beads coated with anti-EpCAM did not improve the yield of the TMA-amplified RNAs. The authors opined that this could be due to stochastic differences between the splitting of a sample containing very dilute number of CTCs. Lastly, the immunomagnetic bead-antibody method yielded a higher amplicon copy number than did analysis of cell-free plasma samples from the same patients, demonstrating that the higher complexity of this assay results in greater sensitivity. The authors concluded that, since this system lends itself well to automation and improves upon detecting RNA in plasma, it might have value in the molecular diagnostics of PCa. Additionally, they concluded that further studies are needed with larger patient samples to validify this technique.

9.4.3.5 PCA3 Utility in PCA Molecular Testing

Presently, the TMA-based PCA3 system is now commercially available in the US and Europe [68]. There are many studies demonstrating the value of PCA3 in the molecular diagnostic testing of PCa. In addition, many other studies have shown interesting biological correlates with PCA3 status. For example, Whitman et al. [69] found that a high PCA3:PSA amplicon ratio correlated with an increased incidence of extracapsular extension and greater tumor volume, with the PCA3 score having a 94 % specificity and 80 % positive predictive value for these events. Interestingly, this represents the first molecular marker to predict PCa extracapsular extension. Many of the studies dealing with new molecular diagnostic techniques will require extensive validation studies if they are to be implemented for routine PCa molecular

diagnostics. Other techniques are presently too complex, time-consuming, poorly informative, and/or expensive to have clinical use.

Meta-analyses of PCA3 have demonstrated that PCA3 detection techniques have accepted diagnostics accuracy rates and can be used in the diagnosis of PCa. For example, Ruiz-Aragón et al. [70] performed a meta-analysis of 14 PCA3 studies done between 2000 and 2009 reported in MedLine, Embase, Cochrane Library, CRD, EGRI, Hayes, and cancer and urology databases and journals. The quality of the studies was evaluated by QUADAS criteria to determine the quality of the studies selected. Fourteen studies were selected that met the criteria for moderate to high quality. Of these, the study sensitivities were between 46.9 % and 82.3 %, and the specificities were between 56.3 % and 89 %. Combined with other parameters, the authors concluded PCA3 molecular testing would be a useful method for early PCA3 detection and would significantly reduce the number of repeat biopsies in patients with a PSA >3.0 ng/mL, who could be monitored by PCA3 testing. It should be remembered, however, that a low percentage of patients could have PCa with low to undetectable PCA3 levels.

9.4.4 Gene Fusions in PCa

Tomlins et al. [71] developed a bioinformatics approach to the discovery of candidate oncogenic chromosomal aberrations based on outlier gene expression, termed Cancer Outlier Profile Analysis (COPA). This analysis applies a simple numerical transformation based on the median and median absolute deviation of a gene expression profile. A compendium of 132 gene expression data sets representing 10,486 microarray experiments was analyzed via COPA analysis. COPA correctly identified several outlier profiles for genes in specific malignancies previously found to have recurrent gene rearrangements or high-level gene amplifications. When applied to PCa, COPA identified two erythroblast transformation-specific (*ETS)* family members, *ERG* (21q22.3) and *ETV1* (7p21.2), as strong outlier profiles. The authors reasoned that, because gene fusions involving the highly conserved DNA binding domains of the ETS family, such as *ERG* and *ETV1*, as found in Ewing sarcoma, are functionally redundant, they are therefore seldomly found together within a specific tumor. The same may be true for *ERG* and *ETV1* expressions in PCa. COPA analysis demonstrated that *ERG* and *ETV1* expression across several PCa data sets had invariably mutually exclusive outlier profiles, consistent with the hypothesis that *ERG* and *ETV1* expressions likely play functionally redundant roles in PCa carcinogenesis.

Further analysis of *ERG* and *ETV1* expression in cell lines and human metastatic PCa tumors revealed over- and underexpression of specific *ERG* and *ETV1* exons. For example, cells derived from metastatic PCa showed >90 % reduced *ETV1* exon 2 expression compared to exons 3–7. This selective exon expression suggested that gene rearrangements might be involved in the altered *ERG* and *ETV1* expression patterns. 5′-RNA ligase mediated rapid amplification of cDNA ends was employed to identify the complete 5′ transcripts of the overexpressed *ERG* and *ETV1* exons.

In this process, an RNA is collected that contains the target of interest. The sample is first treated with calf intestinal phosphatase to remove all 5′-phosphates of the RNA species that lack a cap structure, such as rRNA, tRNA, and all fragmented RNA species. The RNA polyermase II transcripts that have cap structures retain their 5′-phosphates. The calf intestinal phosphatase is removed, and the RNA is treated with tobacco acid pyrophosphatase to remove the RNA cap structure and leave an intact 5′-phosphate. A synthetic RNA oligonucleotide (adaptor sequence) is added to the reaction and ligated to the calf intestinal phosphatase/tobacco acid pyrophosphatase treated RNA. The ligation reaction can only happen on RNA with an intact 5′-phosphate, so only RNA polymerase II transcripts are ligated. The resulting RNA is then converted into cDNA with random primers and reverse transcriptase. The cDNAs were amplified with adaptor sequence primers and *ERG*/*ETV1* primers. The products were cloned and sequenced, revealing that the *ERG* and *ETV1* exons were fused with the prostate-specific and androgen-regulated transmembrane-serine protease gene (*TMPRSS2*, 21q22.2). Specifically, several different fusions were identified, including the following: (1) exon 1 of *TMPRSS2* was fused to exon 4 of *ERG,* (2) exon 1 of *TMPRSS2* with the beginning of *ETV1* exon 4, and (3) exons 1 and 2 of *TMPRSS2* with the beginning of *ETV1* exon 4.

Primers specific for these transcripts were then designed to exon 1 of *TMPRSS2* and exons 4 of *ERG* and *ETV1*, as well as exon 6 of *ERG* and exon 7 of *ETV1*. Amplicons were quantified with SYBR Green. Forty-two cases of clinically localized and metastatic PCas were chosen for *ERG* and *ETV1* overexpression as measured by previous microarray analyses. *ERG* and *ETV1* fusions were readily identified, with transcript size variations seen. Similarly, interphase fluorescent in situ hybridization was performed with red or green-labeled probes for *TMPRSS2, ERG* and *ETV1*. While peripheral lymphocytes showed two separate signals, some prostate cell lines and PCa cases showed *TMPRSS2:ETV1* or *TMPRSS2:ERG* rearrangements, confirming the RNA ligase mediated rapid amplification/PCR-derived data. Lastly, the *TMPRSS2:ERG* fusion was examined for androgen responsiveness. Quantitative PCR analysis of the *TMPRSS2:ERG* fusion was performed on the VCaP and LNCaP cell lines, with only the former expressing the fusion transcript. Although both cell lines responded to androgen exposure with increased PSA expression, the VCaP expressed 2,000-fold more *ERG* than the LNCaP cells and expressed increased *ERG* upon androgen exposure. Similar results were obtained with other *TMPRSS2:ERG* fusion positive cell lines, saved for those that express low androgen receptor levels. Thus, the *TMPRSS2:ERG* fusion is under androgen control and the aberrant *ERG* or *ETV1* expression seen in subsets of PCa may in part be explained by fusion with the androgen-responsive *TMPRSS2* gene.

Presently, the molecular effects exerted by the PCa gene fusions are incompletely understood. Transfection of different *TMPRSS2:ERG* fusion splice forms into primary prostatic epithelial cells or a PCa cell line promotes cell proliferation and increases cell motility and invasiveness. The relative activity of the fusions depended upon the 5′ structure and the presence or absence of *ERG* exon 8. Knockdown of the fusion inhibited cell proliferation and slowed tumor progression in an in vivo orthotopic murine model [72]. Other studies have examined altered gene expression

patterns secondary to the presence of the *TMPRSS2:ERG* fusion. Barwick et al. [73] compared *TMPRSS2:ERG* fusion positive vs. fusion negative PCas with a 502 cancer-related gene microarray system using cDNA-mediated, annealing, selection, extension, and ligation to identify fusion-associated gene expression patterns. Sixty-nine patients with the fusion positive PCas were compared to 70 patients with fusion negative PCas. Fifty-one genes were identified as differentially regulated due to the presence of the fusion. Of these genes, 16 were associated with recurrence following radical prostatectomy and 11 were associated with non-recurrence. Recurrence associated genes included those involved in deoxyribosylthymine monophosphate biosynthesis, negative leukocyte regulation, and inhibition of cell matrix adhesion. Genes associated with non-recurrence generally increased cell-matrix adhesion and collagen binding.

Lastly, like other PCa markers [14], *TMPRSS2:ERG* gene fusions patterns show different expression patterns in different ethnicities. For example, Magi-Galluzzi et al. [74], found *TMPRSS2:ERG* gene fusions in 50 %, 31.3 %, and 15.9 % of African American, Caucasian, and Japanese men with PCa, respectively. Interestingly, the patterns by which the gene fusion arose was different in each group, with fusions arising from translocation, deletion, or both events seen in 61.9 %, 38.1 %, and 0 % of Caucasian men, 20 %, 60 %, and 20 % of African American men, and 71.4 %, 28.6 %, and 0 % of Japanese men.

9.4.5 Diversity of PCa Translocations

The ETS transcription factor family consists of 25 genes that share a conserved winged helix-turn-helix DNA binding domain which binds GGAA/T promoter/regulatory elements of over 200 different target genes. The ETS transcription factors function as positive or negative regulators of genes involved in cellular proliferation, differentiation, hematopoietic, apoptosis, tissue remodeling, angiogenesis, and malignant transformation [75]. Since the ETS transcription factors exert many shared and often-redundant effects, different ETS family genes have been found fused to the same 5′-transcript. For example, in Ewing sarcoma, *EWS* is found fused to the ETS family genes *FLI1, ERG, ETV1, ETV4,* and *FEV* [76]. Clark et al. [77] first demonstrated a high diversity within the *TMPRSS2:ERG* fusion transcripts by analyzing 26 PCas, 17 morphologically normal prostate, 31 BPH, and 20 samples of normal, non-prostate tissue for fusions with PCR primers to *5′-TMPRSS2* exon 1, *3′-ERG* exon 6, as previously employed by Tomlins et al. [71]. Of these samples, 14 distinct *TMPRSS2:ERG* fusion transcripts were detected. Included within these 14 samples were fusions of exons 1, 2, and 3 of *TMPRSS2* to exons 2, 3, 4, 5, or 6 of *ERG*. Five of these fusions contained in-frame stops and were unlikely to encode proteins. The most commonly detected fusions were *TMPRSS2* exon 1 fused to *ERG* exons 4 and 5 (designated T1/E4 and T1/E5, respectively), previously identified by Tomlins et al. [71]. Unlike most fusions, T1/E4 and T1/E5 occurred together within individual samples, indicating that they were probably alternative splices from the

same fusion. Interestingly of the BPH samples examined, 6 % were fusion positive and shown to harbor PCa upon repeat histologic examination. Also of interest, Clark et al. [77] found that different separate regions of the same prostate with PCa can harbor different gene fusions, suggesting multifocal origins for PCa.

More recently over 20 different gene fusions have been identified in PCa, many of which have unique characteristics and biological behaviors. The 3′ *ETS* fusion genes identified in the fusions are *ERG, ETV1, RTV4,* and *ETV5*. All of these promote cellular self-renewal and proliferation [75]. The 5′ fusion partners are *TMPRSS2, SLC45A3, ACSL3, HERV-K, FOXP1, EST14, Chromosome 14(q13.3-21.1), C15orf21, HNRPA2B1, KLK2, CANT1,* and *DDX5*. *ERG* is nearly always paired with *TMPRSS2,* while *ETV1, RTV4,* and *ETV5* have multiple fusion partners. The 3′ portion of the fusion usually retains its *ETS* DNA binding capacity and is responsible for the transforming effects of the fusion. Most 5′ fusion partners are androgen regulated, placing the transforming effects under the control of androgen stimulation [78].

9.4.6 PCa Translocations in PCa Diagnosis and Prognosis

TMPRSS2:ETS fusions occur in about 50 % of PCas, making them the most prevalent gene fusions identified in any solid tumor [62, 71, 74, 78, 79]. Since some gene fusions are associated with specific clinical outcomes in other malignancies, there is considerable interest in the possible value of the PCa gene fusions as prognostic biomarkers. Multiple studies have addressed the importance of *TMPRSS2:ETS* fusions in PCa aggressiveness and clinical outcome; however, no consistent results have so far been obtained. For example, *TMPRSS2:ETS* fusions have been associated with a favorable [80–83], unfavorable [64, 84–93], and no effect on the clinical course and prognosis of PCa [94–98]. The reason for these divergent findings probably comes from several factors, such as different patient populations, different parameters and endpoints employed between different studies, and the molecular heterogeneity found in the *TMPRSS2:ETS* fusions [14, 62, 71, 77–98]. However, several specific gene fusion patterns have been found to correlate with a poor PCa clinical course and prognosis;

9.4.7 Concurrent TMPRSS2-ERG and PTEN Loss in PCa

King et al. [99] examined 121 *ERG* rearrangement positive and negative human PCas for *PTEN* copy number loss and *MYC* amplification. Gene loss or amplification was measured by high-resolution array-based comparative genome hybridization. *PTEN* loss and *ERG* rearrangements were significantly associated in PCa ($p < 0.002$), while *MYC* (commonly amplified in PCa) was not associated with *ERG* rearrangements ($p < 0.56$). Interestingly, transgenic mice that expressed high levels of the *TMPRSS2:ERG* fusion (~20-fold increased *ERG* expression) did not show higher

rates of PCa than control mice. To further analyze this, transgenic mice bearing a *TMPRSS2:ERG* fusion were crossed with *Pten*$^{+/-}$ mice. At 6 months of age all 8 of 8 *TMPRSS2:ERG*/*Pten*$^{+/-}$ mice had clear histologic evidence of high-grade PIN while only one focus of low grade PIN was identified in one of the eight *Pten*$^{+/-}$ littermate controls. Similar crosses of *TMPRSS2:ERG* fusion mice with mice expressing high *MYC* transcripts did not show cooperativity in PCa development. Cooperativity was identified when *TMPRSS2:ERG* fusion mice were crossed with prostate-specific AKT overexpressing transgenic mice, where 8 of 8 bigenic mice developed florid PCa lesions by 10–12 months, while all of the AKT overexpressing mice developed low-grade PIN and did not progress to PCa. Other investigators found similar results [100], finding that *ERG* overexpression promoted cell migration, without affecting proliferation, and that *PTEN* loss was needed for increased cell proliferation, mediated by increased AKT activity. Interestingly, microarray expression analysis demonstrated that *ERG* overexpression increased *CXCR4* and *ADAMTS1* expression, two genes whose products promote cell migration, invasion, and angiogenesis.

Yoshimoto et al. [93] performed a retrospective study on 125 PCa cases with known clinical outcomes and examined *TMPRSS2:ERG* fusions and *PTEN* genomic deletions by fluorescent in situ hybridization (FISH). Forty-eight percent of the PCas had *TMPRSS2:ERG* fusions. Gleason grade ($p=0.0002$)/score ($p=0.001$), median tumor volume ($p=0.15$), preoperative PSA ($p=0.001$), and perineural invasion ($p=0.0304$) were associated with biochemical reoccurrence, while *TMPRSS2:ERG* fusion almost reached significance ($p=0.0523$). Interestingly, multivariate analysis revealed *PTEN* homozygous deletion ($p=0.013$) and concurrent *TMPRSS2:ERG* fusion with *PTEN* homozygous deletion ($p=0.036$). Kaplan-Meier and multivariate analysis both indicated that concurrent *TMPRSS2:ERG* fusion and *PTEN* loss predict earlier PCa biochemical recurrence.

9.4.8 *Loss of the TMPRSS2 3′ Genomic Region of PCa with Concurrent Fusion Duplication*

TMPRSS2 and ERG are located ~3 Mb apart on chromosome 21, allowing the *TMPRSS2:ERG* fusion to form via a translocation between chromosomes 21, or by interstitial deletions [86, 101]. Several studies have demonstrated that *TMPRSS2:ERG* fusions resulting from interstitial deletions have a worse prognosis than those arising from translocations. For example, Attard et al. [89] analyzed 445 PCas from conservatively managed patients. Individuals lacking *ERG* alterations exhibited a favorable clinical course, with a 90 % survival at 8 years. Conversely, individuals with duplications of the *TMPRSS2:ERG* fusions (2+Edel) resulting from interstitial 3′ deletions had an extremely poor clinical course, with 25 % survival at 8 years. Multivariant analysis revealed 2+Edel provided significant prognostic information ($p=0.003$), in addition to that provided by Gleason score and PSA levels.

In another study, Gopalan et al. [87] analyzed 521 patients who underwent radical prostatectomies using FISH probes to 3′ *ERG*, 5′*TMPRSS2*, and 3′ *TMPRSS2* clones. Since FISH measures genomic structural alterations and is therefore an indirect test for gene fusions, 67 PCas previously analyzed for fusions by oligonucleotide arrays were analyzed by FISH. FISH was less sensitive than oligonucleotide arrays and preferentially detected PCas with higher *ERG* expression. Of the 521 PCas analyzed, 217 (42 %) had *TMPRSS2:ERG* alterations; of these, 128 (59 %) were 3′ *TMPRSS2* deletions, or a total of 12 % of the 521 PCas. *ERG* expression alone was not significantly different between patients with and without biochemical recurrence, but did correlate with overall survival and metastases, and was associated with a lower Gleason score. Interestingly, increased *ERG* or *TMPRSS2* copy number without fusion was significantly associated with high (≥7) Gleason scores, while increased copy number of the *TMPRSS2:ERG* deletion fusion was significantly ($p<0.01$) associated with increased metastases and poorer outcomes. Lastly, *ETV1* rearrangements failed to show an association with poorer survival [102].

Wang et al. [88] found that different *TMPRSS2:ERG* isoforms are associated with aggressive PCa. Specifically, *TMPRSS2:ERG* fusion isoforms in which the native ATG of *TMPRSS2* is in frame with exon 4 of *ERG* was associated with early biochemical recurrence following radical prostatectomy, aggressive disease, and death from PCa. Similarly, fusions with the native *ERG* ATG in exon 3 were associated with seminal vesicle invasion and poor outcome following radical prostatectomy. Interestingly, PCas not having these fusions isoforms tended to have higher overall fusion mRNA expression. Wang et al. [88] concluded that both the level of fusion mRNA expression and the specific isoform likely effect PCa progression.

9.5 Specific TMPRSS2:ETS Gene Fusion Detection Techniques

Several different molecular techniques have proven useful in the detection of *TMPRSS2:ETS* gene fusions. Among them are many variations of PCR, FISH, microarray, DNA sequencing techniques, and immunohistochemistry. Here we will review a few of these techniques.

9.5.1 PCR

Many *TMPRSS2:ETS* fusion detection methods utilize PCR, with amplicon detection achieved via various detection methods. Wang et al. [88] extracted RNA from 19 normal (peripheral zone), 18 early recurrence (less than 1 year), 16 late recurrence (recurrence after 1–5 years), and 20 non-recurrent radical prostatectomy samples. Five micrograms of RNA were reverse transcribed in cDNA in a total volume of 100 μL in an iScript cDNA synthesis kit, according to the manufacturer's protocol.

Five μL of cDNA template were amplified with *TMPRSS2* RT forward 5′-CAGGAGGCGGAGGCGGA-3′ and *TMPRSS2:ERG* RT reverse 5′-GGCGTT GTAGCTGGGGGTGAG-3′ in a volume of 25 μL, using a standard three-step protocol with an annealing temperature of 65 °C. The resulting amplicons were detected on a 1.2 % agarose gel stained ethidium bromide. The different sized amplicons were cloned into a PCR 2.1-Topo vector cloning kit. Plasmids were prepared using a Qiagen Spin Mini-Prep kit and sequenced by M13R and M13F primers. The sequences were analyzed by National Center for Biotechnology Information BLAST software. Additionally, quantitative real-time PCR was performed using β-actin as an amplification control and SYBR green fluorescence as the detection method. Fifty-nine percent of the prostatectomies showed *TMPRSS2:ERG* fusions. As mentioned above, those fusions carrying native *TMPRSS2* or *ERG* translation initiation codons had a significantly worse prognosis.

9.5.2 FISH

FISH has commonly been used to detect *TMPRSS2:ERG* fusions and has the advantage of giving information concerning chromosomal structure that PCR does not give. Attard et al. [89] used overlapping bacterial artificial chromosome probes to the centromeric 3′-end (red) and probes to the telomeric 5′-end (green) to analyze different *TMPRSS2:ETS* fusion isoforms. By this assay, unrearranged *ERG* loci appeared as immediately adjacent green and red signals in interphase nuclei. When the *TMPRSS2:ERG* fusion isoform was *TMPRSS2* exons 1 or 2 fused to *ERG* exons 2, 3, or 4 (common isoforms) [71, 77], *ERG* occurred as separated red and green signals. As previously mentioned, duplication of these deletion isoforms (2+Edel) resulted in an extremely poor clinical course.

9.5.3 Gene Fusion Detected in PCa by Immunohistochemistry

Park et al. [103] employed tissue microarrays to analyze 131 men who underwent radical prostatectomies. The tissue microassays were constructed from benign and malignant formalin-fixed paraffin-embedded tissue blocks from the prostatectomies. The selection of tissue samples and review of each case's pathological findings were conducted and performed by the study pathologists. The primary rabbit antibody was obtained from Epitomics (San Diego, CA) and was carefully characterized for use in tissue microarray studies compared to two color interphase FISH. Immunohistochemical tissue microarray reactivity was highly concordant with two color interphase FISH. The tissue microarray was carried out using an automated DiscoveryXT staining platform from Ventana Medical Systems. Antigen recovery was conducted using heat retrieval with the slides incubated with 1:100 primary *ERG* antibody for 1 h at room temperature. The primary antibody was detected

using a ChromoMapDAB anti-rabbit horseradish peroxidase conjugated antibody that was applied for 16 min followed by washing and detection. ERG protein expression was subjectively evaluated using a negative (0), weakly positive (+1), moderately positive (+2), and strongly positive (+3) scale.

ERG expression was confined to the PCa cells and high-grade PIN that was associated with *ERG*-positive PCa. It was also identified in blood vessels and lymphocytes where it has a known biologic role. Analysis of the 131 cases compared to FISH revealed 95.7 % sensitivity and 96.5 % specificity. Since immunohistochemical studies are technically easier and less expensive than FISH analyses, molecular PCa subtyping by immunohistochemistry may have clinical utility, especially for prostate needle core biopsies.

9.5.4 SPINK1 Overexpression in PCa

Tomlins et al. [104] employed COPA analysis on seven PCa profiling studies in the Oncomine database to identify candidate outlier genes in *ETS* negative PCas. Twenty-nine genes were identified as possible outliers in three of the seven databases, with 11 genes identified in at least four of the databases. The first outlier out of all of the databases was *ERG*, with the COPA analysis performed as previously described [71]. One gene, *SPINK1* (serine peptidase inhibitor, Kazal type 1) was ranked as the second outlier, exhibiting overexpression in PCa compared to benign tissue and additionally having overexpression mutually exclusive of *ERG* and *ETV1*.

To confirm that *SPINK1* expression did not occur in *ETS* positive PCas, *SPINK1, ERG,* and *ETV1* expression was analyzed in 10 benign and 61 malignant prostate samples using quantitative PCR. Total RNA was isolated from the samples with Trizol, quantified, and 3–5 μg of total RNA was reversed transcribed into DNA using Superscript III in the presence of random primers. The cDNA was amplified with Power SYBR Green Master Mix in the presence of 25 ng SPINK1 f-CAAAAATCTG GGCCTTGCTGAGAAC and SPINK1 r- AGGCCTCGCGGTGACCTGAT and forward and reverse primers for *ERG, ETV1,* and *GAPDH* and *HMBS* as housekeeping gene controls. The primers for these last four genes were the same as those employed by Tomlins et al. [71]. Serial dilutions of the primers on pooled PCA cDNA demonstrated approximately equal primers efficiencies. All reactions were subjected to melt curve analyses. *ERG, ETV1,* and *SPINK1* were expressed in 41 %, 6.5 %, and 6.5 % of the 61 PCas and none of the 10 benign samples and exhibited mutually exclusive expression patterns. Additionally, FISH analysis for *SPINK1* and *TMPRSS2:ERG* fusion was performed on two tissue microarrays representing 392 PCa cases. *SPINK1* overexpression was identified in 13.3 % of cases, while *TMPRSS2:ERG* fusion overexpression was identified in 48 % of cases; again, no concurrent *SPINK1-TMPRSS2:ERG* fusion was seen.

Examination of three independent cohorts consisting of 79, 75, and 817 PCa cases that underwent radical prostatectomies revealed that *SPINK1* overexpressing cases had a significantly higher risk for PCa recurrence than those cases without overexpression.

Multivariate Cox proportional-hazards regression analysis confirmed that *SPINK1* outlier status was a significant predictor of PCa recurrence and was independent of Gleason score, lymph node status, surgical margin status, seminal vesicle invasion, extraprostatic extension, and preoperative PSA levels. Tomlins et al. [104] hypothesized that in *ETS* negativity may predispose PCas to *SPINK1* overexpression. Although not commonly used in the molecular diagnostic of PCa, *SPINK1* overexpression shows promise as a predictor of a more aggressive PCa clinical course and an increased recurrence rate following radical prostatectomy.

9.5.5 t(4;6)(q22;q15) in PCa

A t(4;6)(q22;q15) chromosomal translocation has been indentified in PCa lines and human PCas using multiplex FISH and FISH for this translocation on tissue microarrays [105, 106]. Of 667 PCas analyzed by tissue microarray, 11.7 % were positive for the t(4;6)(q22;q15) translocation [105]. The presence of this translocation did not affect cause-specific or overall survival. Its presence did, however, correlate with a higher Gleason score, clinical stage, baseline PSA, and tumor volume upon biopsy. Adding the t(4;6)(q22;q15) translocation to a Multivariate Cox model, which included the Gleason score, baseline PSA, extent of disease, and age at diagnosis, did not significantly improve the model. Interestingly, t(4;6)(q22;q15) did occur more frequently in 2+Edel positive samples than negative samples, although the correlation did not reach significance ($p = 0.0628$) [105]. The authors concluded that, while this translocation does not independently affect patient outcome, it may define specific cohort of PCas, thus warranting further investigation.

Since the work by Tomlins et al. [71], PCa has been found to harbor over 20 different translocations, some of which are known to alter the clinical course of PCa, while others either have little effect, or require further study [62, 64, 71–106]. While extremely complex, some of these translocations appear to have value in the molecular diagnostics of PCa and are likely be incorporated into clinical testing within the next few years.

9.6 Somatic Mitochondrial DNA Mutations in PCa

Mitochondrial mutations have been identified in many different malignancies, including, breast, ovarian, colorectal, gastric, hepatic, brain, thyroid, esophageal, pancreatic, renal, and PCas [107]. In a recent study, Parr et al. [108] examined somatic DNA mitochondrial mutations in PCa via full mitochondrial genome sequencing. Thirty-six patients were selected for the study. Twenty-four patients had undergone radical prostatectomy for advanced PCa, and 12 patients were symptomatic (i.e., had elevated PSA) but free of detectable PCa by biopsy. DNA was recovered from histologically malignant and benign paraffin-embedded prostate

glands by laser capture microdissection. DNA was also isolated from whole blood draws taken from the 36 patients and a subset of the patients' maternal relatives recruited for the study. The complete mitochondrial genome was PCR amplified with 34 primer sets, sequenced, and analyzed by Sequencher Software (Gene Codes Corp.). Sequence polymorphic variations were identified by comparing the blood samples to the revised Cambridge reference sequence. Additionally, nuclear-embedded mitochondrial pseudogenes sequences that co-amplified were prepared from an osteosarcoma cell line. The resulting amplicons were extensively analyzed and compared to hetero- and homoplasmic sites in the sequence data to preclude any inclusions of paralogue nuclear mutations in the mitochondrial final data set.

Sixteen of the 24 PCa samples (66.7 %) had mitochondrial mutations in all three examined prostatic compartments. 91.7 % had mutations in the histologically malignant prostatic glands, 79.2 % had mutations in histologically benign prostatic glands near the PCA, and 91.7 % had mutations in distant, histologically benign prostatic glands. The average mitochondrial genome sequence coverage was 78 % which leaved 3.6 kb unexamined. A total of 273 somatic mitochondrial mutations were identified. The maternal relative blood samples showed few differences between genomes, indicating that the mutations were not merely age-associated events.

Since mutations were found in histologically benign glands, the disease specificity of the mutations was examined. The symptomatic benign group had far fewer mutations, most of which occurred in known polymorphic loci on non-coding genomic regions. The histologically malignant glands did not differ in the non-coding genomic region mutations, but had significantly increased coding region mutations compared to all the histologically benign glands. The authors concluded that genomic mutations precede malignant histology and accumulate with malignancy progression. Additionally, with tumor progression, very specific mutations became increasingly common, with six linked mutations seen in 44 % of the PCas and one (mutation 15,527) identified in 74 % of PCas. Since cells typically carry a high mitochondrial genome copy number and mitochondrial DNA can be easily isolated and analyzed for specific mutations, the use of high-frequency mitochondrial genome mutations associated with PCa may have eventual utility in PCa molecular diagnostics. Methods such as microarray and allele-specific PCR would allow for high-throughput analysis of mutations. Presently, further work is required to analyze and validify PCa mitochondrial mutation frequencies and association with PCa.

9.6.1 C-MYC Amplification in PCa

C-*MYC* is found at 8q24.12 to 8q24.13 and belongs to the Myc family that also includes *Myc/MAD/MAX* basic helix-loop-helix leucine zipper domain transcription factors. *C-MYC* is a multifunctional nuclear phosphoprotein that plays a role in regulating cell cycle entry and progression, transcription, differentiation, apoptosis, and cellular motility. *C-MYC* is dysregulated in several malignancies, including breast, colon, gynecological, and hepatocellular malignancies, and those of

hematopoietic lineage. *C-MYC* appears to bind DNA nonspecifically, although it also recognizes the core sequence 5′-CAC[GA]TG-3′ where it activates the expression of growth-related genes [109, 110]. Fleming et al. [111] first demonstrated *C-MYC* overexpression in PCa vs. BPH with Northern blotting. Since then, data derived from FISH analyses have shown that *C-MYC* is expressed in PIN, PCa, and metastatic PCa, with the *C-MYC* levels increasing with tumor progression [112–114]. Additionally, Sato et al. [114] used dual-probe FISH with a centromere-specific probe for chromosome 8 and one region-specific probe for *C-MYC* to examine changes in 50 Japanese PCa patients who had undergone retropubic prostatectomies. *C-MYC* expression correlated strongly with higher histopathologic grade, Gleason score, earlier disease progression, and earlier PCa-related death. Lastly, *C-MYC* overexpression was a predictor of biochemical recurrence, and higher *C-MYC* mRNA levels significantly correlated with positive surgical margins upon prostatectomy, higher Gleason score, and pathologic stage [115]. Interestingly, *C-MYC* expression varies in different neoplastic lesions within the same prostate, similar to many of the other molecular alterations identified in PCa carcinogenesis and progression [62, 64, 71–106, 115].

C-MYC levels in PCa have been quantified via FISH, RT-PCR, and microarray [112–116]. While all these methods have worked well and reliably, RT-PCR is comparatively low-cost, has a short turn-around time, and high sensitivity. Hawksworth et al. [115] employed RT-PCR quantify C-MYC expression in 182 specimens taken from 91 retropubic prostatectomies, using laser capture microdissection to isolate benign and neoplastic prostatic epithelial cells. Quantitative gene expression was performed by extracting total RNA with a MicroRNA kit, quantifying the RNA yield with RiboGreen dye and a VersaLour fluorimeter, converting to cDNA by Sensiscript (Qiagen oligonucleotide primers with reverse transcriptase), and finally PCR amplifying with a forward primer, 5′-ACCACCAGCAGCGACTCTGA-3′, reverse primer, 5′-TCC AGCAGAAGGTGATCCAGA CT-3′, and a probe 5′-ACCTTTTGCCAGGAGCCTGCCTCT-3′. The reactions were carried out with a TaqMan-based QRT-PRC on an ABI 7700 with 50 cycles; 95 °C for 15 s and 60 °C for 1 min. *GAPDH* was amplified as a control gene. Gene-expression results were obtained as average threshold values (C_t value) of duplicate samples. As mentioned above, higher *C-MYC* expression significantly correlated with biochemical recurrence, positive surgical margins, and higher Gleason score and pathologic stage [115]. Interestingly, the common *TMPRSS2:ERG* fusion activates *C-MYC* expression and not surprisingly, *C-MYC* overexpression and the *TMPRSS2:ERG* fusion commonly occur together [117].

9.6.2 Bcl-2

Bcl-2, or B-cell lymphoma 2, is found at 18q21.3 and plays a central role in regulating apoptosis and is induced by a wide variety of divergent stimuli. *Bcl-2* is part of a larger protein family that is involved in regulating cell death by promoting or

inhibiting apoptosis. The ratio of the pro-apoptotic to anti-apoptotic determines cell survival and vulnerability to toxic stimuli by inhibiting the activation of the pro-apoptotic proteins *Bax* and *Bak* on the outer mitochondrial membrane. Thus, *Bcl-2* overexpression confers a survival advantage [118–120]. In normal prostate, *Bcl-2* expression is limited to the basilar glandular epithelium, where its expression is androgen independent. In PCa, *Bcl-2* expression is significantly increased in androgen-independent PCa, but to a far lesser degree in androgen-dependent PCa. Based on this, it is thought that *Bcl-2* overexpression is one factor in allowing the survival and spread of androgen-independent PCa [121].

Although not commonly employed in the molecular diagnostics of PCa, *Bcl-2* overexpression confers are worse PCa prognosis. For example, Concato et al. [122] analyzed 1,007 PCa cases for Bcl-2 expression, with an average patient age of 72 years at PCa diagnosis. The patients were followed for over 15 years. At the end of this period, 71.8 % had died of any cause, with 21.5 % due to PCa. The median overall survival was 7.7 years. Multivariate analysis of this cohort revealed that increasing age, higher PSA levels (over 20 μg/mL), and moderately to poorly differentiated PCa were strongly associated with shorter survival. *Bcl-2* overexpression was associated with an increased long-term death risk from PCa, with an adjusted hazard ratio of 1.61. Interestingly, the same study revealed that p53 overexpression and the upper three quartiles of microvessel density were also associated with a long-term death risk from PCa, having adjusted hazard ratios of 1.48 and 3.20, respectively. *Bcl-2* expression has mainly been examined by immunohistochemical staining [121–124].

9.6.3 Kallikrein

In addition to PSA, another member of the kallikrein family, human glandular kallikrein 2 (hK2), may also be useful in detecting early PCa. In the bloodstream, hK2 is present at 1–2 % of the total PSA serum concentration with up to 20 % of which can be complexed with α_1-antichymotrypsin [125]. Of detectable hK2 in blood, 80–90 % occurs as free, non-complexed 30-kDa form. Both hK2 and PSA are produced mainly in the prostate under the control of androgen stimulation [126, 127]. hK2 shows an age dependency, but less than is seen with PSA. Men in their 40s and 50s seem to have similar hK2 concentrations, while men in the 60s show an increase in hK2, but once again less than that seen for PSA. hK2 functions as a trypsin-like protease and among the kallikrein variants it has the highest homology to PSA, having about 80 % sequence identity. Because of this high level of homology, previous assays utilizing monoclonal antibodies that were raised against PSA could also detect hK2 [128]. The physiological link between these two proteins may be the ability of hK2 to cleave the propeptide of inactive pro-PSA, converting it into its mature active form [129, 130].

hK2 and PSA expression differ in that PSA is more intensely expressed in BPH than in PCa. Additionally, PSA expression is higher in low-grade than in high-grade PCas.

In contrast, hK2 shows higher staining intensity in high-grade PCa and lymph node metastasis than it does in low-grade PCa or BPH [131, 132]. Similar to PSA, hK2 exists in different molecular forms. In vitro, it complexes with several protease inhibitors, including α2-antiplasmin, AT, antithrombin III, α2-macroglobulin, C1-inactivator, and plasminogen activator inhibitor-1 [133–135]. Both free and complexed forms have been found in biological fluids. In seminal fluid, the protein is mainly complexed to protein C inhibitor [136]. However, in the bloodstream, 80–95 % is uncomplexed [127, 137] while in PCa patients with high hK2 levels, up to 20 % is complexed to ACT [125]. As previously mentioned, an inactive zymogen form (pro-hK2) is found in blood [138].

Because of its low circulating levels, immunoassays for hK2 must have low detection limits with excellent reproducibility. The first assay developed to measure hK2 employed an immunoflurometric technique using two separate incubations, one to inhibit subsequent reactions of both free and complexed PSA using MAb2H11 (which does not cross react with hK2) and a second using biotinylated MabH50 (which cross reacts with hK2 by epitope overlapping with Mab2H11, binding only hK2 on streptavidin microtitration wells). A third step utilized eu-labeled MAb117, which cross reacted with hK2 and detected the immobilized hK2. The assay was calibrated with a recombinant hK2, giving a detection limit of 0.1 μg/L, and cross reactivity with PSA of <0.7 %. However, up to 57 % of samples had hK2 concentrations below the detection limit [126].

Since then, more sensitive and specific assays have been developed using hK2 specific monoclonal antibodies. These, with the addition of ultrasensitive immunoassays for measuring free and total hK2 in serum, yield detection limits of 0.0008 μg/mL for total hK2 and 0.001 μg/mL for the free hK2. This technique was developed to achieve the required sensitivity to measure blood hK2. The limit of quantification (intraassay CV <15 %) in serum matrix for total hK2 was 0.003 and 0.010 μg/L for free hK2. The results revealed hK2 concentrations of 1–5 % of the total PSA (range, 0.1–58 %; median, 3.6 %), with a median total hK2 concentration of 0.022 μg/L [139]. Previous studies revealed hK2 concentrations to be 1–4 % of PSA concentrations [126, 140, 141]. The median hK2 concentration was lower than the reported value of 0.026 μg/L [142] but higher than the value of 0.016 μg/L reported by other authors [141]. In addition, the correlation coefficient was low between the two proteins, particularly in the "gray diagnostic area" for PSA (4–10 ng/mL), suggesting PSA and hK2 may have different elimination patterns and that hK2 may therefore be able to provide clinical information independent from PSA [139].

Interestingly, while hK2 concentrations did not significantly differ between PCa and BPH patients, the ratio of hK2 to fPSA (percent hK2) did allow the discrimination of PCa from BPH within the 4–10-ng/mL PSA range over percent fPSA alone [143]. Similar findings of a nonsignificant relationship of hK2 concentrations and PCa have been reported [144–146]; however, later studies found significantly higher levels of hK2 in PCa versus BPH or non-PCa samples [147–154]. The percent hK2 enhanced the discrimination between PCa and non-PCa patients in multiple studies [144–146, 148, 150–152]. Within the 2.5–10-ng/mL PSA range, the predictive value of percent hK2 was greater than percent fPSA in most studies [144, 145, 151, 152],

although a different large study found a higher predictive value for percent fPSA [145]. In addition, other studies found that for PSA levels <4 ng/mL, the predictive value of percent hK2 performed worse than percent fPSA [145, 152].

Several studies examined the relationship between PCa stage and grade, revealing that hK2 can differentiate high- from low-grade tumors and organ versus non-organ confined disease [155–159]. Several studies also revealed that hK2 levels were significantly higher in organ confined versus non-organ confined disease, although after Gleason grade and clinical stage were added to a multivariate logistic regression model, the improvements were only marginal [182–184]. A current study has confirmed these findings [153]. In contrast, others showed that hK2 was of no to little value in predicting tumor stage and grade [160, 161]. In summary, most studies show significantly elevated levels of hK2 in men with PCa than in men without PCa, although several other studies did not. An increased predictive value for percent hK2 over percent fPSA alone is supported by most studies. Most studies show a limited increase in predicting stage and tumor grade after addition of hK2 or hK2 incorporated ratios with fPSA or total PSA.

9.6.4 Urokinase Plasminogen Activator

Most patients that experience relapsed or advanced disease respond initially to androgen ablation therapy because early PCa is often androgen dependent. However, many PCas eventually progress to hormone refractory PCa, which is refractory to androgen ablation, thus limiting treatment options thus far for metastatic PCa.

Metastatic PCa often follows lymphatic and hematogenous routes to regional lymph nodes and bone, especially the spine. Although the mechanism(s) of tumor progression is incompletely understood, several proteolytic enzymes are involved in degrading the basement membrane and extracellular matrix (ECM), thereby increasing PCa metastatic potential. One proteolytic enzyme and receptor involved in this process are the urokinase plasminogen activator (uPA) and its receptor (uPAR). uPA is a serine protease family member linked to many different malignancies. It is synthesized as a pro-enzyme, a single chain known as pro-uPA, which binds to uPAR with high affinity. Binding converts inactive plasminogen into plasmin, which then can cleave various ECM components including fibrin, collagen, laminin, fibronectin, and vitronectin. Plasmin can also activate matrix metalloproteinases (MMPs) and elastase, causing further ECM degradation. It is thought that these processes lead to increased tumor invasiveness and metastatic potential. Binding of uPA to uPAR also increases the affinity of uPAR for the ECM protein vitronectin and for integrin adhesion receptors [162].

Activity of uPA is regulated by two serine protease inhibitors (PAI1 and PAI2) and by uPAR. PAI1 and PAI2 form SDS-stable 1:1 complexes with uPA. PAI1 is a 43-kDa single chain glycoprotein and is likely the primary inhibitor of the uPA. PAI2 has 2 forms, one 47-kDa form that is intracellular and non-glycosolated

and one 60-kDa form that is extracellular and glycosolated. The interaction of PAI1/uPA/uPAR leads to internalization of the ternary complex, which causes cell proliferation. In PCa cells, this complex enters the cell via receptor-mediated endocytosis mediated by the low-density lipoprotein receptor-related protein. The discovery of this mechanism may facilitate the transfer of cytotoxic therapies to uPA-positive cancer cells, as inhibiting uPA by plasminogen activator inhibitor-2 results in accelerated clearance of uPA from the cell membrane [163].

uPAR is not only responsible for lateral protein-protein interactions that contribute to cell adhesion and migration via the integrin family but also contributes to signal transduction cascades within uPAR expressing cells, including the JAK-STAT, mitogen-activated, and Ras/extracellular signal regulated kinase pathway proteins, which regulate genes responsible for proliferation, survival, and apoptosis [164–166]. Interestingly, in a murine model, anti-sense oligonucleotides down-regulated uPAR expression, decreasing FAK/JNK/Jun phosphorylation, inhibiting cyclins A, B, D1 and D3 synthesis, and causing a G_2 phase cell cycle checkpoint, resulting in unaffected adhesion, but impaired proliferation and invasion, and a subsequent decrease in PCa bone metastasis [167].

The uPAR protein consists of three homologous three-finger domains, in which the amino terminal domain I is required in order for uPA binding. Domain 1 contains the N-terminal. Removal of this domain from the cell surface drastically reduces the capacity of cells to bind to uPA. When bound, uPA cleaves uPAR between domains I and II freeing domain I (uPAR1), leaving domains II and III (uPARII-III) attached to the cell surface. Additionally, uPAR can be cleaved from the cell's surface at its lipid anchor by either phospholipases or proteases, generating a full-length uPAR(I-III) and/or uPAR(II-III) [168, 169]. Immunoassays capable of detecting intact and cleaved forms of the receptor have been developed, and certain forms of the cleaved receptor, such as uPAR1 and uPARII-III, are found at higher levels in PCa than in benign disease. In men with a serum PSA of 2–10 μg/L, the combination of percent fPSA with the uPAR1/uPARI-III ratio, had a greater area under the ROC curve (0.73), compared to percent fPSA alone (0.68) [170, 171].

A number of studies have demonstrated increased levels of uPA and uPAR in prostate PCa [172, 173]. Others have shown an association of uPA overexpression with extracapsular extension [174]. Elevated levels of both have also been demonstrated in high-grade tumors (Gleason > 7), using immunohistochemistry and tissue microarrays. Elevated levels have also been seen to be associated with metastasis [175–177]. Stromal expression of uPA and its receptor have also been found to have significance. TMAs have demonstrated expression not only in PCa cells, but also in stromal cells in high Gleason score cancers [177]. By releasing chemotactic factors, the PCa epithelia can induce stromal cells to contribute to an environment conducive to metastasis through their release of cytokines, growth factors, and proteolytic enzymes. MMPs are synthesized by both cancer and stromal cells [178], and elevations in uPA and uPAR have been associated with an increase in these enzymes [179–181]. These findings suggest that increased uPA/uPAR in PCa may stimulate stromal paracine MMP synthesis, increasing PCa metastatic potential.

uPA/uPAR are the targets of several new potential treatments. The tyrosine kinase inhibitors, genistein and tyrphostin AG-1478, profoundly reduce uPA/uPAR production and mRNA levels in PCa cells and inhibit invasive potential [182–185]. Because PAI2 is stable in vitro and is unlikely to be subject to inactivation in oxidizing environments such as inflammation and laboratory testing, it is a potential vector for targeted therapy. By forming radioimmunoconjugates through the labeling of PAI2 with α-emitting radionucleotide ^{213}Bi-uPA positive cancer cells were induced to apoptosis in vitro. This could be done on monolayer PCa cells alone or in conjunction with other radioimmunoconjugates as multi-targeted alpha therapy. In vivo studies using nude mice showed that this strategy leads to a dose-dependent complete regression or delay of tumor growth, along with a decreased incidence of lymph node metastasis [186–188]. RNA interference is a sequence specific, post-transcriptional gene-silencing mechanism that utilizes double stranded RNA homologous to the target gene sequence. Silencing of the uPA/uPAR genes was performed using single plasmid constructs expressing small hairpin RNAs for both uPA and uPAR. This leads to reduced cell viability, apoptosis, and interference of downstream signaling of the ERK1/2 pathway (responsible for cancer cell invasion, proliferation, and survival) and Stat 3. This substantiates the theory that complex signaling networks downstream of uPA/uPAR are responsible for tumor cell invasion, proliferation, and survival of PCa cells [189].

9.6.5 GSTP-1 Promoter Hypermethylation

The detoxifying glutathione-S-transferases (GSTs) comprise a large supergene family that includes members of eight classes based on sequence similarity: Alpha, Mu, Theta, Kappa, Zeta, Omega, and Sigma. GSTs detoxify a wide variety of exogenous and endogenous compounds, such as reactive oxygen species produced by normal cellular functions, and exogenous toxins, such as polycyclic aromatic hydrocarbons. GSTs function by catalyzing the nucleophilic attack of glutathione on electrophilic substrates, reducing their reactivity with cellular constituents [190, 191]. Interestingly, some data indicate that GSTs may have non-detoxifying activities, such as selective inhibition of oncogenic ras-p21-induced mitogenic signaling via Jun kinase inhibition [192].

Because GSTs play an important role in the detoxification and hence inhibition of endogenous and exogenous toxins, loss of these proteins is pro-mutagenic [193]. In fact, the GSTs are lost in a large number of different malignancies, including squamous cell carcinoma, nasopharyngeal carcinoma, non-small cell lung cancers, and PCa [193–196]. Within PCa, hypermethylation of glutathione-S-transferase P1 (GSTP1) promoter CpG islands are the most consistent DNA alterations identified in PCas, having been identified in ~70 % of high-grade PIN and over 90 % of PCas [196]. GSTP1 CpG island promoter methylation has been detected by a variety of different methods, such as digestion of normal and PCa DNA with endonucleases that cut at non-methylated, but not methylated cytosine residues, followed by PRC amplification, or methylation-specific PCR techniques [196, 197].

9.6.6 Methylation-Specific PCR

Methylation-specific PCR (MSP) is commonly employed in the analysis of CpG promoter hypermethylation. In this technique, genomic DNA (~1 μg) is isolated and denatured in NaOH for 10 min at 37 °C. Samples with low (nanogram) quantities of DNA often have carrier DNA, such as salmon sperm DNA, added to the subsequent reactions. Next, fresh hydroquinone and sodium bisulfite are added to the reaction at pH 5, and the samples are mixed and incubated for ~16 h at 50 °C. The bisulfite treatment changes cytosine to uracil while not altering methyl-cytosine. Thus, following bisulfite treatment, methylated and unmethylated cytosines can be distinguished by binding to either guanine (methyl-cytosine) or adenine (uracil). The modified DNA is then purified, and different sets of oliogonucleotide primers are employed to analyze the DNA for the presence or absence of methyl-cytosine(s) [198]. Esteller et al. [199] employed bisulfite-modified DNA from more than 300 primary tumors for GSTP1 aberrant promoter methylation and included tumor DNA from endometrial, ovarian, bladder, pancreatic, lung, head and neck, leukemias, melanomas, meningiomas, and PCas. Primers for the unmethylated reactions were *5′-GATGTTTGGGOTGTA*GTGGTTGTT-3′and5′-CCACCCCAATACTAAATCAC AACA-3′ while primers for the methylated reactions were 5′-TTCGGGGTGTAG CGCTCGTC-3′ and 5′-GCCCCAATA CTAAATCAGACG-3′. The annealing PCR temperature was 59 °C, and the resulting amplicons were analyzed on nondenaturing polyacrylamide gels stained with ethidium bromide and visualized under UV-illumination. Normal lymphocytes, breast, kidney, and liver had completely unmethylated GSTP1 promoters, while PCas showed a very high level of methylation. Much lower methylation levels were identified in lung and colon cancers and non-Hodgkin lymphomas. The endometrial, ovarian, bladder, pancreatic, head and neck cancers, melanomas, and meningiomas showed no GSTP1 promoter methylation. GSTP1 methylation has been analyzed in urinary sediments following prostatic massage and found to have an overall sensitivity of 73 % and a specificity of 98 % in a relatively small patient cohort [200].

Although there is utility for promoter methylation analysis, including GSTP1 PCa promoter methylation, it is relatively cumbersome, time-consuming, complex to perform, and often difficult to get to work reliably, as ~90 % of the DNA is often lost in the bisulfite treatment step. The loss of so much DNA with the bisulfite treatment step makes this assay difficult to apply to human testing, as often very little DNA is obtained from sources such as blood or urine sediments. Lastly, methylation-specific promoter design can be very complex. For these reasons, MSP is not often used routinely in molecular diagnostics.

9.6.7 Alpha-Methylacyl-CoA Racemase (AMACR)

Alpha-methylacyl-CoA racemase (AMACR) is encoded in the p504S gene found at 5p13. It is localized to the peroxisomes and mitochondria, where it interconverts a large variety of *(R)*- and *(S)*-2-methyl-branched-chain-fatty acyl-CoAs, an activity

necessary for the degradation of these substrates by peroxisomal β-oxidation [201–203]. AMACR levels are increased in PCa compared to benign prostatic epithelium, as measured by immunohistochemistry, Western blotting, microarray, and RT-PCR [204–206]. With immunohistochemical staining, AMACR shows 97 % sensitivity and 100 % specificity for PCa detection [206]. The major drawback of employing AMACR in PCa testing is that AMACR levels are increased in other malignancies and normal tissues. Increased AMACR can be seen in lesions such as metanephric adenomas and papillary renal cell carcinoma [207, 208]. Presently, AMACR immunohistochemical staining, often combined with p63 immunohistochemical staining, is mainly employed in the interpretation of diagnostically difficult prostate needle core biopsies, where p63 staining identifies basal myoepithelial cells and AMACR identifies PCa [205].

9.6.8 MicroRNA Expression in PCa

MicroRNAs (miRNAs) are small noncoding RNAs that regulate the translational efficiency of target mRNAs. They typically bind to 3′ or 5′ untranslated regions of the target mRNAs, leading to lowered stability or decreased translation efficiency [209]. It is estimated that ~30 % of gene expression is regulated by miRNAs, and they have been demonstrated to be dysregulated in a variety of human malignancies. Interestingly, some miRNAs function as tumor suppressors and others act as oncogenes, with the former suppressed and the latter overexpressed in human malignancies [209–212]. Multiple studies have shown that PCa shows an altered miRNA expression pattern, with more than 50 miRNA species dysregulated in this disease [213]. Interestingly, some of the dysregulated miRNAs give biochemical information about the specific PCa. For example, miR-21 expression is significantly higher in androgen-independent than in androgen-dependent PCa cells while expression of miR-203 correlates with PCa progression and metastases [214, 215]. Although presently mainly a research tool, miRNA expression profiling in PCa may eventually have value in the diagnosis and treatment of PCa. miRNA expression appears to lend itself more easily to profiling analysis, as miRNA typically regulates about 200 different mRNAs [209–212]. Thus, a relatively low number of miRNA species studied may be superior to profiling the expression of hundreds of different mRNAs in PCa. One study supports this contention, where miRNA was highly informative in analyzing human tumors, whereas mRNA profiling was highly inaccurate when applied to the same system [216].

9.6.9 Single Nucleotide Polymorphisms

A large number of single nucleotide polymorphisms (SNPs) have been implicated in the risk of developing PCa, including SNPs in the ATM, AR, E-cadherin, ICAM-1s, -4, and -5, APE1, VDR, p53, CDKN1A, CCND1, MDM2, hOGG1,

VEGF, EGF, and HIF-1α genes [14, 229–238]. Presently, SNP analysis is largely confined to basic research and not used in the clinical molecular diagnostics of PCa. It is likely within the next few years that SNP analysis will be employed as part of an algorithm to establish an individual's relative risk for the development of PCa and other pathologies. Such an analysis would be facilitated by accurate and low-cost whole genome sequencing technologies [217].

9.6.10 Golgi Phosphoprotein 2 (GOLPH2)

Golgi phosphoprotein 2 (GOLPH2) is a type II Golgi membrane phosphoprotein that plays a role in liver disease and may be a potential serum marker for hepatocellular carcinoma [218–221]. Laxman et al. [222] obtained urine samples from 276 men prior to prostrate needle biopsy (n = 216) or radical prostatectomy (n = 60). The urine was collected following mild digital pressure to the prostate, and RNA was isolated and analyzed via quantitative PCR. *GOLPH2* mRNA expression analysis outperformed serum PSA as a predictor of PCa. Similarly, other investigators found *GOLPH2* overexpression in PCa by immunohistochemistry, microarray, and RT-PCR [223, 224]. Although not presently used in the molecular diagnostic of PCa, *GOLPH2* expression analysis may be used in PCa diagnosis in the future, particularly as part of a panel [222, 225] (see below).

9.6.11 Biomarker Panels in PCa Molecular Diagnostics

The above data indicate that molecular pathology underlying PCa is exceptionally complex, with many different genetic events playing a role in the development and progression of PCa. Many of the molecular alterations seen in PCa are present only in a subset of PCas or carry altered prognostic significance only when accompanied by other concomitant molecular alterations; the poorer prognosis seen with concomitant *TMPRSS2:ERG* fusion and *PTEN* loss are examples [93]. Several studies have addressed this issue. For example, Laxman et al. [222] found by univariant analysis that increased *GOLPH2, SPINK1, PCA3*, and *TMPRSS2-ERG* fusion all were significant predictors of PCa. However, multivariate regression analysis demonstrated that a multiplexed model incorporating all of the biomarkers outperformed both serum PSA and PCA3 by itself in detecting PCa. Similarly, Vener et al. [225] developed a multiplexed, methylation-specific PCR array consisting of three methylation markers for *GSTP1, RARB,* and *APC,* along with one endogenous control *ACTB*, in a closed-tube, homogeneous assay format. A total of 234 urine samples were examined following digital rectal examinations in two independent cohorts of men with PSA ≥2.5 μg/L. Combined with factors such as race, patient age, and serum PSA, the addition of these markers improved PCa detection. It is likely that future PCa molecular diagnostic testing will often involve gene expression/mutation panels to increase assay sensitivity and specificity.

9.7 Conclusion

The spectrum of PCa ranges from relatively indolent tumors, where an active surveillance approach offers maximum benefit to the patient, to PCa cases with metastatic progression and death occurring within a matter of months. Obviously, many cases require aggressive clinical intervention [226–228]. Presently, our ability to differentiate between these distinct entities is poor, thus leaving some patients overtreated and others tragically undertreated. Although current PCa molecular diagnostics are extremely limited, our understanding of this disease has vastly increased in a relatively short period of time. Within the next few years, molecular diagnostics will very likely be applied to PCa on a routine basis and prove a useful tool for patient risk stratification and a personalized therapeutic approach to each individual patient.

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Chapter 10
Molecular Diagnosis of Bladder and Kidney Cancer

Marino E. Leon, Dahui Qin, and Debra L. Zynger

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Abstract This chapter will review the molecular diagnosis of bladder and kidney cancer. In current clinical practice, early detection and diagnosis of urothelial carcinoma is based on urinary cytology. UroVysion™ bladder cancer fluorescence in situ hybridization (FISH) has become a useful ancillary test in the detection of urothelial carcinoma for initial diagnosis and recurrence; this test is applicable to routine cytologic urine specimens. Multiple studies have shown that UroVysion™ FISH in voided urine and washing specimens can help in patient management due to its superior sensitivity over cytology in certain situations. Within the renal cortex,

M.E. Leon, M.D. (✉)
Department of Anatomic Pathology, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: marino.leon@moffitt.org

D. Qin, M.D., Ph.D.
Department of Hematopathology and Laboratory Medicine and Anatomic Pathology, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida College of Medicine, 12902 Magnolia Drive, Tampa, FL 33612, USA

D.L. Zynger, M.D.
Department of Pathology, The Ohio State University, Wexner Medical Center, Columbus, OH, USA

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molecular studies have not achieved routine use; the most widely available tests are used to identify Xp11.2 translocations/TFE fusions for the subclassification of renal cell carcinoma.

Keywords Carcinoma • Bladder • Diagnosis • Molecular • Urothelial

Abbreviations

BCG	Bacillus Calmette-Guerin
BTA	Bladder Tumor Antigen
CEP	Chromosome Enumeration Probes
FDP	Fibrinogen Degradation Products
FDP	Fibrinogen Degradation
FGFR3	Fibroblast Growth Factor Receptor 3
FISH	Fluorescent in Situ Hybridization
hCFHrp	Human Complement Factor H Related Protein
LSI	Locus Specific Identifier
TPS	Tissue Polypeptide Specific

10.1 Introduction

This chapter will focus on the current clinical molecular diagnosis of neoplasms arising in the urothelium, the specialized form of transitional epithelium that lines the renal pelvis, ureters, bladder, and urethra. The urothelium is composed of a surface layer of large cells, the umbrella cells, and several layers of cells with smaller oval nuclei resting on a well developed basement membrane.

Urothelial carcinoma is more common in males than females [1]. This latter predominance may be explained by a higher prevalence of cigarette smoking in men. Tobacco use, especially cigarette smoking, is one of the leading causes of urothelial carcinoma in the Western hemisphere [1–3]. Additionally, other implicated toxins have been identified including 4-aminobiphenyl, benzidine, 2-naphthylamine and phenacetin-containing analgesics, and cancer chemotherapeutic agents, such as phosphoramide mustards [3]. In the Middle East and parts of Africa, Schistosoma bladder infection is the main cause of squamous cell carcinoma of the bladder [1, 3].

About 70 % of urothelial carcinomas are superficial including non-invasive low-grade papillary (Ta), carcinoma in situ (Tis) [4] and urothelial carcinomas with invasion of subepithelial connective tissue (T1) [5–7]. The remaining 30 % present as muscularis propria invasive disease (≥T2) and are associated with a poor prognosis [6]. Of the superficial urothelial carcinomas, most patients with non-invasive low-grade papillary urothelial neoplasms have a good prognosis [5, 8, 9] yet approximately 70 % of these patients have recurrences [8, 10], and less than 5 % of cases progress to invasive carcinoma [8, 10]. Patients with low-grade and lower

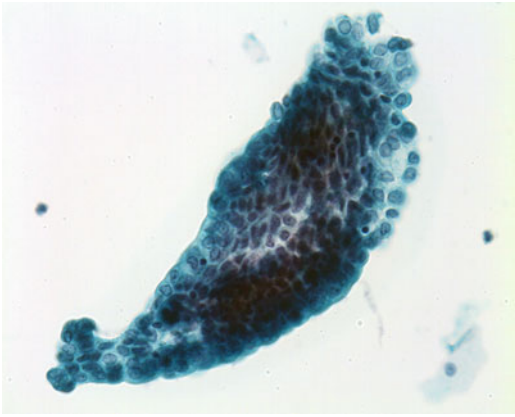

Fig. 10.1 Low-grade urothelial neoplasm with papillary architecture. Urine cytology specimen stained with Papanicolaou staining (original magnification 400×)

pathologic T stage tumors tend to have lower rates of recurrence, progression, and mortality than high-grade and/or higher pathologic stage, suggesting a different biology in tumors that are prone to recurrence [9].

Two main pathways are believed to be involved in urothelial carcinogenesis [11, 12]. One pathway involves the mutation of the *Fibroblast Growth Factor receptor 3* gene (*FGFR3*); this appears to be a favorable pathway giving rise to low-grade non-invasive papillary tumors that may recur but rarely invade [11–13]. The second pathway involves mutation and deletions in the *TP53* gene; tumors that harbor *TP53* gene mutations are high-grade, including high-grade papillary urothelial carcinoma and urothelial carcinoma in situ [11–13]. Supporting this genetic profile, expression array (cDNA) analysis shows specific mRNA signatures associated with either one of these two pathways [14]. Additionally, several other chromosomal abnormalities are observed in high-grade urothelial carcinoma. Alterations that involve chromosome 9 are frequent, including monosomy, and deletions of 9p and 9q [4, 15–19]. Loss of chromosomes 4 and 8, deletions of 2q, 8p, 11p, 13q, 14q, and 17p, and gains of chromosomes, 3, 7, and 17 are also reported [15, 16, 18–20]. Furthermore, papillary tumors and intraepithelial urothelial neoplasia show deletions of chromosome 9 [16, 18, 21]. Specifically, 9p21 deletion that affects the tumor suppressor gene p16 *(INK4a)* is seen [22]. A detailed discussion of the molecular mechanisms of urothelial carcinoma formation is beyond the scope of this review; recent publications are suggested for this purpose [12, 13, 23].

Urinary tract carcinoma presents with painless hematuria making early identification sometimes difficult [6, 24]. Patients with carcinoma in situ present with dysuria, nocturia, urinary frequency and urgency with microscopic hematuria [25]. In the current clinical practice, early detection and diagnosis is based on urinary cytology [6, 26, 27]. Urine cytology is the initial test of choice for screening and follow-up of urothelial carcinoma [27–32]. Cytology is a highly reliable, sensitive, and specific test in the diagnosis of high-grade urothelial carcinoma [27]. However, given the cytomorphologic features of a low-grade urothelial neoplasm, the findings may be equivocal or negative in a large proportion of patients yielding a low sensitivity for low-grade urothelial tumors (Fig. 10.1) [27]. Due to the low sensitivity of

a single urine cytology study, several specimens may be needed to detect a tumor, justifying the use of three consecutive urine specimens [27]. Additionally, the diagnosis is difficult in patients with upper urinary tract neoplasms and in patients with urothelial carcinoma treated with bacillus Calmette-Guerin (BCG) [33–35]. Many ancillary studies used in conjunction with urine cytology have been explored to further characterize equivocal cytology [30, 32]. Unfortunately, an ideal ancillary test for screening and monitoring has yet to be created [30, 32, 36, 37].

Once the initial evaluation and detection with cytology is performed using voided urine specimens, the patients undergo cystoscopy with a confirmatory biopsy [32]. However, cystoscopy is invasive and expensive. Therefore, there is a need to the develop markers capable of identifying urothelial carcinoma by a more economical and/or less invasive methodology [8, 28, 31, 32].

10.2 UroVysion™ Fluorescent In Situ Hybridization (FISH) Test for Urothelial Carcinoma

The FISH technique allows for the visualization of a specific nucleic acid sequence area within a cell by the precise annealing of a fluorescent labeled single strand of DNA fragment, called a probe, to its complementary DNA target sequence on the chromosomes. The hybridization of the probes is visible in a fluorescent microscope as fluorescent dots. Each dot represents a copy of the targeted sequence.

Currently, FISH analysis of urothelial cells from urine has emerged as powerful ancillary technique addressing the limitations of cytopathology [33]. The commercially available UroVysion™ FISH multiprobe (Abbot, DesPlaines, IL) is a molecular test with proven clinical value [22, 33, 34, 38, 39] and it has become a well known and established test to further characterize urine cytology specimens [20, 22, 34, 38–41]. UroVysion™ is Food and Drug Administration (FDA)-approved for the detection of urothelial carcinoma in voided urine specimens from patients with gross or microscopic hematuria and no previous history of urothelial carcinoma and for the detection of recurrent urothelial carcinoma in voided urine specimens from patients with a prior history of urothelial carcinoma [39, 42].

UroVysion™ bladder cancer FISH kit is applicable to routine cytologic urine specimens fixed on slides. This kit consists of a four color, four probe mixture of DNA sequences to determined regions on chromosomes 3, 7, 9, and 17 [22, 39] including three Chromosome Enumeration Probes (CEP), CEP 3 SpectrumRed, CEP 7 SpectrumGreen, and CEP 17 SpectrumAqua, and a Locus Specific Identifier (LSI) 9p21 SpectrumGold (Fig. 10.2) [43]. Therefore, this test is capable of detecting increased copy numbers (polysomy) of the chromosomes 3, 7 and 17 and deletion of 9p21 (Figs. 10.3 and 10.4) [33, 39].

The criteria for a FISH positive result as suggested by the manufacturer of UroVysion™ are as follows: abnormal cells show a gain of multiple chromosomes (three or more signals of CEP 3, CEP 7, or CEP 17) or a homozygous loss of 9p21. This assay requires that a minimum of 25 morphologically abnormal cells are to be

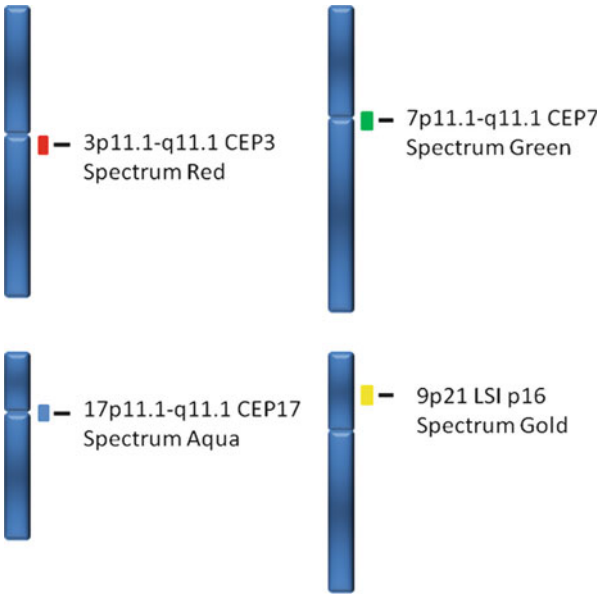

Fig. 10.2 The specific markers used on the UroVysion™ kit are shown in this graph. Note that chromosome enumeration probes (CEP) are used for chromosomes 3 (*Spectrum Red*), 7 (*Spectrum Green*) and 17 (*Spectrum Aqua*); a locus specific identifier is used in chromosome 9p21 (*Spectrum Gold*)

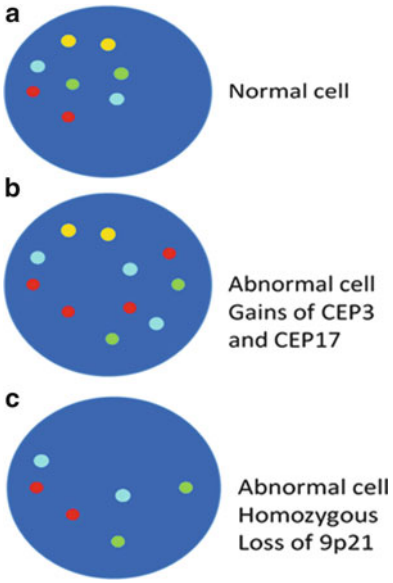

Fig. 10.3 (**a**) Diagram of a chromosomally normal cell nucleus with presence of all the tested probes; two of each CEP 3 *red*, CEP 7 *green*, CEP 17 *aqua*, and LIS 9p21 *gold* respectively. (**b**) Diagram of a chromosomally abnormal cell with additional signals for CEP 3, four signals, and CEP17, three signals. (**c**) Diagram of a chromosomally abnormal cell with homozygous loss of LSI 9p21 *gold* signals

analyzed [44]. Specifically, "analysis should continue until either ≥4 cells with gains of multiple chromosomes or ≥12 cells with homozygous loss of 9p21 are detected or the entire sample is analyzed. The total number of chromosomally abnormal cells, i.e. cells with gain of multiple chromosomes or homozygous loss of

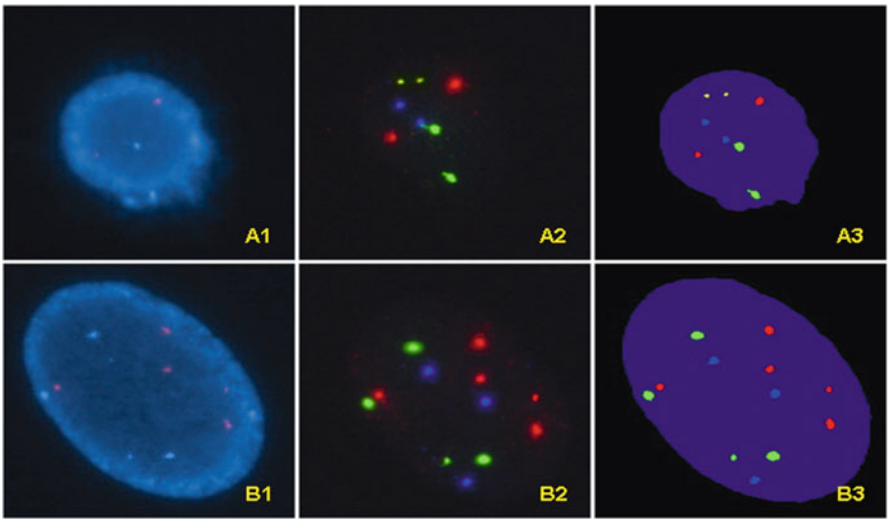

Fig. 10.4 *A1*. An UroVysion negative ("normal") cell observed with DAPI filter. *A2*. The same UroVysion negative cell observed with filters for *gold*, *green*, *red*, and *aqua* respectively; the actual photographs from different filters are overlapped using software. The results show normal 9p21 locus (two *gold signals*) and normal number of chromosomes 3 (two *red signals*), 7 (two *green signals*) and 17 (two *aqua signals*). *A3*. The overlapping images of the same UroVysion negative cell with computer generated pseudo-color. *B1*. An UroVysion abnormal cell observed with DAPI filter. *B2*. The same UroVysion abnormal cell observed with filters for *gold*, *red*, *green*, and *aqua* respectively; the actual photographs from different filters are overlapped using software; gains in chromosome 3 (five *red signals*), 7 (four *green signals*) and 17 (three *aqua signals*) are noted. Note that the signal for 9p21 (*gold*) is not present due to homozygous loss of 9p21. *B3*. The overlapping images of the same UroVysion abnormal cell with computer generated pseudo-color

9p21, are determined; results are reported as positive or negative" [44]. As such, a positive result is reported when either four or more cells with multiple chromosomal gains or 12 or more cells with loss of both signals for 9p21 are observed. When there is less than four cells with chromosome gain or less than 12 cells with 9q21 loss, a negative result is reported with a comment, indicating how many cells with chromosome gain or how many cells with 9q21 loss. The study may be aided using either FDA cleared systems BioView Duet™ System for Automated FISH scanning of UroVysion™ (Rehovot, Israel) or the *onco*FISH® bladder for automated UroVysion™ by Ikoniscope® Digital Microscopy System (Ikonisys, New Haven, Connecticut) [45–47]. When an automated system is used, the assay requires the screening of more cells before a negative result can be issued [48, 49]. In the case of BioView Duet™ System, at least 100 cells are needed to be screened for a negative case [48, 49]. These systems enhance the practice; however, they do not replace the microscopic evaluation by cytology professionals.

UroVysion™ may detect more abnormalities than cytology [20, 50]. Multiple studies have shown that UroVysion™ FISH in voided urine and washing specimens can help in patient management due to its superior sensitivity over cytology in

different situations [50]. It can be particularly useful to clarify equivocal cytological findings [33]. UroVysion™ can aid in the clinical follow-up after BCG treatment to identify patients with therapy failure. In this clinical circumstance, UroVysion™ is also better than cytology [20, 41]. UroVysion™ FISH is more sensitive than cytology in diagnosing high-grade tumors than low-grade tumors and flat intraepithelial lesions [51], yet as with any test, clinical correlation is always recommended. Additionally, UroVysion™ may clarify cases with reactive epithelial atypia [35]. In these specimens, the overall sensitivity of cytology improves when coupled with UroVysion™ [52]. In patients followed with UroVysion™ for urothelial carcinoma, 27 % of patients without clinical evidence of tumor will demonstrate a positive UroVysion™ FISH result [53]. Interestingly, about 63 % of these patients will develop a clinically and pathologically recognizable recurrent tumor within 2 years [20, 53]. Although UroVysion™ FISH has a higher sensitivity than cytology, the specificity is reported as similar or higher for cytology [50, 51].

Further molecular applications, such as coupling UroVysion™ FISH and FGFR3 mutational status are in investigation [54]. Although the following tests are not clinically used at this time, they represent a glimpse of future possibilities. Among these are the testing of epigenetic alterations in bladder carcinogenesis involving promoter hypermethylation and aberrant expression of microRNA [14]. Notably, aberrant expression of specific microRNAs is associated with the FGFR3 mutant bladder carcinogenesis pathway [14].

10.3 Other Assays for Urothelial Carcinoma

DNA ploidy has been used as an ancillary study in urine cytology [27, 31, 55–59]. Initially, DNA ploidy was determined using flow cytometry [60–63]. Later, DNA ploidy was performed using image analysis automated systems on Feulgen stained cytology preparations [55, 57, 58]. Of these methods, DNA ploidy by image analysis was found superior to DNA ploidy by flow cytometry [57]. This method also has some value in the follow-up detection of recurrent disease in patients with urinary diversion [55]. UroVysion™ is more specific than DNA ploidy image cytometry for the detection of aneuploid neoplastic cells, identifying twice as many abnormalities, and as a result has become a more popular ancillary test in the detection of urothelial carcinoma [52].

Other tests described in the literature are Bladder Tumor Antigen (BTA *stat*®, and BTA TRAK®), nuclear matrix protein 22 (NMP22® BladderCheck®), urinary fibrinogen degradation products (FDP), ImmunoCyt™/uCyt+™, Quanticyt, Hb-dipstick, LewisX, Telomerase, Microsatellite LOH, cytokeratin 19 fragment enzyme link immunoabsorbent assay (CYFRA21-1 ELISA), urinary bladder cancer enzyme link immunoabsorbent assay (UBC ELISA), rt-PCR to determine the expression of cytokeratin 20, hyaluronic acid and hyaluronidase (HA-HAase), urine tissue polypeptide specific antigens (TPS), cDNA microarray and detection of certain microRNAs [13, 43, 64–70].

In addition to UroVysion™ FISH multiprobe (Abbot, DesPlaines, IL), BTA *stat*® and BTA-TRAK® (polyMedco, Radmond, WA), NMP22® BladderCheck® (Inverness Medical Innovation, Bedford, UK) and ImmunoCyt™/uCyt+™ (Scimedx, Denville, New Jersey) also have FDA approval for bladder cancer surveillance [68, 69, 71, 72].

ImmunoCyt™/uCyt+™ is an immunofluorescent test that uses three monoclonal antibodies labeled with fluorescent markers; two antibodies, M344 and LDQ10, react with a mucin glycoprotein and a third antibody, 19A211, reacts with a glycosylated form of carcinoembryonic antigen [73]. BTA *stat*® is an immunoassay using two different monoclonal antibodies to human complement factor H related protein (hCFHrp) [25, 72, 74–76]. NMP22® BladderCheck® is based on a lateral flow immunochromatographic method detecting a nuclear matrix protein 22 utilizing two different monoclonal antibodies, one as a capture antibody and one as a reporter antibody [71, 72, 77]. Although numerous other molecular studies have been published and are available, their use is still limited to research studies as their clinical relevance has not been established [13].

Recent advances in the knowledge of the molecular pathways and the pathogenesis of bladder cancer could make targeted therapy in urothelial carcinoma possible and the beginning of personalized or individualized therapy directed towards a specific molecular profile of the tumor [78]. The future in bladder carcinoma may involve the use of molecular studies to determine the diagnosis, individual therapy and prognosis [79, 80]. However, in bladder carcinoma, the era of personalized medicine is yet to begin.

10.4 The Molecular Diagnosis of Kidney Tumors

Currently, the molecular diagnosis of kidney tumors is still evolving. Studies mainly involve the molecular characterization of renal carcinomas to identify Xp11.2 translocations/TFE fusions. These carcinomas typically affect children and young adults and show papillary architecture with clear cells and nested patterns, but may also have cells with granular eosinophilic cytoplasm. Renal carcinomas with the *ASPL-TFE3* fusion contain cells with abundant clear to eosinophilic cytoplasm, discrete cell borders, prominent vesicular chromatin, and nucleoli. Psammoma bodies and hyaline nodules are usually present. Renal carcinomas with the *PRCC-TFE3* fusion reveal a more nested architecture with cells with a moderate amount of cytoplasm, hyaline nodules and fewer psammoma bodies. Using immunohistochemistry, these tumors have expression of the TFE3 protein, renal cell carcinoma marker antigen and CD10 and they may be cytokeratin and epithelial membrane antigen positive [76, 81]. The diagnosis of these tumors can be established based on morphology with confirmation by immunohistochemistry for TFE3. Tumors with the Xp11.2 translocation, all involving the *TFE3* gene [82, 83], include:

(a) t(X;1)(p11.2;q21) with the fusion of *PRCC* and *TFE3* genes
(b) t(X;17)(p11.2;p25) with the fusion of *ASPL (RCC17)* and *TFE3* genes

(c) t(X;1)(p11.2;p34) with the fusion of *PSF* and *TFE3* genes
(d) inv(X)(p11;q12) with the fusion of *NonO* (*p54^{nrb}*) and *TFE3* genes

These fusion proteins act as aberrant transcription factors. FISH and other molecular methods may be applied to confirm the specific molecular change in these tumors, although these tests are not widely available [82].

Molecular studies have shown some utility in pediatric tumors such as the cellular variant of congenital mesoblastic nephroma (but not the classic variant); this tumor shows the same t(12;15)(p13;q25) and *ETV6-NTRK3* genes fusion as infantile fibrosarcoma [84]. Rhabdoid tumor of the kidney shows a deletion of the hSNF5/INI1 gene [84]. As with molecular diagnostics for renal cell carcinoma, these tests are not in routine clinical use.

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Chapter 11
Molecular-Genetic Testing in Penile, Scrotal, and Testicular Cancer

Nicholas A. Whitling, Sharon M. Hirsh, and Rodney E. Shackelford

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N.A. Whitling • S.M. Hirsh • R.E. Shackelford (✉)
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, SL-79, New Orleans, LA 70112-2699, USA
e-mail: rshackel@tulane.edu

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Abstract Malignant tumors of the penis, scrotum, and testes are rare, accounting for roughly 0.5 % of all male tumors. Presently the diagnosis of these tumors depends mainly on histologic examination, supplemented with immunohistochemistry. Over the past 20 years, however, many genetic changes have been identified that are largely specific to different tumors and tumor classes, such as 12p gains in testicular germ cell tumors. Although not commonly employed in the analysis of these tumors, molecular genetic testing is now available for many of these tumors and is becoming an increasingly important adjunct in their diagnosis and treatment. Here, we summarize what is known about the molecular pathology of these tumors and some of the molecular testing modalities currently available. We also briefly discuss the possible future molecular diagnostics of these tumors.

Keywords Germ cell tumor • Testes • Seminoma • Granulosa cell tumor • Leydig cell tumor • HPV • Genotyping • Cryptorchidism • Mesothelioma

Abbreviations

AAM	Aggressive angiomyxoma
AFP	α-fetoprotein
CGH	Comparative genomic hybridization
DSRCT	Desmoplastic small round cell tumor
FISH	Fluorescent in situ hybridization
hCG	Human chorionic gonadotropin
HPV	Human papilloma virus
ITGCNU	Intratubular germ cell neoplasia, unclassified
LCCSCT	Large cell calcifying Sertoli cell tumors
LCT	Leydig cell tumors
NOS	Not otherwise specified
PLAP	Placental-like alkaline phosphatase
RMS	Rhabdomyosarcoma
SCC	Squamous cell carcinoma
YST	Yolk sac tumors

11.1 Introduction

Penile cancer is an uncommon malignancy in the US and Europe, with approximately 1,400 cases reported each year in the US and 4,000 in Europe. This accounts for less than 0.5 % of all male cancers and has a peak incidence in men of over 70 years of age [1, 2]. The incidence of this malignancy shows considerable geographic variation, accounting for 10–20 % of male cancer in parts of Asia, Africa, and South America [3–5]. Multiple risk factors have been identified for penile

cancer including tobacco use, human papilloma virus (HPV) infection, phimosis, balanitis, and poor hygiene [6–8]. Cessation of tobacco use and neonatal or early childhood, but not adult, circumcision lowers the incidence of penile cancer, most likely due to the elimination of chronic inflammation and toxic exposure [8–10]. Approximately 95 % of penile cancer is squamous cell carcinoma (SCC), with the remaining penile malignancies mainly being urothelial, basal, and adenocarcinomas, melanoma, and Kaposi sarcoma. Primary sarcomas of the penis, such as leiomyosarcoma, mesothelioma, and malignant fibrohistiocytic sarcomatous tumors are very rare [1–3, 8, 11–13].

11.2 HVP and Penile Cancer

Approximately 40 % of penile SCC is caused by HPV infection, with HPV types 16 and 18 accounting for ~70 % of penile SCCs [9, 11]. Interestingly, uncircumcised men exhibit a reduced clearance of HPV following infection and not surprisingly, the sex partners of uncircumcised men show an elevated cervical cancer incidence [14, 15]. More than 100 different HPV types have been identified, and it has been estimated that up to 200 types exist. Approximately 40 different HPV viruses infect the anogenital tract and other mucosal areas of the body, with infection being necessary, but not sufficient to cause nearly all (99 %) cervical cancers, and a large portion of anogential neoplasms and oral non-keratinizing SCCs. Significant subsets of 13–18 HPV types have been identified as conferring a probable or definite high-oncogenic risk. The molecular mechanisms by which HPV causes transformation are complex, involving host factors, such as chronic inflammation and immunosuppression, and viral-induced cell cycle progression, apoptosis inhibition, checkpoint ablation, and genomic instability induction [16–19]. Many of these events are mediated through the actions of the HPV E6 and E7 gene products, which bind and inactivate p53 and pRb, respectively [18, 19]. Interestingly, in most HPV infections, the viral genome remains episomal with the E6 and E7 proteins expressed at low levels. High-oncogenic risk HPV types, however, integrate into the epithelial basal cell genome and express high levels of E6 and E7, promoting malignancy [18, 19].

11.2.1 HPV Molecular Diagnostics

Although successful HPV vaccines have been developed, HPV infection remains common, with some 270,000 annual deaths worldwide from cervical cancer alone [1, 20–22]. Thus molecular diagnostics for HPV, especially the high-oncogenic risk types, is often clinically warranted. The detection and analysis of HPV types are largely done on cervical biopsies and cytological specimens, with very few studies examining HPV infection in men [16–19, 23, 24]. Like the analysis of cervical HPV infections, the detection of HPV proteins alone is not clinically significant, as many

HPV infections are benign and produce no significant pathology. Several methods have been employed to detect penile HPV. Here, we will review the most commonly used tests.

11.2.2 Histology

Cellular HPV infections produce specific cytologic changes that can often be seen by examination of hematoxylin-eosin stained slides. Although there is some variation with different anatomic sites, HPV infection are typically superficial, displaying enlarged, hyperchromatic nuclei, with irregular contours, and perinuclear cytoplasmic halos (koilocytotic atypia) [25, 26]. Although histologic analysis is relatively inexpensive and has a short turn-around time, interobserver variability is often high, leading to over-diagnosis of dysplasia over diagnosis in some biopsies, mainly due to non-viropathic changes that mimic koilocytic atypia (pseudokoilocytes) [27, 28].

11.2.3 Surrogate Markers for HPV Infection in Penile Cancer

Similar to the analysis of HPV-induced cervical dysplasia, p16INK4A and Ki-67 (MIB-1) have been used as surrogate markers for HPV-induced dysplasia in penile cancer [29–31]. Interestingly, Ki-67 nuclear staining correlates with penile cancer tumor grade and metastatic potential [31]. The utility of p16INK4A and Ki-67 as markers of dysplasia is covered in detail in the gynecologic malignancies chapter.

11.2.4 HPV PCR Testing

Due to the extremely high precision of nucleic acid hybridization and PCR amplification, both reactions are commonly used for HPV nucleic acid amplification and subtype identification. Although useful for detecting HPV nucleic acid, PCR amplification cannot distinguish between clinically inconsequential transient infections and infections related to significant pathology and oncogenesis [32–35]. For this reason, like all clinical testing modalities, PCR-based HPV analysis must be interpreted in the context of histologic findings and patient history and physical examination.

Flores et al. [36] employed a combination of PCR and hybridization to examine HPV status and type in 463 men between 18 and 40 years old, who had no current sexually transmissible infection and no history of genital warts. Samples were taken from six different anogenital regions, as well as from semen, urine, and venous

blood. Samples were taken by genital swabbing, and the DNA was isolated using a QIAamp MiniKit. The samples were lysed and digested with proteinase K, and the DNA was eluted with nuclease-free water and stored at −20 °C until use. PCR was performed with a PGMY09/11 consensus primer pair for a portion of the HPV L1 gene using AmpliTaq Gold in 50 μL volume, with an amplification profile of 95 °C Hot start for 9 min, 95 °C denaturation for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min for 40 cycles, followed by a final extension period of 5 min at 72 °C. Specimen adequacy was determined by co-amplifying human β-globin gene with B_PC04 and B_GH20 primers and a negative water-only control that lacked primers.

The resulting amplicons were biotin-labeled, denatured, hybridized to an HPV genotype strip, washed, probed with strepavidin-horseradish peroxidase conjugate, and washed. The hybridization/strepavidin binding was detected by a 5-min incubation in a mixture of hydrogen peroxide in sodium citrate buffer and tetramethylbenzidine in dimethylformamide. The genotype strip contained the β-globin gene control and L1 gene sequences for HPV types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51–56, 58, 59, 61, 62, 64, 66–73, 81–84, IS39, and CP6108. Strip interpretation was done by labeled overlay, with lines indicating the position of specific HPV probes types, relative to the reference mark. By this method, HPV was identified on the penile shaft, scrotum, and glands in 62.5 %, 50.0 %, and 44.8 % of the cases, respectively, with the oncogenic HPV types found in approximately ~10–20 % of the HPV-positive patients. The assay was highly reproducible, demonstrating that such testing can work on anatomic areas other than the cervix, and revealed a high rate of HPV infection rate in asymptomatic men.

11.2.5 Sanger and Pyrosequencing for HPV Genotyping

Both Sanger and pyrosequencing have been used to analyze cervical HPV infections but, so far, have not been applied to penile or scrotal patient samples. Due to the very low volume of these samples compared with the large number of cervical samples, clinical testing of penile and scrotal tissues for HPV by sequencing is unlikely to be implemented.

11.3 Scrotal/Paratesticular Cancer

Scrotal cancer is an uncommon malignancy, occurring in 1 per 1 million men in the US and Western Europe. SCC is most common (~27 %), with basal cell carcinoma (19 %), Bowen's disease (15 %), various sarcomas (13 %), extramammary Paget's disease (12 %), malignant melanoma (8 %), lymphoma (2 %), and mesothelioma (1 %) making up most of the remainder. The remaining 3 % are very rare tumors (see below, [36]). Percivall Pott carried out the initial epidemiological studies of

scrotal cancer in 1775 and first described a strong correlation between being the profession of chimney sweep and scrotal cancer [37]:

> It is a disease which always makes its first attack on, and its first appearance in, the inferior part of the scrotum; where it produces a superficial, painful, ragged, ill-looking sore, with hard and rising edges. The trade call it the soot-wart. I never saw it under the age of puberty, which is, I suppose, one reason, why it is generally taken, both by patient and surgeon, for venereal, and being treated with mercurials, is thereby soon and much exasperated: in no great length of time, it pervades the skin, dartos, and membranes of the scrotum, and seizes the testicle, which it inlarges, hardens, and renders truly and thoroughly distempered; from whence it makes its way up the spermatic process into the abdomen, most frequently indurating, and spoiling the inguinal glands: when arrived within the abdomen, it affects some of the viscera, and then very soon becomes painfully destructive [sic].

Since this time, scrotal cancer has been related to many different environmental carcinogens, especially petrochemicals and HPV infection [36–39]. PCR combined with restriction-length polymorphism analysis for identifying the HPV type has been employed to analyze scrotal cancer; however, it is not commonly employed in clinical molecular diagnostics [39].

11.4 Rare Scrotal Tumors

A number of rare scrotal tumors show relatively specific molecular alterations. Due to the rarity of these tumors, clinical molecular diagnostics is not performed on them.

11.4.1 Aggressive Angiomyxoma (AAM)

In men, 38 % of aggressive angiomyxomas (AAMs) are derived from the pelviperineal interstitial tissue of the scrotum. Other sites of involvement include the spermatic cord (33 %), perineum (13 %), and intrapelvis organs, such as the urinary bladder (8 %) [41]. Despite this, AAMs primary to the scrotum are very rare, with only a few cases reported. AAMs are rare, locally aggressive, myxoid mesenchymal tumors found almost solely in adult females, usually arising in the soft tissues of the pelvis, perineum, buttocks, and vulva [40–45]. Due to the extreme rarity of scrotal AAM, the tumor is often misdiagnosed as entities such as a hernia sac or hydrocele [41, 46]. No studies have been performed on the molecular pathology underlying scrotal AAMs; however, one study revealed that vulvar AAMs carry a t(8;12)(p12;q15) that causes aberrant expression of the HMGIC transcription factor [47]. Testing for this translocation is not currently performed on AAMs, due to the rarity of this tumor. However, it has been suggested that this translocation might be useful in identifying residual disease [47].

11.4.2 Desmoplastic Small Round Cell Tumor (DSRCT)

Desmoplastic small round cell tumors (DSRCTs) are rare, highly aggressive tumors that confer a poor prognosis; they are found mainly in the pediatric age group and young adults. Nearly all cases are initially confined to the abdomen, with a low percentage found in other anatomic sites, including the scrotum. Histologically, DSRCTs are characterized by small round blue cells embedded within a densely fibrotic, desmoplastic stroma. These tumors are typically reactive for epithelial, mesenchymal, and neural markers [48]. Over 95 % of DSRCTs carry a t(11;22)(p13;q12) that fuse the EWS and WT1 genes [48–50]. Histologically the DSRCT can closely resemble several other rare small blue cell tumors, such as Ewing's sarcoma/primitive neuroectodermal tumor, and some rhabdomyosarcomas (RMS); thus molecular diagnostic testing is often useful in establishing a diagnosis [48–51].

Chromosomal translocation t(11;22)(p13;q12) has been detected by a variety of different molecular techniques. Most commonly, reverse transcriptase-PCR is employed to amplify the nucleic acids regions immediately surrounding the unique EWS and WT1 gene fusion point. Ladanyi et al. [50] isolated RNA from DSRCT snap-frozen tissue and reverse transcription was performed on 1 μg RNA for 30 min at 42 °C using a GeneAmp RNA PCR kit. The reverse transcriptase was denatured at 99 °C for 5 min, and the PCR reagents were added using a forward primer corresponding to EWS exon 7 (5′-TCCTACAGCCAAGCTCCAAGTC-3′) and two reverse primers corresponding to WT1 exons 8 and 9 (5′-ACCTTCGTTCACAGTCCTTTG-3′ and 5′-GACCAGGAGAACTTTCGCTGAC-3′). The fusion sequence was amplified by 40 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. The resulting amplicons were analyzed by gel electrophoresis followed by transfer onto nitrocellulose and probing for the EWS/WT1 fusion product [50, 52].

11.4.3 Liposarcoma

Scrotal/paratesticular liposarcomas are rare malignancies usually found in men in the fourth to sixth decade and include different histologic subtypes, such as well differentiated, myxoid/round cell, pleomorphic, and dedifferentiated liposarcomas. Liposarcomas are usually diagnosed based on the clinical presentation, imaging studies, and histologic analysis [53]. In cases where these modalities do not allow a clear diagnosis, molecular diagnostics can be employed as many liposarcoma subtypes show unique molecular alterations. For example, the myxoid/round cell liposarcomas usually carry a t(12;16)(q13;p11) that can be analyzed by reverse transcriptase-PCR as is done for the EWS/WT1 fusion product [50, 52–54]. Other liposarcoma subtypes carry identifiable molecular alterations, such as 12q13-q21 amplification seen in well- and dedifferentiated liposarcomas [55].

11.4.4 Malignant Mesothelioma

Malignant mesothelioma is a rare tumor that arises in mesothelial-lined body cavities, most commonly in the pleura and peritoneum and far less commonly in the tunica vaginalis and pericardium. Malignant mesothelioma can exhibit a wide spectrum of histologic subtypes making diagnosis based on histology alone difficult. Although specific molecular alterations have been identified in malignant mesothelioma such as a unique microRNA expression pattern, immunohistochemical analysis is typically used in cases were a diagnosis is not possible based on histology alone [56, 57].

11.4.5 Rhabdomyosarcoma (RMS)

Rhabdomyosarcoma (RMS) can occur anywhere in the body, with ~7 % occurring in the paratesticular tissue. In this location, embryonal RMS is most common with other types, such as the alveolar and pleomorphic RMS types being less common [58–60]. Most RMSs occur in the pediatric group and in young adults, with a bimodal age distribution showing peaks at 5 and 16 years of age [60]. The diagnosis of RMS depends on the clinical presentation, histology, and immunohistochemistry [58–60]. Seventy to eighty percentage alveolar RMS carry t(2;13)(q35;q14) (~70 %) or t(1;13)(p36;q14) (~10 %) translocation fusing portions of the PAX3-FKHR or PAX7-FKHR genes, respectively. Analysis of these translocations usually involves reverse transcriptase-PCR employing primers spanning the gene fusion region [50, 52, 61, 62].

11.4.6 Leiomyosarcoma

Leiomyosarcomas rarely occur in the scrotal/paratesticular area [63]. Although these tumors often carry identifiable molecular changes, they are typically complex and do not lend themselves to clinical molecular analysis [64]. The diagnosis of these tumors typically depends on histologic analysis, supplemented with immunohistochemistry [63].

11.5 Testicular Germ Cell Tumors

Although uncommon, testicular neoplasms are the most common cancers among men aged 15–44 years, with 98 % being germ cell tumors. The highest incidence of testicular tumors (1.5/100,000) is found in individuals with Northern European ancestry, with the incidence in Denmark and Norway up to 10 times higher than in

African and Asian men [65–67]. In 2006, the American Cancer Society estimated that 8,250 US men developed testicular, with 370 men dying of these tumors. Although these tumors represent ~0.5 % of all Caucasian male cancer, they account for only ~0.1 % of cancer-related deaths, due to many of these tumors being curable. Interestingly, their incidence has increased substantially over the past four decades [66–68].

Roughly 90 % of testicular neoplasm arise from germ cells, with seminomas being most common, accounting for ~50 % of all testicular germ cell tumors and often making a large percentage of mixed germ cell tumors. With the exception of the spermatocytic seminoma, germ cell tumors typically arise from intratubular germ cell neoplasia, unclassified (ITGCNU) [69, 70]. The non-seminomatous germ cell tumors include embryonal carcinoma, yolk sac carcinoma, choriocarcinoma, teratomas, mixed germ cell tumors, spermatocytic seminomas, polyembryomas, and diffuse embryomas [71]. The genetics, histology, and usual clinical course of these cancers are diverse, with tumors, such as seminomas, having a relatively good prognosis (even when widely metastatic), while other tumors, such as choriocarcinoma, having a much poorer prognosis [72]. Several risk factors have been identified for testicular germ cell cancers. These are summarized below.

11.5.1 Genetic Inheritance

Having an effected father or brother with testicular cancer confers a relative risk for testicular cancer of 6–10, consistent with a heritable genetic predisposition [73]. The risk factor is higher in twins than in non-twins, as well as higher in dizygotic than in monozygotic twins, possibly due to higher maternal estrogen levels secondary to two placentas [74]. Several genetic loci conferring a testicular cancer predisposition have been identified. These include the phosphodiesterase 11A gene variants known to reduce the gene product's enzymatic activity and gr/gr deletions on the Y chromosome [75, 76]. Several genome-wide association studies have demonstrated that alterations at 12q22 (KITLG), 5q31.1 (SPRY4), 6p21.31 (BAK1), 5p15 (TERT), and 12p13 (ATF7IP) confer an increased risk for testicular cancer. Interestingly, spermatocytic seminoma, which does not arise from ITGCN, shows a different set of mutations involving DMRT1 and RET [77].

11.5.2 Cryptorchidism

Cryptorchidism (undescended testes) is closely associated with ITGCNU. The incidence of in situ testicular carcinoma is 2–3 % in cryptorchordic testes compared to 0.43 % in bilaterally descended testes – a 32-fold increased risk with cryptorchidism [78–80]. The highest risk is seen with intra-abdominal cryptorchidism, which develops cancer five-times more often than inguinal cryptorchidism

[78]. Additionally, bilateral undescended testicles develop in situ carcinoma at a higher rate than unilateral undescended testicles, and, interestingly, the descended testes in men with unilateral cryptorchidism show an elevated carcinoma incidence of 20–30 % compared to bilaterally descended testes [78, 81]. Delayed orchiopexy increases the risk for cancer, supporting a casual role for cryptorchidism leading to cancer [82, 83]. However, Kallmann syndrome, characterized by congenital hypogonadotropic hypogonadism and cryptorchidism, does not shown an elevated testicular cancer incidence [84]. Additionally, although different ethnic groups have roughly the same incidence of cryptorchidism, testicular cancer between different ethnic groups shows enormous differences [85]. Last, both conditions are associated with premature birth, low birth weight, and other gonadal abnormalities [86]. Presently, it is not clear whether cryptorchidism confers an increased cancer risk or whether cryptorchidism and cancer share common risk factors. They are however closely interrelated.

11.5.3 Sexual Development Disorders

Sexual development disorders are congenital conditions in which sexual development is atypical at the anatomic, gonadal, or chromosomal/genetic levels(s). Many show an elevated incidence of testicular cancer. The highest increased incidence is seen in testes-specific protein Y-encoded positive gonadal dysgenesis, which shows a 15–35 % increase in germ cell tumors. Individuals with this condition overexpress testes-specific protein Y-encoded, the main candidate for the so-called gonadoblastoma locus on Y chromosome. Testes-specific protein Y-encoded is highly expressed in ITGCNU and seminomas (coexpressing with placental-like alkaline phosphatase [PLAP], c-KIT, OCT4, Ki-67, and cyclin B), indicating a possible role in carcinogenesis, Additionally, its ectopic expression in cultured cells up-regulates progrowth genes frequently overexpressed in ITGCNU [87, 88]. Other sexual development disorders, including partial androgen insensitivity syndrome, complete androgen insensitivity, and ovotesticular sexual development, show increased testicular cancer incidences of 15 %, 0.8 %, and 2.6 %, respectively [87, 88].

11.5.4 Other Factors

Many other factors may be involved in the pathogenesis of testicular cancer. These include low birth weight, exogenous hormone and disrupting endocrine chemical exposures, maternal age, impaired male fertility, height, body mass index, occupational exposure, childhood physical activity, and testicular trauma. For many of these factors, the evidence is either weak, or presently equivocal, and further studies are needed define the specific roles of these factors in testicular carcinogenesis [89].

11.5.5 Molecular Testing for Testicular Germ Cell Neoplasms

The diagnosis of most testicular tumors is accomplished by the histologic analysis of hematoxylin-eosin-stained slides, often supplemented with immunohistochemistry. In fact, only three laboratories in the US currently offer molecular diagnostic testing for testicular tumors [90]. Here, we will review some of the molecular techniques applied to each germ cell neoplasm type.

11.5.5.1 Intratubular Germ Cell Neoplasia Unclassified

ITGCNU is the precursor lesion for most germ cell neoplasms, with the exception of the spermatocytic seminoma, and is found at increased rates in conditions with an increased germ cell tumor incidence ([69–71], see above). Histologically, ITGCU is characterized by seminiferous tubules containing large atypical primitive appearing cells, with clear cytoplasm, enlarged vesicular nuclei, and prominent nucleoli. There is no spermatogenesis present, and the tubular lamina propria is often thickened and hyalinized. The cells typically stain for PLAP, CD117, OCT4, and p53. ITGCNU usually does not spontaneously regress and most cases will eventually progress to an invasive germ cell tumor. The treatment is typically radiotherapy or orchiectomy, with the former preserving testicular endocrine functions (but not fertility), and the latter providing the greatest success rates [71].

Very few molecular studies of ITGCNU have been performed, although comparative genomic hybridization (CGH) studies have demonstrated that ITGCNU carries many of the chromosomal alterations seen in seminomas and other germ cell tumors, including gains of material from chromosomes 1, 5, 7, 8, 12q, and X, with loss of material from chromosome 18. Interestingly, the commonly seen gains of 12p in seminomas and other germ cell tumors are not present, indicating that gains at 12p may be required for the progression of ITGCNU to invasive neoplasm [91, 92]. Presently, besides histologic analyses, immunohistochemistry is used to diagnose ITGCNU, where immunostains for p53, PLAP, OCT4, and CD117 are helpful is establishing a diagnosis, as is periodic acid-Schiff staining [71, 93].

11.5.5.2 Seminomas

Seminomas are the most common post-pubertal testicular germ cell neoplasms, with the World Health Organization recognizing a seminoma with syncytiotrophoblastic giant cells variant. Seminomas have several histologic variants characterized by different growth patterns including cribiform/microcystic, tubular, intertubular, and the anaplastic or atypical seminoma characterized by an increased mitotic rate, greater nuclear pleomorphism, and a lower number of associated infiltrating lymphocytes. Immunohistochemically, seminomas are PLAP, OCT4, and CD117 positive, focally/weakly positive for cytokeratin 40 % of the time, and negative for CD30, α-fetoprotein (AFP), and human chorionic gonadotropin (hCG) [71].

Histologically seminomas generally show a pattern of sheets of tumor cells with clear cytoplasm separated by fibrous trabeculae rich in T-lymphocytes. The tumor cells are large, uniform, round to polyhedral cells having distinct plasma membranes, clear cytoplasm, and large nuclei with "salt and pepper" chromatin and one to several prominent nucleoli. Mitotic activity is usually low, and about 10 % of seminomas will have a significant non-seminomatous component [61].

Although multiple genetic alterations have been identified in testicular seminomas, 12p overexpression often secondary to isochromosome formation is the only consistent change found in seminomas and other testicular germ cell neoplasms [94–96]. Analysis of ITGCNU following laser capture microdissection with degenerate oligonucleotide primed PCR reveal gains in 12p in ITGCNU adjacent to histologically identifiable invasive germ cell tumors, while ITGCNU without a nearby invasive component either had no 12p gains or only "diminutive" 12p gains. Thus, 12p gains appear to be required for the development of germ cell neoplasia invasion [97]. Comparative expressed sequence hybridization studies have revealed overexpressed genomic sequences at 12p11.2-p12.1, 4q12, and 12p13, with the KRAS2, CMAS, EKI1, LRMP, C12orf2, ITPR2, FLJ10637, TM7SF3, SURB7, CCND2, MLF2, PTMS, GLU3, HPH1, c-KIT, and LRP6 genes commonly overexpressed [98–100].

11.5.5.3 Germ Cell Tumor Molecular Testing

CGH array and fluorescent in situ hybridization (FISH) are presently the main molecular tests used to detect seminomas and other testicular germ cell tumors, with other testing modalities presently limited to research applications. Both of these techniques work well in the identification of 12p amplification and have utility in the analysis of testicular germ cell tumors where histology and immunohistochemistry give ambiguous results.

11.5.5.3.1 CGH

In CGH reference (normal) and test (tumor) tissues are compared to identify deleted or amplified genomic tumor regions in the test sample. The reference and test genomic DNAs are isolated and labeled with different fluorochromes. Care is taken such that the two fluorochromes do not have any spectral overlap. Additionally, the reference sample is always from healthy tissue, often from sources such as peripheral lymphocytes. The two labeled samples are then hybridized together on an array that covers the genomic area being interrogated. Typically, the sequences being interrogated are attached to a solid support, such as glass or nitrocellulose that does not interfere with hybridization. The hybridization is performed under maximally stringent temperature and buffer conditions to lessen non-Watson-Crick (non-specific) hybridizations as much as possible. Following hybridization and multiple washing steps, the signal intensity on the solid support is calculated using the

background signal intensity against the intensity of each fluorochrome emission. The ratios of each fluorochrome on each specific hybridization spot is calculated and averaged over several replicate spots. The test to reference sample intensities are often standardized by dividing the median of all the hybridization spots in the sample. Often chromosomal areas, such as the X chromosome, known to not undergo amplification or loss, are used as an internal control [98–100]. CGH has high specificity and under the right conditions is an excellent testing modality. However, it can be more complex, costly, and time-consuming than other molecular tests.

11.5.5.3.2 FISH

FISH is also employed to identify 12p amplification in testicular germ cell neoplasms. Most often, FISH probes specifically designed to hybridize with the regions of 12p, commonly amplified, are used in conjunction with FISH probes to unamplified chromosomal regions. Each FISH probe carries a different fluorochrome. 12p amplification is identified by an increased ratio of 12p FISH probe hybridization to control probe hybridization. FISH assays are relatively easy to perform and over one million assays are done worldwide each year.

11.5.5.4 Testicular Choriocarcinomas

Choriocarcinoma is a rare tumor, representing only about 1 % of testicular tumors in its pure form and more commonly occurs as a component of a mixed tumor. Choriocarcinoma is typically clinically aggressive, often presenting with metastases to the lungs, liver, and brain. Histologically, choriocarcinomas are characterized by an attempt to replicate the architecture of immature placental villi, with a cap of syncytiotrophoblasts (large multinuclear cell with eosinophilic and vacuolated cytoplasm) surrounding cytotrophoblasts consisting of round cells with distinct cell borders, clear cytoplasm, and unremarkable, bland nuclei. Usually, the syncytiotrophoblasts are positive for hCG, HPL, and EMA [71]. Due to the rarity of these tumors, very little molecular testing is performed on them and their analysis rests upon histology, supplemented with immunohistochemistry.

11.5.5.5 Testicular Embryonal Carcinomas

Embryonal carcinoma is rare, accounting for about 2 % of testicular tumors in its pure form and is a component in about 85 % of non-seminomatous testicular germ cell tumors. About two thirds of embryonal carcinomas present with metastases, typically to the lungs. Histologically, embryonal carcinomas typically consist of sheets of epithelioid pleomorphic cells with indistinct cell borders, eosinophilic to amphophilic cytoplasm, with large pleomorphic nuclei having prominent nucleoli and overlapping nuclei. Often many apoptotic bodies and mitotic figures are

present. Embryonal carcinomas are positive for CD30, PLAP, and OCT4 [71]. Like other testicular germ cell tumors, embryonal carcinomas often show 12p amplification [71]. Molecular testing is seldom performed on these tumors and when done is limited to 12p analysis.

11.5.5.6 Testicular Yolk Sac Tumors

Yolk sac tumors (YST), also known as endodermal sinus tumors, recapitulate primary embryonic yolk sac tissue and are usually a component of non-seminomatous testicular germ cell tumors in adults. In the pediatric age group, YST is the most common testicular tumor, with incidence peaking before 2 years old. About 95 % of individuals with a YST component in the tumor show elevated serum AFP. Histologically, YST shows many different architectural patterns, most commonly a "microcystic pattern" consisting of interconnecting cords and ribbons of tumor cells within a myxoid stroma. About 50 % of YCT have Schiller-Duvall bodies, a glomerulus-like structure pathognomonic for YSTs. The tumors cells usually show bland nuclei and eosinophilic hyaline globules that are α-1-antitrypsin and diastase-PAS staining positive, not usually seen in other testicular germ cell tumors. YSTs are usually positive for cytokeratin and AFP, showing diffuse cytoplasmic staining, while CD30 staining is variable. Like other testicular germ cell tumors, YSTs show 12p amplification, which is sometimes analyzed by molecular diagnostics testing [71, 98–101]. Interestingly, pediatric YSTs do not show 12p amplification, but show structural alterations of chromosomes 1, 3, and 6, and RUNX3 gene promoter hypermethylation [102, 103]. These molecular alterations are not commonly analyzed in the diagnosis of pediatric YSTs.

11.5.5.7 Spermatocytic Seminoma

Spermatocytic seminomas are testicular germ cell tumors that do not arise from ITGCN and are unrelated to seminomas. Spermatocytic seminomas are uncommon, representing 1–4 % of testicular tumors, occurring at an average age of 55 years. The histologic analysis of the spermatocytic seminoma lies in identifying three separate cell types: small, intermediate, and multinucleated giant cells. The small cells are 6–8 μm in diameter and have a narrow rim of eosinophilic cytoplasm. Intermediate cells are 15–18 μm in diameter, have round nuclei with filamentous chromatin and prominent nucleoli, and have a more voluminous eosinophilic cytoplasm. The giant cells are more pleomorphic, 50–100 μm in diameter, and can have multiple nuclei. Spermatocytic seminomas usually have a high mitotic index, and lack fibrous septae and lymphocytes. They are positive for CAM5.2 and are usually negative for keratin, EMA, PLAP, OCT4, HCG, CD30, and AFP [71]. Molecular-genetic analyses of spermatocytic seminomas have shown consistent gains on chromosome 9p, with DMRT1 (at 9p24.3) overexpression likely playing a role in spermatocytic seminoma development [71, 77, 98–100, 104]. Presently, molecular diagnostic testing is not performed on this rare tumor.

11.5.5.8 Other Malignant Testicular Germ Cell Tumors

Testicular teratomas, teratocarcinoma, and polyembryomas often show 12p amplification [71]. Due to the rarity of these tumors and their distinctive histology, molecular testing is seldom performed on them.

11.6 Testicular Sex Cord Tumors

Sex cord tumors make up ~5 % of all testicular tumors and are derived from cord-like masses which invaginate from the germinal epithelium during embryogenesis and give rise to the rete testes and seminiferous tubules [105]. In the testes, the sex cord tumors are the Sertoli, Leydig, Sertoli-Leydig, and granulosa cell tumors, which are named by the cell types from which they arise. For many of these tumors, specific subtypes exist that will be discussed in each section on a specific tumor [105–107]. Many of these tumors are very rare. For example, although the ovarian granulosa cell tumor is fairly common, its testicular counterpart is very rare, with only about 28 recorded cases [108]. Thus for most of these tumors, molecular diagnostics is not performed and the molecular alterations underlying these tumors are poorly understood.

11.6.1 Sertoli Cell Tumors

Sertoli cell tumors, also called androblastomas, include the Sertoli cell tumor not otherwise specified (NOS), Sclerosing Sertoli cell tumors, Sertoli-Leydig cell tumors, and large cell calcifying Sertoli cell tumors (LCCSCTs). The latter tumor can be associated with Peutz-Jeger and Carney syndromes. The Lipid-Rich Sertoli cell tumor is found almost exclusively in the ovary [107].

11.6.1.1 Sertoli Cell Tumor NOS

Sertoli cell tumors NOS of the testes make up approximately 1 % of all testicular tumors, appearing at a mean age of 45 years over a wide age range (15–80 years) [109, 110]. About 20 % are malignant and most typically present as a unilateral slowly enlarging well-circumscribed mass. Histologically, the tumor cells typically have moderate amount of pale to slightly eosinophilic cytoplasm, often with large cytoplasmic vacuoles. They exhibit minimal nuclear atypia, a low mitotic rate, and a general resemblance to Sertoli cells. The tumor cells form diffuse sheets or large nodular aggregates and may display a solid, trabecular, or tubular architecture that resemble immature seminiferous tubules [110].

Due to the relative rarity of Sertoli cell tumor NOS of the testes, little is known about the molecular alterations underlying these tumors. CGH studies have shown

multiple molecular changes in Sertoli cell tumor NOS that typically varied from cases to case, indicating a high degree of tumor heterogeneity. Gains in the X chromosome were common. Less frequently, losses of all or portions of chromosome 2 and 19 are seen [111]. Additionally, animal studies have shown that increased β-catenin may play a role in the development of these tumors [112]. Presently, there is insufficient knowledge about the molecular alterations underlying the Sertoli cell tumors, and, due to the rarity of these tumors, clinical molecular testing is rarely performed on them.

11.6.1.2 Sclerosing Sertoli Cell Tumors

Sclerosing Sertoli cell tumors are very rare, with one overview recording only 14 cases reported in the literature [113]. Histologically these tumors resemble those of Sertoli cell tumors NOS, but exhibit extensive hypocellular, collagenous stroma separating clusters of Sertoli cells. The average age of presentation is about 35 years, are usually unilateral, and are rarely malignant [113, 114]. Due to the extreme rarity of these tumors, molecular analyses have not been performed on them.

11.6.1.3 Large Cell Calcifying Sertoli Cell Tumors (LCCSCT)

Large cell calcifying Sertoli cell tumors LCCSCTs are rare tumors with only about 60 cases recorded in the literature. Approximately 25 % of these tumors are malignant and may be associated with the Peutz-Jeghers syndrome (see Chap. 5, Colorectal Carcinoma) or the Carney complex. Histologically, LCCSCTs show large tumor cells with abundant eosinophilic cytoplasm, extensive amorphous calcifications, and tubule formation with focal areas of trabecular or solid differentiation [115]. Malignant tumors typically have two or more of the following features: (1) a size >4 cm, (2) greater than three mitoses/10 high power fields, (3) significant nuclear atypia, (4) necrosis, or (5) lymphovascular invasion [116].

About one third of males with the Carney complex will have LCCSCTs. The Carney complex was first described in 1985 by Carney et al. [117] and is characterized by abnormal skin pigmentation, usually pale brown to black lentigines that are often the presenting feature, myxomas of the heart, skin, breast, oropharynx, and female genital tract, primary pigmented nodular adrenocortical disease often causing Cushing syndrome, multiple thyroid adenomas, and psammomatous schwannomas in approximately 10 % of individuals. The Carney complex is usually diagnosed based on the clinical presentation; however, molecular testing is clinically performed. Approximately 62 % of individuals with Carneys complex have identifiable mutations at 17q23-24, where the tumor suppressor gene, the regulatory subunit type 1A (RIα) of cAMP-dependent protein kinase (PRKAR1A) gene is located [118]. Additionally, about 20 % of individuals with Carney complex have

genetic alterations linked to 2p16, where loss of heterozygosity and copy number gains have been detected [119].

Molecular clinical testing for the Carney Complex usually involves PCR amplification of the 12 exons and proximal intronic regions of the PRKAR1A gene, followed by sequencing of the resulting amplicons. Bertherat et al. [118] analyzed 353 patients who met the diagnostic criteria for Carney complex, had primary pigmented nodular adrenocortical disease, or had previously identified PRKAR1A gene mutations; 62 % of the tested individuals carried detectable PRKAR1A gene mutations, of which 82 % resulted in no detectable protein expression due to nonsense mRNA mediated decay. Interestingly, a frequency of 491-492delTG was found to be associated with lentigines, cardiac myxomas, and thyroid tumors.

11.6.1.4 Sertoli-Leydeg Cell Tumor

The Sertoli-Leydeg cell tumor is exceptionally rare, with less than 10 cases described in the testes [120]. Little is known about the molecular alterations underlying these tumors.

11.6.2 Leydig Cell Tumors (LCT)

Leydig cell tumors (LCTs) represent approximately 0.8–3 % of all testicular tumors and are the most common interstitial neoplasms of the testes [105, 106]. LCTs may occur at any age, but are most commonly shown in those between the ages of 5 and 10 years and between 30 and 60 years. Roughly 10 % of LCTs are malignant, while almost all-pediatric LCTs are benign. Grossly, LCTs are sharply delineated solid masses within the testicular stroma. Microscopically the tumor cells form nest or sheets of large polygonal cells with abundant granular eosinophilic cytoplasm, with scattered lipofuscin pigment and Reinke crystals seen in less than 50 % of these tumors. LCT can be hormonally active, producing either androgens or less commonly estrogens, which lead to masculinizing or femininizing effects [105, 106, 121].

Leydig cell tumorigenesis appears to depend on alterations in the hypothalamic-pituitary-testicular axis leading to excessive Leydig cell stimulation by luteinizing hormone [122]. One study on three pediatric testicular LCTs showed three different mutations in the luteinizing hormone receptor, two of which increased receptor expression and one that increased basal cAMP production 28 ± 3-fold. All three mutations caused increased cell growth when transiently transfected into COS-7 cells. Additionally, a low percentage of adult LCTs show fumarate hydratase mutations [123]. Because little is known about the molecular alterations in LCTs and many of these alterations occur in a low percentage of these tumors [123], the diagnosis of LCTs is presently based on the clinical presentation and histology, supplemented with immunohistochemistry.

11.6.3 Granulosa Cell Tumors

Granulosa cell tumors of the testes are rare, with only 28 cases reported in the literature [108]. The tumor usually presents as a slowly growing testicular mass that histologically resembles its more common ovarian counterpart; usually being well-circumscribed with uniform to mildly pleomorphic cells with medium-sized ovoid or elongated nuclei with fine chromatin, inconspicuous nuclei, and scattered nuclear grooves. In the review by Hanson et al. [108], 21 % of testicular granulosa cell tumors metastasized. Due to the extreme rarity of these tumors, clinical molecular diagnostics is not performed on them. However, one study demonstrated that these tumors often have dysregulated WNT/CTNNB1 and PI3K/AKT signaling pathways [124].

11.7 Conclusion

Clinical molecular diagnostics are not commonly performed on penile, scrotal and testicular malignancies. This is partly due to the rarity of some of these tumors but also because diagnosis can usually be successfully rendered by the combination of clinical presentation, histology, and immunohistochemistry. This is especially true for germ cell tumors of the testes. As new molecular therapies are developed, the identification of specific molecular alterations will become necessary prior to the initiation of therapy.

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Chapter 12
Molecular Pathology and Diagnostics of Gynecologic Malignancies

Rodney E. Shackelford, Masoumeh Ghayouri, Domenico Coppola, and Ardeshir Hakam

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R.E. Shackelford
Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, 1430 Tulane Avenue, SL-79, New Orleans, LA 70112, USA

M. Ghayouri • D. Coppola • A. Hakam, M.D. (✉)
Department of Pathology, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: Ardeshir.Hakam@moffitt.org

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Abstract Gynecologic malignancies account for roughly 13 % of solid tissue tumors in women. Many of these malignancies, such as many ovarian tumors, first present at an advanced stage, making them difficult to treat. Until recently the analysis of these malignancies was largely limited to hematoxylin-eosin-stained slide examination, supplemented with immunohistochemical stains. Recently, molecular diagnostic techniques have been applied to these tumors with some success, especially with BRCA1/2 mutations and Lynch syndrome analyses. Here, we describe the present molecular diagnostic tests applied to gynecologic malignancies and discuss possible future developments in this field.

Keywords Gynecologic malignancies • HPV • BRCA1 • BRCA2 • Mole • Nampt • Ovary • Vulva • Fallopian tubes • Vagina • Lynch syndrome • Endometrium

Abbreviations

CDK	Cyclin-dependent protein kinase
dsDNA	Double-stranded DNA
EST	Endometrial stromal tumor
FDA	US Food and Drug Administration
HNPCC	Hereditary nonpolyposis colorectal cancer
HPV	Human papilloma virus
HRM	High-resolution melting analysis
MMMT	Malignant mixed mullerian tumor
Nampt	Nicotinamide phosphoribosyltransferase
PCR	Polymerase chain reaction
PJS	Peutz-Jeghers syndrome
UCCC	Uterine clear cell carcinoma
UEA	Uterine endometrial adenocarcinoma
USC	Uterine serous carcinoma

12.1 Introduction

Gynecologic malignancies include cervical, endometrial, uterine, vaginal, vulvar, ovarian, and fallopian tube cancers. Together, these malignancies account for 13 % of all solid tumors in women, following breast, lung, and colon cancers, with cervical,

uterine, and ovarian cancers making up ~95 % of gynecologic malignancies [1–3]. Until recently, the diagnosis and analysis of prognostic indicator(s) in gynecologic malignancies largely depended on examination of hematoxylin-eosin-stained slides, supplemented with immunohistochemical analyses. Over the past 15 years, molecular genetic studies of gynecologic malignancies have significantly contributed to our understanding of the pathogenesis and molecular differences between different gynecologic malignancies.

Currently, molecular studies are employed in the diagnosis, prognosis, and management of some types of gynecologic malignancies and of heritable gynecologic cancer syndromes. As molecular testing becomes more common, the cost of testing falls, and its implementation will undoubtedly help our understanding of the molecular pathology underlying gynecologic malignancies [4].

12.2 Cervical Cancer

12.2.1 Epidemiology, HPV, and the Molecular Pathogenesis of Cervical Cancer

Cervical cancer is the second most common gynecologic cancer and the most common cause of cancer-related death in women world-wide, with some 400,000 new cases and 200,000 deaths yearly [2]. In the United States, roughly 12,000 new cases are diagnosed yearly with 4,000 women dying of the disease. In addition, the associated medical costs are now over $2 billion [1–3, 5]. Clinical, molecular, and epidemiologic studies show that cervical cancer is overwhelmingly caused by epitheliotropic human papilloma virus (HPV) infection, with other causes such as diethylstilbestrol exposure, counting for a minority of cases [6–8]. HPV consists of more than 150 related DNA viruses, many capable of infecting multiple sites within the body, including the cervix, vulva, anus, vagina, mouth, esophagus, penis, and more rarely, the lungs, larynx, and stomach [6, 7, 9, 10]. The cervix is most the commonly infected site, with HPV detected in up to 95 % of high-grade cervical dysplasias and cancers [11]. Different HPV types have been classified into two categories based on their potential tumorigenicity. For example, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82 confer a high risk for cervical cancer, whereas types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6 108 carry a low cancer risk, with infection by these associated with condylomatas. Types 16 and 18 are by far the most common high-risk HPV types [6].

In general, the presence of a high-risk HPV type is insufficient to cause cancer. For example, only ~10 % of women infected with high-risk HPV types progress to precancerous lesions, with 20–30 % of those developing invasive cancer [12]. In general, other factors need to be present for progression to cancer, including tobacco use (which interferes with the cervical physical barrier), the presence of other sexually transmitted diseases (for example, HIV or herpes virus), alterations of hormonal status, promiscuity, β-carotene deficiency, and immunodeficient conditions such as pregnancy, AIDS, steroid treatment, and certain malignancies [11–13].

HPV is a small double-stranded DNA (dsDNA) virus of 7,900 base pairs that infects differentiating basal cells, requiring minor epithelial trauma for initial infection. The virus replicates in terminally differentiated epithelial cells, exiting within infected cells as both extrachromosomic multicopy nuclear plasmids and as integrated viral genomes [14]. Functionally, the HPV genome is divided into the Long Control Region and the Open Reading Frames, with the former containing regulatory sequences (promoters and enhancers) and target sequences for transcription factors that regulate HPV gene expression. The Open Reading Frames code for "early" and "late region" proteins, with the early region consisting of six nonstructural proteins (E1, E2, E4, E5, E6, and E7) and the late region coding for two structural proteins (L1 and L2) [14].

The mechanisms by which HPV causes cancer are not completely understood. Viral integration into the host genome is required for keratinocyte immortality. Upon integration, E6 and E7 protein expression is increased, conferring a growth advantage usually resulting in clonal expansion [14–19]. E6 and E7 bind and inhibit p53 and pRb activities, respectively. p53 is a transcription factor required for G_1 checkpoint and apoptotic functions, and induction of p21 and GADD45, which delay S phase entry and promote DNA repair. pRb inhibition increases protein synthesis which promotes S-phase cell cycle entry and also inhibits apoptosis [14–18, 20]. Together, HPV E6 and E7 proteins promote genomic instability and cell cycle entry [20]. Additionally, HPV integration and replication induce DNA damage, resulting in chromosomal translocations and host genomic rearrangements adjacent to the HPV integration site [14–19]. Together, HPV-induced genomic damage, combined with E6 and E7 expression, lead to dysplasia and eventual invasive carcinoma [21].

12.2.2 HPV Testing Methods

12.2.2.1 Histology

HPV is detected in cervical, vaginal, and vulvar biopsies mainly via histologic examination of hematoxylin-eosin-stained slides. Typically, HPV-infected cells are superficial, displaying enlarged, hyperchromatic nuclei, with cytoplasmic halos (koilocytotic atypia) [22, 23]. Although histologic examination is inexpensive and relatively rapid, there is considerable interobserver variation in the diagnosis of these lesions, leading to overdiagnosis. For example, a review of cervical, vaginal, and vulvar biopsies histologically diagnosed as low-grade squamous intraepithelial lesions or condylomas, revealed a ~33 % over-diagnosis in all three mucosal sites, mainly due to mimics of koilocytotic atypia (pseudokoilocytes) [24, 25]. Based on this, ancillary HPV testing in equivocal cases would be useful, preventing overdiagnosis with its attendant unnecessary medical treatment and costs. Unfortunately, many HPV detection techniques such as in situ hybridization and immunohistochemistry for viral capsid proteins have low sensitivity. Additionally, as yet, no US

Food and Drug Administration (FDA)-approved HPV tests applicable to tissue sections exist. Lastly, while HPV polymerase chain reaction (PCR) testing is sensitive, the specificity of this testing is less so, especially in women under 30 years, due to the prevalence of transient clinically benign infections [26–29].

Due to these problems, several surrogate markers for HPV infection are now in use. Both have proven useful in lowering the number of false positives in low-grade squamous intraepithelial lesions.

12.2.2.2 Ki-67

Ki-67 is a commercial monoclonal antibody that binds an antigen on a nuclear protein referred to as Ki-67 or MKI67. *Ki-67* is located at 10q25 and has 15 exons, encoding several transcripts that are translated into proteins ranging from 320 to 359 kDa. It is readily detected in G_1, S, G_2, and M phase, but not in G_0, making Ki-67 a good immunohistochemical marker for proliferating cells [30, 31]. Antisense oligonucleotides to Ki-67 inhibit cell proliferation in a dose-dependent manner in IM-9 cells, indicating that Ki-67 expression is likely an absolute requirement for cell proliferation [32].

HPV infection increases cellular proliferative activity; hence, Ki-67 expression is often altered where HPV-related pathology exists. Ki-67 is normally expressed only in the parabasal nuclei in normal cervical squamous epithelium. With HPV infection, Ki-67 staining extends to the intermediate and superficial epithelial layers, with increased and more superficial staining seen in higher-grade lesions and condylomas. Interestingly, apoptosis is seen in the higher grade lesions (high-grade squamous intraepithelial lesions and squamous cell carcinomas) [33, 34]. The sensitivity and specificity of Ki-67 as a marker for low-grade and high-grade squamous intraepithelial lesions are high for cervical, vaginal, and vulvar lesions. This stain is especially useful in making a diagnosis in equivocal cases [24, 25, 35]. Ki-67 staining is not 100 % specific for dysplastic epithelium, as staining above the parabasal cell layer may be seen in immature and regenerating epithelium, the edge of ulcers, and in vulgar squamous cell hyperplasia [36]. Assessment of the histology of the specific lesion is important in differentiating between these conditions and HPV-induced neoplastic conditions.

12.2.2.3 p16

p16, also known as CDKN2A or MTS1, is located at 9p21.3 and consists of multiple exons that encode multiple transcripts of the 16.5-kDa p16 protein and by an alternative promoter and reading frame, another protein, INK4a. p16 belongs to a group of tumor suppressor genes that includes p15INK4b, p21waf1, and p27KIP1, all of which negatively regulate the G_1 phase of cell cycle [37]. Specifically, p16 binds the cyclin-dependent protein kinases CDK4 and CDK6, inhibiting their interactions with cyclin D1. As a result, pRb phosphorylation is inhibited and the E2F

transcription factors are sequestered, blocking G1 → S phase progression. p16 expression loss allows cells to escape G_1 arrest [20, 38]. The HPV E7 oncoprotein binds pRb causing E2F release, resulting in G1 → S progression despite high p16 expression [39–41]. Thus high p16 expression is a sensitive marker for HPV E7 protein expression [42]. Cuschieri et al. [42] reviewed 9 studies, including 2,178 cases, and found that 7 % of nondysplastic, 54 % of CIN1 cases, 86 % of CIN2 cases, and 96 % of CIN3 and invasive cancers showed diffuse p16 expression, demonstrating high specificity and sensitivity of p16 staining as a measure of HPV infection. As with Ki-67, p16 staining is not entirely specific for a neoplastic process or HPV infection. For example, benign cervical tubal metaplasia shows p16 positivity [43].

12.2.2.4 Other Markers

The catalytic subunit of *telomerase* is located at 5p15.33 and functions as an endogenous human telomerase reverse trancriptase that employs its integral RNA as a template for adding repeats to chromosomal ends [44, 45]. The telomerase RNA component is located at 3q26.2 and primes 5′-TTAGGG-3′ telomere repeats. Telomerase is up-regulated and activated as part of carcinogenesis [46]. Kailash et al. used reverse transcriptase-PCR to measure telomerase RNA expression changes in cervical cytology samples and compared these changes to high-risk HPV viral expression [47]. There is an excellent correlation between high-risk HPV types 16 and 18 and telomerase RNA expression, with sensitivity of 88.1 % and 100 % for high-risk HPV and telomerase RNA expression, respectively. Telomerase RNA weak positivity was seen in 12.7 % of normal cervical epithelium samples, in 63.6 % of those of mild squamous dysplasia, in 100 % of cervical samples affected by moderate and severe squamous cell dysplasia, and in 100 % of cases of squamous cell carcinoma in situ. Thus, telomerase RNA expression may be used as an adjunct to cytology and HPV DNA testing in triaging women with cervical lesions. Support for the above work also comes from the observation that the human telomerase RNA gene is often up-regulated in cervical intraepithelial neoplasia and carcinoma [48].

E-cadherin is an adhesion molecule involved in normal tissue morphogenesis, differentiation, signaling, and proliferation [49]. Ancuta et al. found that, of cervical carcinomas with E-cadherin loss, 75 % relapsed within 5 years, while only 37.5 % of cervical carcinomas with positive E-cadherin staining relapsed within 5 years [49]. Interestingly, of all the relapsed patients, 62.1 % of those with E-cadherin loss died of their disease, while only 25 % of those who maintained E-cadherin expression died of their disease. Thus initial E-cadherin cervical carcinoma staining may have potential as a prognostic marker.

p63 is located at 3q27-29 and encodes a protein with strong homology to p53 and the related gene p73. p63 is expressed in many tissues, including the basal cells of the epidermis, prostate, urothelium, and cervix [50]. Shirendeb et al. found

basal cell p63 expression in benign cervical tissue, with staining extending to the intermediate and superficial epithelial layers with CINI → III dysplastic progression [51]. The p63 staining may also be a useful adjunctive stain for histologically equivocal cases.

12.2.2.5 PCR HPV Testing–Emphasize Typing

The majority of DNA-based HPV testing PCR amplify a portion of the highly conserved major structural gene L1, limiting the test to the presence or absence of viral DNA. Such testing cannot distinguish between transient, clinically insignificant HPV infections and those related to HPV-induced pathology and transformation [26–29]. There is also good evidence that HPV16 and other high-risk category viruses confer a significantly higher probability for HPV-induced transformation than do other viral types [6, 52]. Additionally, factors such as co-infection with different viral subtypes and the viral load are significant in determining transformation risk [53]. The extreme precision of PCR makes it ideal for identification of different HPC types, as well as the specific amplification and analysis of different HPV gene expression patterns. This has been used in several different systems to aid in the diagnosis of HPV infections.

1. *Analysis of HPV Early Gene Expression* via *Real-Time Quantitative Reverse Transcriptase PCR:* Analysis of specific HPV transcripts likely to be up regulated in dysplastic/malignant cells may be more specific for HPV-related pathologic changes than the presence of the HPV viral genome. The HPV E7 RNA transcript is a good candidate for such testing, as it plays a central role in inducing dysregulated cell cycle progression and transformation. Lamarcq et al. [54] analyzed HPV 16 and 18 E7 RNA expression via real-time quantitative reverse transcriptase PCR. RNA was isolated in cervical cytology samples and reverse transcribed in DNA via random heximer priming, and the resulting DNA was amplified with primers specific for each viral 57 type. Labeled probes were employed to detect real-time amplification. Zero percent of cytologically normal cervical samples expressed E7 RNA, with 47 % and 5 % of the high-grade lesions expressing HPV types 16 and 18 E7 RNAs, respectively. The remaining 53 % of high-grade lesions were thought to harbor other HPV type infections. The authors concluded that the detection of the E7 transcripts may be more specific than HPV DNA detection and may therefore have use in triaging women with abnormal Pap smears.
2. *Pyrosequencing for HPV Genotyping:* DNA sequencing is often considered the gold standard in molecular testing, particularly for viral testing. One problem with employing sequencing technologies is that many specimens harbor multiple HPV types, often yielding non-specific and uninterruptible sequence data. This problem has been solved through the use of HPV type-specific multiple sequencing primers followed by sequencing. For example, Gharizadeh et al. [55] used such

primers to first specifically PCR-amplify sequences of the seven most prevalent HPV types (6, 11, 16, 18, 31, 33, and 45) and analyze the resulting amplicons via pyrosequencing. The resulting amplicons were both specific and nonspecific, allowing the identification of specific sequences by the use of multiple sequencing primers, which in turn allowed the identification of the specific sequence signal. This technique has the advantage of eliminating the need for nested PCR reactions, cloning, and very high stringency PCR techniques. The disadvantage is that it is relatively costly and complex for routine cervical HPV screening. It does have value, as with other molecular diagnostic tests, in identifying women at greater risk to the presence of high-risk HPV types.

3. *HPV DNA Testing by Hybrid Capture:* Hybrid capture assays involve the isolation of sample DNA, followed by denaturation, mixing, and hybridization with labeled multiple RNA probes, each specific for one HPV type. The resulting RNA/DNA hybrids are captured by a specific antibody attached to a solid support (often the wall of a 96 well plate), the sample is washed, and the bound RNA/DNA hybrids are reacted with an alkaline phosphatase-conjugated antibody directed against RNA-DNA hybrids. After extensive washing, a chemiluminescent substrate is added that emits when it reacts with alkaline phosphatase. The light produced is measured via a luminometer and expressed in relative light units, allowing semiquantitative determination of viral DNA quantity in the specimen. In general, as little as 1 pg viral DNA can be detected with this technique [56, 57]. The method is largely reproducible, relatively inexpensive, and accurate. As with most molecular testing methods, long-term specimen storage results in poorer results secondary to DNA degradation [57]. This technique is currently FDA approved for cytologic preparations and is available through Digene.

12.3 Adenoma Malignum, Ovarian Sex Cord Tumor with Annular Tubules, and Peutz-Jeghers Syndrome

Peutz-Jeghers Syndrome (PJS) is a rare autosomal dominate disease characterized by pigmented mucocutaneous lesions, hamartomatous polyps mainly seen in the small intestine, and increased malignancies in multiple organs. Women with PJS often have a high incidence of cervical adenoma malignum and ovarian sex cord tumor with annular tubules, two rare, well-differentiated neoplasms [58–60]. The diagnosis of PJS and these two well-differentiated neoplasms rests upon the clinical presentation and histology supplemented with immunohistochemical analyses, respectively [58–63]. Molecular diagnostic testing in PJS is employed for prenatal diagnosis, kindred testing, and clinical diagnosis confirmation. Molecular testing for PJS involves the analysis of two genes, *BMPR1A* and *SMAD4,* via multiple molecular techniques such as DNA sequencing and PCR-based denaturing gradient gel electrophoresis [64–66].

12.4 Ovarian Cancer

12.4.1 Epidemiology, Types, and Molecular Pathogenesis

Ovarian cancer is comprises 15 distinct tumor types, each with a different histology, prognosis, underlying molecular pathology, and often surgical-chemotherapeutic treatment options [67, 68]. Approximately 90 % of these neoplasms are epithelial derived, with ~70 % being high-grade serous carcinomas [69]. Ovarian cancer is the fifth leading cause of female cancer-related deaths, with its yearly treatment costing ~$2.2 billion/year in the US [67, 68, 70]. Each of the major ovarian malignancies is characterized by specific mutations, which also correlate with their histologic and their malignant progression. Some of the major molecular lesions for each of the major ovarian malignancies are summarized below.

12.4.2 Ovarian Endometrioid Adenocarcinoma

Endometrioid adenocarcinoma accounts for ~10 % of ovarian tumors; it is often associated with endometriosis and typically presents as stage I or II [71]. The most common mutations found in this tumor are *KRAS*, *BRAF*, *PTEN* and *β-catenin* (*CTNNB1*) mutations.

12.4.2.1 KRAS

KRAS is the human homologue of the Kirsten rat sarcoma virus. KRAS transduces signals from several surface growth factor receptors (EGF-, HGF- and IGF-1 receptors), activating down-stream signaling proteins, including Raf, Braf, mTOR, MEK, ERK, and PIK3CA. The resulting gene expression patterns promote cell growth and division, while exerting anti-apoptotic and pro-angiogenesis effects. KRAS is a GTPase that becomes inactive upon hydrolysis of bound GTP to GDP. Activating KRAS mutations typically ablate KRAS GTPase activity, resulting in constitutive KRAS activation and tumor promotion [72–77]. KRAS mutations are seen in ~10 % of these malignancies; interestingly, ovarian endometriosis, a precursor lesion of endometrioid adenocarcinoma, shows a higher frequency of chromosomal abnormalities than extragonadal endometriosis [78, 79].

12.4.2.2 BRAF

BRAF is a serine/threonine kinase which functions downstream of KRAS. Following phosphorylation and activation by KRAS, BRAF activity increases cyclins D1-3, mdm2, and c-myc expression, promoting cell cycle entrance, increasing cell growth

potential, and inhibiting apoptosis [80–82]. Many of the BRAF mutations in endometrioid ovarian carcinoma are the common V600E (valine → glutamate substitution) activating mutation seen in other malignancies [83, 84]. BRAF mutations are seen in ~10 % of endometrioid adenocarcinomas [78].

12.4.2.3 PTEN

PTEN also known as *MMAC1* or *TEP1*, is a phosphatidylinositol 3,4,5-triphosphate phosphatase. Phosphatidylinositol 3,4,5-triphosphate activates the PI3K/AKT signaling pathway, promoting cell growth and survival [85]. Inactivating *PTEN* mutations activate the PI3K/AKT signaling pathway and are seen in ~20 % of endometrioid ovarian carcinomas [68]. They have also been described in typical and atypical endometriosis, possibly functioning as early events in carcinogenesis [86, 87].

12.4.2.4 β-Catenin

Beta-catenin is the mammalian homologue of *Drosophila armadillo* and is mutated in at least 60 % of endometrioid ovarian carcinomas [88]. β-*catenin* exists in two major cellular pools, each exerting a different function. First, it is found on the plasma membrane where it provides a mechanical link between E-cadherin and β-catenin, forming a trimer that binds the intracellular actin cytoskeleton, promoting intercellular adherens junctions formation and likely suppressing tumor invasion. Second, in tumors, β-catenin is often found in the cytoplasm and nucleus where it is part of a large protein complex that includes AXIN1, AXIN2, APC, CSNK1A1, and GSK3B. This complex phosphorylates β-catenin and leads to ubiquitination and proteasomal degradation, keeping β-catenin levels low in normal cells. In many tumors, β-catenin carries mutations in codons 32, 33, 37, and 41 of exon 3. These mutations inhibit β-catenin phosphorylation and degradation, resulting in association with TCF/LEF-1 family members and WNT, leading to the transcription of pro-mitotic proteins including c-Myc and cyclin D1, and promoting transformation [89–91]. Roughly 80 % of ovarian endometrioid adenocarcinomas show nuclear β-*catenin* accumulation [68]. Finally, the combinations of *PTEN*/*KRAS* and β-*catenin*/*PTEN* mutations have been demonstrated to be sufficient to induce ovarian endometrioid carcinoma in engineered mouse models, demonstrating the importance of these mutations in the carcinogenesis of these malignancies [92, 93].

Due to this malignancy commonly presenting at stage I or II and carrying a low mortality and morbidity burden, molecular diagnostics is seldom applied to these tumors outside of the research settings. Additionally, BRCA mutations are not seen in endometrioid ovarian carcinomas [68].

12.4.3 Ovarian Mucinous Carcinoma

Ovarian mucinous carcinoma accounts for only 3–4 % of ovarian malignancies [68]. KRAS codon 12 and 13 mutations have been found in up to 55.7–75 % of these tumors [94, 95]. Low frequency BRAF mutations are also found in this tumor type [83]. Last, 15–20 % of these tumors expressed estrogen and progesterone receptors, CA19-9, CA-125, and HER2, with the last two markers expressed in a small percentage of these tumors [96].

12.4.4 Ovarian Clear Cell Carcinoma

Clear cell carcinoma is a high-grade ovarian malignant tumor that accounts for ~10 % of ovarian malignancies and usually presents with low-stage disease [68]. Kuo et al. [97] examined 97 ovarian clear cell carcinomas for *PIK3CA*, *TP53*, *KRAS*, *PTEN, CTNNB1*, and *BRAF* sequence mutations, finding 33 %, 15 %, 7 %, 5 %, 3 %, and 1 % mutant sequences, respectively. Samples taken from tumors with *PIK3CA* mutations showed intense phosphorylated AKT immunoreactivity. Thus, although arising from endometriosis like the endometrioid adenocarcinoma, the low *CTNNB1* mutation levels combined with the high *PIK3CA* mutation levels differentiate this tumor from endometrioid adenocarcinomas. Interestingly, although clear cell carcinomas are high-grade, they have DNA copy number changes resembling those reported for low-grade ovarian serous tumors, suggesting a relatively high level of genomic stability in these tumors [98].

12.4.5 Dysgerminoma

Dysgerminomas account for less than 1 % of ovarian malignancies and, like its more common testicular counterpart seminoma, these tumors have high (~60 %) isochromosome 12p and c-kit mutations [99–102].

12.4.6 Ovarian Granulosa Cell Tumor

Granulosa cell tumors account for 1–2 % of ovarian tumors and ~2 % of ovarian malignancies [68]. Ninety-five percent or more of these tumors have been shown to harbor a G → C mutation at nucleotide 402 (Cys134Trp) in the *FOXl2* gene [103–105]. FOXl2 is a member of the Forkhead/Winged-Helix family of transcription factors associated with blepharophimosis ptosis epicanthus inversus syndrome and in its unmutated form is normally found in ovarian granulosa cell nuclei [106, 107].

12.4.7 Low and High-Grade Ovarian Serous Carcinoma

Ovarian serous carcinomas have recently been divided into low- and high-grade tumors. Interestingly, the two tumor types appear to have different precursor lesions, with the low-grade tumors arising from serous borderline ovarian tumors or adenofibromas, and high-grade tumors arising from the Fallopian tube epithelium. Low-grade serous carcinomas are relatively rare, making up about 2 % of ovarian carcinomas [68]. Typically ~60 % will harbor *KRAS* codons 12 and 13 or the V600E *BRAF* activating mutations with KRAS [78, 94, 95]. A lower level of ERBB2 mutation is also seen (~6 %) [108]. Since KRAS, BRAF, and ERBB2 all activate the RAS-MAPK pathway, activation of this pathway is likely required for low-grade serous ovarian carcinoma genesis and progression [108]. These tumor types also commonly express estrogen and/or progesterone receptors, are not associated with BRCA1 and BRCA2 mutations, and only rarely have p53 mutations [68, 109].

In contrast, high-grade serous ovarian carcinomas show nearly 100 % p53 mutations (96.7 %), with the remaining cases showing molecular evidence of p53 dysfunction via MDM2 or MDM4 amplifications [110]. Additionally, while *BRCA* mutations are strongly associated with hereditary ovarian cancers, more than 50 % of high-grade serous carcinomas carry some *BRCA1* and *BRCA2* abnormalities, including promoter hypermethylation and gene mutations [111–113].

12.4.8 Nicotinamide Phosphoribosyltransferase Expression in Ovarian Tumors

Interestingly, nicotinamide phosphoribosyltransferase (Nampt) is overexpressed in many ovarian tumors compared to benign ovarian tissue; including serous adenocarcinoma, clear cell carcinoma, endometrioid carcinoma, yolk sac tumor, and mucinous adenocarcinoma [114]. Nampt catalyzes the rate-limiting step of NAD^+ synthesis [115]. Although apparently not a specific ovarian tumor subtype, Nampt overexpression suggests an increased NAD^+ requirement by these neoplasms. Since a specific Nampt inhibitor, FK866, has shown promising results in the treatment of human malignancies, this inhibitor may eventually have use as an adjunctive treatment for ovarian malignancies [116, 117].

12.4.9 BRCA1/2 Testing

12.4.9.1 Background

Roughly 90 % of ovarian malignancies are sporadic, with 5–10 % due to hereditary causes [118]. Thirty to fifty percent of hereditary ovarian malignancies are due to germline mutations of BRCA1 and BRCA2 located at 17q21 and 13q12,

respectively [118]. These mutations typically follow an autosomal dominant pattern and are highly penetrant, with 80–90 % or more of individuals carrying these mutations developing ovarian cancers without intervention [119, 120]. Often different mutations occur at different frequencies in various populations. For example, ~2.6 % of the Ashkenazi Jewish population carry one of three BRCA mutations: BRCA1-185delAG, BRCA1-5382insC, and BRCA2-6174delT. These mutations have a frequency of ~1 %, 0.13 %, and 1.52 %, respectively, and confer a 64 %, 67 %, and 43 % lifetime cancer risk [121].

12.4.9.2 BRCA1

BRCA1 or "breast cancer 1, early onset" is a 208-kDA nuclear phosphoprotein that plays an important role in maintaining genomic stability, functioning as a tumor suppressor gene. BRCA1 functions in conjunction with MSH2, MSH6, MLH1, ATM, BLM, PMS2, RAD50, MRE11, and NBS, forming a nuclear protein complex involved in the recognition and repair of aberrant DNA structures and DNA breaks [122]. More than 1,000 *BRCA1* mutations have been identified, many either producing an abnormally short, nonfunctional protein, or no protein expression. Other *BRCA1* mutations include deletions and missense and nonsense mutations. *BRCA1* mutations increase breast, ovarian, pancreatic, fallopian tube, and male breast cancer risk [123].

12.4.9.3 BRCA2

BRCA2 or "breast cancer 2, early onset" is a 384-kDa protein that functions in DNA break repair, homologous recombination, and the S phase checkpoint. More than 800 *BRCA2* mutations have been identified, largely consisting of insertions/deletions resulting in small, nonfunctional proteins. *BRCA2* mutations increase the risk for breast, ovarian, prostate, pancreatic, fallopian tube, male breast cancer, and melanoma [124].

12.4.9.4 BRCA Testing

Only 0.2 % of the general population carries *BRCA1* or *BRCA2* mutations; therefore, general screening on women for these mutations is not recommended [121]. Currently, although there are no standard criteria for recommending *BRCA1* or *BRCA2* mutation testing, testing should be considered for women with; (1) breast cancer in a male relative, (2) two first-degree relatives diagnosed with breast cancer, with one diagnosis at age 50 or younger, (3) three or more first-degree relatives diagnosed with breast cancer, regardless of age, (4) a first-degree relative diagnosed with bilateral breast cancer, (5) two or more first- or second-degree relatives diagnosed with ovarian cancer, regardless of age, (6) a first- or second-degree relative diagnosed

with both breast and ovarian cancer, or (7) a combination of first- or second-degree relatives diagnosed with breast cancer and ovarian cancer, with one cancer type per person. Women of Ashkenazi Jewish descent should consider testing if they have any first-degree relative diagnosed with breast or ovarian cancer or if they have two second-degree relatives diagnosed with breast or ovarian cancer [125].

BRCA1 and BRCA2 testing has been based on several molecular techniques, such as direct sequencing, denaturing high-performance liquid chromatography, Southern blotting, and denaturing gradient gel electrophoresis. Each of these techniques has specific advantages, disadvantages, and limitations. Below are the ones currently most commonly used.

Due to the very high number of *BRCA1* and BRCA*2* mutations, *DNA sequencing* is the method of choice when no prior knowledge exists concerning any specific BRCA mutation. Typically, genomic DNA is obtained from whole blood, purified, measured, and amplified via PRC with specific primers. Due to the large size of the BRCA genes, many primer pairs are required to fully amplify all exons and flanking intronic sequences. For example, King et al. [126] employed 78 pairs of M13-tagged PCR primers to amplify *BRCA1*. The resulting amplicons are usually analyzed via a commercial DNA sequencer employing Sanger or pyrosequencing, with software programs employed to facilitate analysis.

DNA sequencing has the advantage of interrogating every nucleotide. It suffers the drawbacks of (1) being relatively costly, (2) missing the loss of gene protein expression secondary to epigenetic silencing, (3) missing nonfunctional regulatory region mutations that are not sequenced, (4) being time consuming, and (5) being unable to detect large genomic deletions/rearrangements. Other reasons for lack of BRCA detection include selection of the wrong proband or the existence of mutations in undiscovered genes [127].

High-Resolution Melting Analysis (HRM) allows mutation analysis to be conducted in the same tube or well in which the sequence was amplified. In HRM, selected portions of the gene being tested are PCR amplified with specific primer sets in the presence of a specific dye. The dye has several properties: (1) it does not interfere with PCR reactions, (2) it absorbs at one light frequency and emits at another when bound to dsDNA, and (3) it emits light relatively poorly in the presence of denatured/ssDNA. HRM has been commonly used in *BRCA1* and *−2* molecular testing. Takano et al. [128] used HRM to analyze two founder Ashkenazi Jewish *BCRA1* 185delAG and BRCA*2* 5382insC mutations, in exons 2 and 20, respectively. Following PCR amplification, the reactions containing the amplicons and dye were cooled and then slowly heated; multiple data acquisitions (usually ~25) are taken per degree of temperature increase [129]. As the temperature increases, the dsDNA denatures to ssDNA, lowering fluorescent signal intensity and producing a sigmoid curve. Amplified *BRCA1* and *−2* sequences with denaturation points different than the wild-type control are mutated [128]. HRM is quick to perform but is also relatively expensive and does not identify specific mutations [128, 129].

Although there are over 1,000 *BRCA1* and 800 *BRCA2* mutations, *BRCA* mutations are often relatively constant within specific ethnic/racial groups [121, 123, 124]. Since whole-gene sequencing is cumbersome and expensive, techniques have

been developed for targeting specific mutations (*Targeted Mutation Testing*). Such techniques are comparatively inexpensive and have a rapid turn-around time compared to sequencing. One common method, referred to as allele-specific PCR, uses two primers, one with a 3′ end with nucleotides that match the wild-type gene sequence and the other primer with a 3′ end that matches the specific gene mutation. The *Taq* polymerase initiates the PCR poorly from mismatched template-primer 3′-termini, with a 10^{-3} to 10^{-6} lower amplification efficiency depending on the mismatch type [130]. Thus in a PCR involving two such primers, only one amplicon would be produced, either wild-type or mutant, as the 3′ primer-template mismatch would not initiate a PRC reaction. An amplicon type, mutant or wild type, can be indentified in a variety of ways. For example, Takano et al. [128] used differences in HRM to identify BRCA mutations specific to Ashkenazi Jews. Similarly, Abbaszadegan et al. [131] employed allele-specific PCR to identify BRCA mutations in the same population. Two primers, one wild-type and the other corresponding to the mutant sequence, were used; each primer was 5′ and 3′ labeled with different fluorescent reporters and quencher dyes. The 5′ nuclease activity of the *Taq* polymerase generates fluorescence by separating the quencher from the fluorescent reporter. As the wild-type and mutant primer were labeled with different dyes, the emission spectra of the released dye identifies the *BRCA* sequence. This testing is highly accurate, low cost, and has a rapid turn-around time.

The *BRCA Protein Truncation Test* has been employed in *BRCA* testing in many labs. In this system, RNA is extracted usually from nucleated peripheral blood cells of the individual being tested. The RNA is converted into DNA, typically via M-MLV reverse transcriptase with random heximers as primers. The resulting cDNA is amplified with specific *BRCA* primers covering portions of the gene suspected of harboring mutations. The amplicons are in turn transcribed and translated in proteins in a reticulocytelysate system. The proteins are often labeled with ^{35}S-labeled proteins in the reticulocytelysate system or can be visualized in a gel via various protein stains. *BRCA* mutations are detected by comparing the migration of the patient-translated product to that of an individual without *BRCA* mutations. A shorter, faster-migrating translated product indicates the presence of a truncated mutation and very likely a nonfunctional protein. Although whole proteins may be translated [132], *BRCA1* and BRCA2 are sufficiently large so that translating smaller portion of either gene facilitates analysis [133]. This testing method only detects truncating mutations, rendering it less sensitive than many other *BRCA* mutation testing methods.

Denaturing High-Performance Liquid Chromatography has been shown to give accurate and reliable results in *BRCA1* testing [133]. Genomic DNA is isolated and PCR amplified. The unpurified amplicons are partially heat denatured and placed within a linear acetonitrile gradient. The amplicon DNA heteroduplexes that form have internal sequence variations that are identified by reduced column retention time(s) relative to their homoduplex counterparts. The elution profiles for such samples are distinct from those having homozygous sequences, making the identification of samples harboring polymorphisms/mutations relatively easy. This technique has been used in many studies and is especially useful in identifying novel *BRCA* mutations rapidly and efficiently [134–136].

To summarize, Gerhardus et al. [133] compared 12 different *BRCA1* testing methods and determined that protein truncation, single-strand conformation polymorphism, micronucleus, conformation-sensitive gel electrophoresis, and two-dimensional gene scanning testing lacked sufficient sensitivity for clinical testing. Additionally, fluorescence-based conformation-sensitive gel electrophoresis, restriction endonuclease fingerprinting-single strand conformation polymorphism, RNA-based sequencing, enzymatic mutation detection, and multiple-dye cleavase fragment length polymorphism testing all required further evaluation. Denaturing high-performance liquid chromatography outperformed most testing methods.

Other than *BRCA1* and *BRCA2* testing, molecular testing for different ovarian malignancies is uncommon. As our knowledge of the molecular pathology underlying these tumors increases, molecular diagnostic testing will likely play an increasingly important role in the clinical management of these tumors.

12.4.10 Management of Patients with BRCA *Mutations*

Women with identified *BRCA* mutations should be clinically followed. In addition, their relatives, who might have *BRCA* mutations, should receive genetic counseling and molecular testing specific for the identified *BRCA* mutation [125]. Specifically, the following tests should be considered.

12.4.10.1 Cancer Screening

Women with identified *BRCA* mutations should undergo regular cancer screening, including mammography and clinical breast exams. Magnetic resonance imaging may also be useful for the early detection of breast cancer. Additionally, ovarian cancer screening should be via blood testing for CA-125 and transvaginal ultrasound.

12.4.10.2 Prophylactic Surgery

Bilateral prophylactic risk-reducing mastectomy and salpingo-oophorectomy may lower cancer risk but not eliminate it. Some data suggest that these risk-reducing prophylactic surgeries may have different efficacies between *BRCA1* and *–2* mutation carriers [137].

12.4.10.3 Chemoprevention

Tamoxifen may lower the breast cancer risk in women with *BRCA1* and BRCA2 mutations [145, 146]. Interestingly, tamoxifen reduced the risk of developing breast cancer in the opposite breast of women being treated for an initial breast cancer [138, 139].

12.4.10.4 Risk Avoidance

Although there is little evidence indicating that avoiding cancer risk factors lowers the risks for breast and ovarian cancers, the National Cancer Institute recommends that women with *BRCA* mutations avoid known risk factors for these malignancies, such as obesity and smoking [125].

12.5 Endometrial Cancer

12.5.1 Epidemiology and Types

Uterine cancer is the most common gynecologic malignancy in the United States, with ~40,100 women diagnosed with endometrial cancer in 2008 and 7,470 dying of the disease [2, 68, 140]. Although the disease presents at an early stage with vaginal bleeding and is usually diagnosed at an earlier stage than ovarian cancers, the mortality rate has not decreased in 15 years for women presenting with distant metastases [140, 141]. Additionally, a subset of endometrial histologic subtypes, such as the serous, clear cell, and malignant mixed Mullerian tumor (MMMT) carry a poor prognosis [142]. Bokhman examined 366 patients with endometrial carcinomas and broadly divided the malignancies into two types based on distinct clinicopathologic features [143].

Type 1 accounts for ~65 % of endometrial carcinomas arising from a background of obesity, hyperlipidemia, and signs of hyperestrogenism such as anovulatory bleeding, infertility, endometrial and ovarian stromal hyperplasia, and late onset menopause. These malignancies are estrogen dependent, usually highly or moderately differentiated tumors with superficial myometrial invasion, and have a favorable prognosis. The prototypic Type 1 endometrial malignancy is endometrioid carcinoma.

Type 2 accounts for ~35 % of endometrial malignancies and does not arise in the clinicophysiologic setting of type 1 malignancies. They are estrogen insensitive, occur in older women, are more likely to be poorly differentiated, deeply invade the myometrium, and have metastases to the pelvic lymph nodes. They have few known risk factors and overall carry a poorer prognosis.

12.5.2 Uterine Endometrial Adenocarcinoma (UEA)

Uterine endometrial adenocarcinoma (UEA) arises in a setting of unopposed estrogen and is typically well-differentiated and has a good prognosis. Tamoxifen has been found to mildly increase the risk of UEA secondary to its weak estrogenic effects on the endometrium. It also increases endometrial cell proliferation, endometrial polyps, and endometrial hyperplasia [144–146].

12.5.2.1 PTEN

Like the ovarian endometrial adenocarcinoma, *PTEN* is commonly mutated in UEA. In fact, up to 83 % of UEAs show inactivating *PTEN* mutations [147–149]. As in the ovary, *PTEN* activity loss results in the activation of the signaling pathway that promotes cell growth and survival [85]. As expected, endometrial hyperplasia also shows PTEN mutations, with complex atypical hyperplasia having ~22 % *PTEN* mutations. 88 % of PTEN mutations occur in exons 5, 7, and 8, with frameshift, nonsense, missense, and silent mutations identified. Some data indicate that PTEN mutation also confers a better prognosis [150–152].

12.5.2.2 PIK3CA

PIK3CA is found at 3q26.32 and is a phosphatidylinositol 3-kinase family member that promotes cell proliferation, motility, and cell invasion, via AKT and PDK1 activation [153–156]. *PIK3CA* is mutated in up to 36 % of UEAs, and, as in colon cancers, it can coexist with *PTEN* and *KRAS* mutations [150]. Interestingly, *PIK3CA* mutation combined with p53 mutation/loss results in malignant transformation and a shorter survival time than in individuals with p53 mutations only [157]. Commonly, *PIK3CA* has missense exon 9 mutations, with mutations in exon 20 correlating with more aggressive tumor behavior [157].

12.5.2.3 KRAS

KRAS mutations are detected in up to 10–30 % of UEAs, occurring with approximately the same frequency in endometrial hyperplasias [150, 158]. *KRAS* mutations do not correlate with age of onset, histology, differentiation, or clinical stage and appear to have no prognostic value [158].

12.5.2.4 β-Catenin

Beta-catenin is mutated in UEAs as is its ovarian counterpart, and its accumulation is easily identified with immunohistochemistry [150]. β-*catenin* is mutated in 31–47 % of UEAs, and 0–3 % of endometrial malignancies without endometrioid histology [149].

12.5.3 Uterine Serous Carcinoma

Uterine serous carcinoma (USC) is a Type 2 malignancy, accounting for 10 % of endometrial carcinomas and pursues an aggressive clinical course compared to UEAs [143, 150].

12.5.3.1 p53

At least 90 % of USCs carry p53 mutations, with p53 overexpression readily identified by immunohistochemistry. Interestingly, p53 overexpression is also often in benign-appearing endometrial glands. Molecular analyses have demonstrated that the specific p53 mutations found are the same as in nearby USC, likely indicating that p53 mutations precede USC [159, 160].

12.5.3.2 HER2/neu

HER2/neu is expressed in ~60 % of USC, often with moderate to strong expression [161].

12.5.3.3 WT1

Approximately 97 % of ovarian serous carcinomas are WT1 positive, while USCs are not. This immunohistochemical difference has been useful in identifying tumor origin in metastases [162].

12.5.3.4 Uterine Clear Cell Carcinoma

Uterine clear cell carcinoma (UCCC) accounts for ~5 % of endometrial carcinomas. *PTEN* and *p53* mutations have been identified in UCCC, although at low frequencies. UCCCs appear to be heterogeneous malignancies that arise by presently unknown molecular pathway(s) [163].

12.5.4 Malignant Mixed Mullerian Tumors

Malignant mixed mullerian tumor (MMMT), also known as a carcinosarcoma, is a highly aggressive Type 2 malignancy. MMMTs are biphasic, having both epithelial and sarcomatous components. MMMTs show high p53 mutation frequency in both tumor components [143, 164].

12.5.5 Endometrial Stromal Tumors

Endometrial stromal tumors (ESTs) are rare uterine malignancies comprising ~15 % of uterine sarcomas and ~1 % of uterine malignancies [165]. Molecular diagnostics plays almost no role in the analysis of these tumors. ESTs have a high degree of morphologic and cytogenetic heterogeneity, making diagnosis difficult in

some cases. Approximately 50 % of low-grade ESTs carry a *JAZF1/JJAZ1* gene fusion [166]. Combined with other established methods, analysis of this gene fusion may have value in the diagnosis of difficult cases [166].

12.6 Lynch Syndrome or Hereditary Nonpolyposis Colorectal Cancer

Hereditary nonpolyposis colorectal cancer (HNPCC) is the most common cause of hereditary colorectal carcinoma [167]. HNPCC shows an autosomal dominant inheritance and results from the loss of one or more DNA mismatched repair genes (*MLH1, MSH2, MSH6n* and *PMS2*), resulting in a specific form of genomic instability [168, 169]. HNPCC-associated extracolonic tumors include endometrial, ovarian, sebaceous, gastric, small intestinal, pancreatic, brain, hepatobiliary tract, and upper urothelial cancers [167–169]. Interestingly, women with *MSH6* mutations have a higher rate of endometrial tumors than colorectal carcinoma [170]. Molecular diagnostic testing for HNPCC is covered in Chap. 5, regarding colorectal carcinoma

12.7 Vulva

Cancer of the vulva is often associated with HPV infection, with the HPV E6 and E7 proteins playing a role in carcinogenesis [10, 14–20]. Molecular diagnostics is seldom employed in the clinical management of vulvar malignancies. p53, CHK2, and PTEN mutations have been identified in subsets of vulvar carcinomas [171, 172]. Last, although aggressive angiomyxomas have translocations involving 12q14-15, this work is in its early phase and as yet is not used in the diagnosis of these rare tumors [173].

12.8 Vagina

Like the vulva, vaginal cancer is often HPV-related, with ~50 % of malignancies having detectable p53 mutations [10, 174, 175]. Molecular diagnostics is seldom used in the management of these malignancies.

12.9 Fallopian Tube

Malignancies of the fallopian tubes seldom require molecular diagnostics for their diagnosis and clinical management. The fallopian tubes appear to be a common location for precursor lesions of high-grade serous ovarian carcinoma [176].

12.10 Hydatidiform Moles

Two different hydatidiform mole types exist, with each one having a different appearance and etiology (partial or complete). Histologically, in the complete mole, the villi are typically cystic and embryonic development is usually absent. In the partial mole, embryonic development is seen and a range of normal and abnormal cystic villi are present. At the molecular level the complete mole is diploid, with all 46 chromosomes being of paternal origin. Partial moles are triploid. The extra haploid chromosome set can have either a maternal origin (digynic triploidy) or a paternal origin (diandric triploidy) [177].

The ability to distinguish between non-moles, complete-, and incomplete moles is important in the clinical management and accurate risk assessment for gestational trophoblastic disease. However, the diagnosis of hydatidiform moles based solely on morphology suffers from poor interobserver reproducibility; therefore, several ancillary techniques are employed to assist in the diagnosis of these tumors [177, 178]. Short tandem repeat genotyping can identify the parental source of polymorphic alleles, allowing a specific diagnosis [178]. Although currently not in widespread use, molar testing is being clinically employed.

12.11 Conclusion

Gynecologic malignancies account for ~13 % of solid tumors in women. Although the diagnosis of these malignancies is largely dependent on histologic examination of hematoxylin-eosin-stained slides, molecular studies are becoming increasingly important in establishing a diagnosis, determining prognosis, and clinically managing these malignancies. Additionally, molecular studies are required for the diagnosis, management, and genetic counseling of individuals with hereditary gynecologic malignancies, such as those secondary to BRCA1 mutations. As the field of molecular diagnostics continues to grow, the expenses associated with testing diminish, and a more complete understanding of the molecular pathology underlying gynecologic malignancies is attained, the role for molecular testing in this area will increase.

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Chapter 13
Molecular Pathology of Bone and Soft Tissue Neoplasms and Potential Targets for Novel Therapy

Evita B. Henderson-Jackson, Anthony Conley, and Marilyn M. Bui

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Abstract Bone and soft tissue tumors encompass a rare but a heterogenous group of mesenchymal neoplasms ranging from benign to malignant. Frequently, the diagnosis of many bone and soft tissue tumors prove to be difficult due to the enormous variety of histologic subtypes, with some of them (especially the tumors of spindle cell morphology) presenting with similar clinical, microscopic, immunohistochemical, and/or radiographic characteristics. With continuous and more advanced understanding of the cytogenetic and molecular genetics of bone and soft tissue tumors, it is hopeful that, eventually, classifying bone and soft tissue tumor types can be based on molecular pathology rather than by traditional histogenesis. More importantly, the forecast for the prognosis or prediction to therapy can also be benefited from molecular pathology. Chapter 13 will review the most

E.B. Henderson-Jackson • M.M. Bui (✉)
Department of Anatomic Pathology, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, College of Medicine, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: Marilyn.bui@moffitt.org

A. Conley
Department of Sarcoma, H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, College of Medicine, 12902 Magnolia Drive, Tampa, FL 33612, USA

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current molecular testing of bone and soft tissue tumors and the biomarkers that have the potential for targeted therapy with focus on the recent developments from the past 5 years.

Keywords Cytogenetic • Molecular pathology • Diagnostic, bone tumor (neoplasm) • Soft tissue tumor (neoplasm) • Genetic • Prognosis • Prediction • Biomarkers • Targeted therapy

Abbreviations

ASPS	Alveolar soft part sarcoma
DFSP	Dermatofibrosarcoma protuberans
FISH	Fluorescent in situ hybridization
GIST	Gastrointestinal stromal tumor
HMO	Hereditary multiple osteochondromas
HSP	Heat-shock protein
IGF1R	Insulin-like growth factor 1 receptor
NTRK3	Neurotrophin 3 receptor gene
PDGFRA	Platelet-derived growth factor receptor alpha
PIK3CA	Phosphotidylinositol-3 kinase catalytic alpha polypeptide
PNET	Primitive neuroectodermal tumor
PPARγ	Peroxisome proliferator-activated receptor-gamma
PVNS	Pigmented villonodular synovitis
RT-PCR	Reverse transcription-polymerase chain reaction
SNP	Single nucleotide polymorphism
VEGFR	Vascular endothelial growth factor receptor
WDLPS/ALT	Well-differentiated liposarcoma/atypical lipomatous tumor

13.1 Introduction

Bone and soft tissue tumors encompass a rare but heterogenous group of mesenchymal neoplasms ranging from benign to malignant. The etiology of most tumors is unknown. There are reported associations with radiation, viral infections, chemical carcinogens, and genetic syndromes. Currently, mesenchymal neoplasms are diagnosed and classified using the criteria published by World Health Organization in 2002 [1]. The tumors are mainly classified by their histogenesis and incorporated cytogenetic and molecular genetic information. Morphologically, they are divided into groups by cell morphology and matrix formation. Spindle, small round cell, epithelioid/polygonal, pleomorphic, myxoid, fatty, giant cell, and chondro-osseous tumors are the main groups. Immunohistochemical studies are important ancillary tools to facilitate a definitive diagnosis by providing information regarding tumor

histogenesis, proliferation index, and some the status of certain predictive markers. However, there are no reliable histochemical markers to determine if the tumor is benign versus malignant.

Genetic analysis of mesenchymal neoplasms has revealed three distinct groups. The first group contains genetically unstable sarcomas associated with complex karyotypes without possessing characteristic chromosomal rearrangements. Osteosarcoma, fibrosarcoma, and leiomyosarcoma [2–4] are such examples. These sarcomas can arise from a less aggressive form and evolve into a more aggressive process with increasing genomic instability and complexity. They can also arise de novo as a high-grade sarcoma. The second group is characterized by specific and recurrent chromosomal numerical or structural abnormalities, such as translocations, deletions, additions, and ring or giant chromosomes. There are usually distinct histopathologic characteristics and clinical scenarios associated with the soft tissue neoplasms with recurrent chromosomal rearrangements [2–4]. The examples are Ewing's sarcoma and myxoid liposarcoma. The third group exhibits molecular abnormalities such as gene amplification, mutations, and loss of heterozygosity. An example includes gastrointestinal stromal tumor (GIST).

13.2 Soft Tissue Tumor-Specific Genetic and Molecular Abnormalities

There are approximately 60 sarcoma subtypes and 52 benign soft tissue tumors. The core mechanisms of the pathogenesis of soft tissue tumors have been elegantly described and illustrated in the review article by Taylor et al. published in *Nature Reviews* [5]. A taxonomy of soft tissue sarcomas illustrate the differences and similarities in lineage for subtypes of sarcomas. A summary of the recurrent translocations in soft tissue sarcomas illustrate the relationship between subtypes.

The most common and recent cytogenetic and molecular abnormalities of soft tissue neoplasms are summarized in Table 13.1. Small round blue cell tumors, a group of primitive appearing neoplasms, are often difficult to classify. However, most of the small round blue cell tumors have an associated tumor-specific chromosomal translocation, which is diagnostically valuable [1]. Ewing's sarcoma, the second most common primary bone sarcoma in children, is associated with the t(11;22)(q24;q12) translocation. This translocation results in the fusion of the *EWSR1* gene, on chromosome 22q12, to a member of the *ETS* gene family of transcription factors, Fli1, on chromosome 11q24 [6]. This translocation is detected in approximately 85 % of Ewing's sarcomas and primitive neuroectodermal tumors (PNET). A second translocation, t(21;22)(q22;q12) found in 5–10 % of Ewing's sarcomas, fuses the *EWSR1* gene to a different *ETS* gene, *ERG*, found on chromosome 21q22 [7]. Many other less frequent translocations involving the *EWSR1* gene and other *ETS* genes and rarely *FUS* gene have been reported [2, 4].

Table 13.1 Characteristic genetic alterations in soft tissue neoplasms

Neoplasm	Translocation	Gene/alteration
Alveolar rhabdomyosarcoma	t(2;13)(q35;q14)	*PAX3-FOXO1*
	t(1;13)(p36;q14)	*PAX7-FOXO1*
	t(X;2)(q13;q35)	*PAX3-AFX*
Alveolar soft part sarcoma	der(17)t(X;17)(p11;q25)	*ASPL-TFE3*
Angiomatoid fibrous histiocytoma	t(12;22)(q13;q12)	*EWSR1-ATF1*
	t(2;22)(q33;q12)	*EWSR1-CREB1*
	t(12;16)(q13;p11)	*FUS-ATF1*
Clear cell sarcoma	t(12;22)(q13;q12)	*EWSR1-ATF1*
	t(2;22)(q33;q12)	*EWSR1-CREB1*
Congenital/infantile fibrosarcoma	t(12;15)(p13;q25)	*ETV6-NTRK3*
DFSP	t(17;22)(q21;q13) derivative ring chromosomes	*COLIA1-PDGFB*
Desmoid tumor (fibromatosis)	Trisomy 8 or 20; loss of 5q21	*CTNNB1 or APC* mutation
Desmoplastic small round cell tumor	t(11;22)(p13;q12)	*EWS-WT1*
Epithelioid hemangioendothelioma	t(1;3)(p36;q25)	*WWTR1-CAMTA1*
Epithelioid sarcoma (proximal type)	Inactivation of INI1	*INI1*
Ewing's sarcoma/PNET family	t(11;22)(q24;q12)	*EWS1-FLI1*
	t(21;22)(q22;q12)	*EWS1-ERG*
	t(7;22)(p22;q12)	*EWSR1-ETV1*
	t(2;22)(q33;q12)	*EWSR1-FEV*
	t(17 ;22)(q12 ;q12)	*EWSR1-E1AF*
	inv(22)(q12 ;q12)	*EWSR1-ZSG*
	t(16 ;21)(p11 ;q22)	*FUS-ERG*
Extrarenal rhabdoid tumor	Inactivation of INI1	*INI1*
Extraskeletal myxoid chondrosarcoma	t(9;22)(q22;q12)	*EWSR1-NR4A3*
	t(9;17)(q22;q11)	*TAF2N-NR4A3*
	t(9;15)(q22;q21)	*TCF12-NR4A3*
	t(3;9)(q11;q22)	*TFG-NR4A3*
GIST (Sporadic and familial)	Activating kinase mutations	*KIT or PDGFRA*
		ETV1 overexpression
		BRAF mutation
Inflammatory myofibroblastic tumor	t(1;2)(q22;p23)	*TPM3-ALK*
	t(2:19)(p23;p13)	*TPM4-ALK*
	t(2;17)(p23;q23)	*CLTC-ALK*
	t(2;2)(p23;q13)	*RANBP2-ALK*
	t(2;11)(p23;p15)	*CARS-ALK*
	inv(2)(p23;q35)	*ATIC-ALK*
Leiomyosarcoma		*MYOCD* amplification
Lipoma	t(3;12)(q27-28,q14-15)	*HMGA2-LPP*
Low-grade fibromyxoid sarcoma	t(7;16)(q33;p11)	*FUS-CREB3L2*
	t(11;16)(p11;p11)	*FUS-CREB3L1*

(contined)

Table 13.1 (continued)

Neoplasm	Translocation	Gene/alteration
Myoepithelioma	t(1;22)(q23;q12)	*EWSR1-PBX1*
Myoepithelial carcinoma	t(19;22)(q13;q12)	*EWSR1-ZNF444*
Myxoid/round cell liposarcoma	t(12;16)(q13;p11)	*FUS-DDIT3*
	t(12:22)(q13;q12)	*EWSR1-DDIT3*
		PIK3CA mutation
Myxofibrosarcoma		*NF1* deletion, point mutation and indel
Pericytoma	t(7;12)(p22;q13)	*ACTB-GLI1*
Pleomorphic liposarcoma		*NF1* deletion, point mutation and indel
Synovial sarcoma	t(X;18)(p11;q11)	*SS18-SSX1*
	t(X;18)(p11;q11)	*SS18-SSX2*
	t(X;18)(p11;q11)	*SS18-SSX4*
TGCT/PVNS	t(1;2)(p13;q35)	*CSF1-COL6A3*
WDLPS/ALT and DDLPS	12q14-15 (supernumerary ring chromosomes ; giant marker chromosomes)	Amplification of *MDM2, CDK4, HMGA2, SAS, GLI* and *JUN*

DFSP dermatofibrosarcoma protuberans, *PNET* peripheral primitive neuroectodermal tumor, *GIST* gastrointestinal stromal tumor, *WDLPS* well-differentiated liposarcoma, *ALT* atypical lipomatous tumor, *DDLPS* dedifferentiated liposarcoma, *TGCT* tenosynovial giant cell tumor, *PVNS* pigmented villonodular synovitis

Cytogenetic analysis of alveolar rhabdomyosarcoma, a common soft tissue neoplasm of young children, has demonstrated two specific chromosomal translocations [8]: the t(2;13)(q35;q14) translocation, which is found in 60 % of cases, and a less common variant t(1;13)(p36;q14) [9, 10]. These involve the *FKHR* gene (*FOXO1A*) on chromosome 13q14 and either *Pax3* (2q35) or *Pax7* (1p36) genes [11]. The Pax7-FKHR tumors are often seen in younger patients and are associated with a lower rate of metastasis and better survival than Pax3-FKHR tumors [12]. There is no specific chromosomal abnormality identified involving the embryonal subtype of rhabdomyosarcoma [13].

Desmoplastic small round cell tumor demonstrates multilineage differentiation and characteristically possesses the t(11;22)(p13;q12) translocation that fuses the *EWSR1* gene to *WT1*, the Wilm's tumor suppressor gene, on chromosome 11p13 [4, 14].

Extraskeletal myxoid chondrosarcoma, an uncommon sarcoma of uncertain lineage, demonstrates the EWS-NR4A3 fusion generated from a t(9;22)(q22;q12) translocation [15]. A second translocation that may be encountered is t(9;17)(q22;q11) [16]. Of note, there is no immunohistochemistry profile that can reliably diagnosis this particular sarcoma, making molecular studies important for the diagnosis of extraskeletal myxoid chondrosarcoma [17].

In addition to the small round blue cell tumors, several spindle cell sarcomas are associated with recurrent translocations. Importantly, synovial sarcoma, an

aggressive neoplasm of uncertain histogenesis, demonstrates a recurrent reciprocal t(X;18)(p11.2; q11.2) translocation [1, 18]. The translocation fuses the *SYT* gene on chromosome 18q11 to one of the three homologous genes on Xp11 (*SSX1*, *SSX2*, or *SSX4*), which is detectable in 95 % of synovial sarcomas [2, 19]. The SYT-SSX1 fusion transcript is reportedly associated with biphasic histology, whereas the SYT-SSX2 fusion transcript is associated with a monophasic histology as well as a better metastasis-free survival [20].

Dermatofibrosarcoma protuberans (DFSP), a low-grade spindle cell neoplasm, possesses a specific chromosomal rearrangement consisting of either supernumerary ring chromosomes or unbalanced derivatives of t(17;22)(q22;q13) translocation. Both aberrations have sequences encoding a COL1A1-PDGFB fusion transcript that is commonly identified by reverse transcription-polymerase chain reaction (RT-PCR) [21–23]. Reportedly, imatinib mesylate (Gleevec, STI571/Glivec) has clinical activity against DFSP with t(17;22); however, fibrosarcomatous variants of DFSP without the translocation may not show a response. Molecular testing would be important in identifying patients with DFSP who have the translocation because they would more likely respond to therapy [24].

Congenital fibrosarcoma, another spindle cell sarcoma, has a characteristic cytogenetic change, t(12;15)(p13;q25) translocation, not found in the adult fibrosarcomas. In addition, the chromosomal rearrangement is identical to that described for congenital mesoblastic nephroma, acute myeloblastic leukemia, and secretory carcinoma of the breast [25–27]. Fusion of the *ETV6* gene on 12p13 to the neurotrophin 3 receptor gene (*NTRK3* or *TRKC*) on chromosome 15q25 is often difficult to identify cytogenetically and is better detected by FISH or PCR [28, 29].

Low-grade fibromyxoid sarcoma, a rare variant of fibrosarcoma, is associated with the FUS-CREB3L2 gene fusion or less frequently the FUS-CREB3L1 gene fusion, the result of translocations t(7;16) or t(11;16), respectively [30, 31]. The chromosomal abnormalities may be detected with either FISH or RT-PCR [32].

Gastrointestinal stromal tumor (GIST), a spindle cell sarcoma, is unlike the previous spindle cell sarcomas discussed in that it is characterized by activating oncogenic mutations instead of specific translocations or fusion genes [3]. KIT mutations are a defining feature of most GISTs, and, to a lesser degree, mutations in platelet-derived growth factor receptor alpha (PDGFRA) can also occur in GIST. These activating mutations lead to constitutive activation of their respective kinases, leading to downstream effects that decrease apoptosis and promote cellular survival [33–36]. A majority of GISTs reveal a mutation in one of three sites: KIT exon 11, KIT exon 9, or PDGFRA exon 18 [35, 37]. These mutation types correlate with the anatomic location and have prognostic implications. The KIT exon 11 mutations include in-frame deletions, point mutations, and internal tandem duplications; however, patients with point mutations fare better prognostically than those with deletions [38]. Small intestinal GISTs usually demonstrate KIT 9 exon mutations, and patients appear to have a higher mortality with small intestinal versus gastric GISTs with KIT 9 exon mutations [36, 39]. PDGFRA mutations are primarily localized to the stomach and have an epithelioid morphology, and they frequently exhibit an

indolent clinical course [40, 41]. GISTs have been successfully treated with tyrosine kinase inhibitors that specifically target c-Kit, such as imatinib mesylate and sunitinib, which further support molecular genotyping of these tumors because the genotype correlates with different treatment outcomes, including progression-free survival and overall survival [35, 42–44]. The majority of laboratories use PCR to amplify the most commonly mutated exons with subsequent direct sequencing analysis of the amplified exon [3].

There are a few notable chromosomal abnormalities and genetic aberrations involving lipomatous neoplasms. Myxoid-round cell liposarcoma is known for its characteristic t(12;16)(q13;p11) reciprocal translocation, resulting in the formation of *FUS-DDIT3* chimeric gene in 95 % of cases [4, 45, 46]. Myxoid-round cell liposarcoma is radiosensitive; with the use of molecular studies as an adjunct to histopathology, patients will be able to get neoadjuvant therapy and reduce the risk of an ablative procedure [32]. Recently, Barretina et al. [47]performed a high-throughput, integrative analysis of DNA, mRNA, and mutational data to show that 18 % of myxoid-round cell liposarcomas contain mutations in the phosphotidylinosital-3 kinase catalytic alpha polypeptide (PIK3CA). Compared to the wild-type version, tumors with PIK3CA exhibited a shorter progression-free survival, suggesting a more aggressive course. On the other hand, well-differentiated liposarcoma/atypical lipomatous tumor (WDLPS/ALT) cytogenetically shows supernumerary ring chromosomes, giant marker chromosomes, and double minutes in 80 % of cases, corresponding to the amplification of the 12q13-15 band [2]. Several tumor-associated genes are localized to that band region, including MDM2 and CDK4 [48]. Pleomorphic liposarcomas, a less common liposarcoma variant, does not exhibit a specific chromosomal translocation, but these tumors have been shown to exhibit mutations in TP53 and NF1 [47].

There are sarcomas of uncertain histogenesis with characteristic translocations. Clear cell sarcoma (malignant melanoma of soft parts) demonstrates t(12;22)(q13;q12) translocation involving the *ATF-1* gene on chromosome 12q13 and *EWSR1* [49, 50]. Alveolar soft part sarcoma (ASPS) has a recurrent der(17)t(X;17)(p11;q25) unbalanced translocation [51]. This translocation involves the fusion of *TFE3* transcription factor gene on Xp11 and the *ASPL* gene on chromosome 17q25 [52]. The *ASPSCR1-TFE* fusion protein has been associated with overexpression of MET, and inhibition of MET by RNA interference has resulted in decrease growth of ASPS cells lines [53].

Extrarenal (and renal) rhabdoid tumors, proximal-type epithelioid sarcoma, and a subset of pediatric undifferentiated soft tissue sarcomas have an inactivating oncogenic mutation, specifically an inactivation of the tumor suppressor gene *SMARCB* (*INI1*) [54–57]. Basically, there is either a loss of both alleles or loss of one allele with an inactivating mutation of the other, resulting in the inactivation of the tumor suppressor gene [3]. SMARCB molecular abnormalities may be detected using conventional karyotyping, FISH, and direct sequencing and more recently with multiplex ligation-dependent probe amplification of single nucleotide polymorphism-array analysis to identify small deletions, duplications, or single base pair mutations within the tumors [3, 58].

13.3 Bone Tumor-Specific Genetic and Molecular Abnormalities

Approximately 0.2 % of bone tumors are primary, which is less frequent than metastasis to the bone and less common than soft tissue counterparts (1:10). There are 19 main subtypes of benign bone tumors and 22 subtypes of bone sarcomas. Table 13.2 summarizes the most common and recent cytogenetic and molecular abnormality of bone neoplasms.

Bone tumors are primarily of mesenchymal origin. Tumors of the bone as classified by the World Health Organization may be cartilaginous, osteogenic, fibrogenic, fibrohistiocytic, vascular, myogenic, lipogenic, neural, or notochordal [1]. However, there are instances when bone is involved by tumors of neuroectodermal origin, such as Ewing Sarcoma, or by tumors of undefined histogenesis, such as aneurysmal bone cyst.

Cartilagenous tumors produce a chondroid matrix and may either be benign or malignant in nature. Osteochondroma, a cartilage capped bony projection arising on the external surface of bone containing a marrow cavity continuous with that of the underlying bone, may form as a solitary lesion or multiple lesions as in hereditary

Table 13.2 Characteristic genetic alterations in bone neoplasms

Neoplasm	Translocation	Gene/alteration
Aneurysmal bone cyst	t(16;17)(q22;p13)	*CDH11-USP6*
	t(1;17)(p34.3;p13)	*THRAP3-USP6*
	t(3;17)(q21;p13)	*CNBP-USP6*
	t(9;17)(q22;p13)	*OMD-USP6*
	t(17 ;17)(q21 ;p13)	*COL1A1-USP6*
Bizarre parosteal osteochondromatous proliferation (Nora lesion)	t(1;17)(q32 ;q21)	*RDC1*
Chondrosarcoma or chondroma		*IDH1 or IDH2* Point mutation
Ewing's sarcoma/PNET family	t(11;22)(q24;q12)	*EWS1-FLI1*
	t(21;22)(q22;q12)	*EWS1-ERG*
	t(7;22)(p22;q12)	*EWSR1-ETV1*
	t(2;22)(q33;q12)	*EWSR1-FEV*
	t(17;22)(q12;q12)	*EWSR1-E1AF*
	inv(22)(q12;q12)	*EWSR1-ZSG*
	t(16;21)(p11;q22)	*FUS-ERG*
Fibrous dysplasia	Activating oncogenic mutations	*GNAS1*
Mesenchymal chondrosarcoma	t(8;8)(q13;q21)	*HEY1-NCOA2*
Osteosarcoma, low grade (parosteal and intramedullary)	12q14-15 (ring chromosomes, giant marker chromosomes)	*Amplification of CDK4, MDM2, HMGA2, GLI and SAS*
Osteosarcoma or chordoma		Multiple structural rearrangements
Subungual exostosis	t(X;6)(q24-q26;q15-21)	*COL12A1-COL4A5*

multiple osteochondromas (HMO) [1]. Cytogenetic aberrations have been identified in the *EXT1* gene located at 8p22-24.1 in sporadic and hereditary osteochondromas. Specifically, germline mutations in the tumor suppressor genes *EXT1* located at 8q24 and *EXT2* located at 11p11-p12 have been found in HMO [59–61]. Enchondroma is a benign hyaline cartilage tumor of medullary bone commonly involving the hands and feet [1]. By conventional cytogenetic analysis, aberrations involving chromosome 12 have been detected [62, 63]. Chondromyxoid fibroma frequently occurs in the long bones, particularly the tibia. It is a benign tumor composed of lobules of spindle-shaped cells with abundant myxoid or chondroid intercellular material [1]. Structural rearrangements of chromosome 6 have been found to be non-random, specifically involving the long arm (q13 and q25) and short arm (p25) [64–68]. Chondrosarcoma is a malignant tumor of hyaline cartilage differentiation that may contain myxoid changes, calcifications, or areas of ossification [1]. There are several subtypes of chondrosarcoma, including conventional, periosteal, dedifferentiated, mesenchymal, and clear cell. Genetic alterations involving chondrosarcoma are heterogeneous. Comparative genomic hybridization studies have revealed extensive genetic alterations, including gains of whole chromosomes or chromosome arms at 20q, 20p, and 14q23 or partial losses [69]. Loss of genetic material from 13q was found to be an independent predictor of metastasis development, regardless of tumor grade or size [68, 70, 71]. Dedifferentiated chondrosarcoma, a subtype of chondrosarcoma composed of a well-differentiated cartilage tumor juxtaposed to a high-grade non-cartilaginous sarcoma; reportedly possesses frequent structural and numerical alterations of chromosome 1 and 9 [1, 66, 72–75]. Identical somatic deletion in exon 7 of p53 have been identified in the cartilaginous, as well as within the dedifferentiated part [1, 66]. Mesenchymal chondrosarcoma is a rare subtype characterized by a biphasic pattern composed of undifferentiated small round cells and islands of well-differentiated hyaline cartilage [1]. In two cases of mesenchymal chondrosarcoma a Robertsonian (13;21) translocation was observed [76].

Osteogenic tumors produce a bony matrix or what is referred to as ‘osteoid’ and, depending on their behavior, are considered either benign or malignant. However, rarely benign osteogenic tumors undergo malignant transformation [1]. Osteoid osteoma is a benign cortically based bone-forming tumor defined by its small size, limited growth potential (seldom exceeds 1.0 cm), and disproportionate pain. Reportedly, there have been two of three cases of osteoid osteoma in which a loss of the distal part of chromosome arm 17q was detected [77]. Osteosarcoma, a highly malignant bone tumor, commonly arises within the long bones of children; however, about 15 % arise in adults secondary to a pre-existing condition, such as Paget disease [1]. The classical or conventional osteosarcoma may histologically show a variable of features and be subtyped as either osteoblastic, chondroblastic, or fibroblastic. There are other more rare subtypes, including telangiectatic osteosarcoma, small cell osteosarcoma, parosteal osteosarcoma, periosteal osteosarcoma, high-grade surface osteosarcoma, and more. Most osteosarcomas possess complex chromosomal alterations. However, there appear to be certain chromosomal regions that display recurrent amplifications. Amplification at 1q21-23 and 17p, together

with co-amplification of 12q13-15, are frequently reported [78–84]. The *MDM2* gene is amplified in 14–27 % of osteosarcomas [82, 85, 86]. Amplification of CDK4 gene is found in aggressive lesions [87–89]. Lastly, the *MYC* gene has been shown to be amplified in 44 % of osteosarcomas (8q24) [90]. Understandably, low-grade variants of osteosarcoma have not been found to harbor complex aberrations. For example, low-grade central osteosarcoma show recurrent gains in minimal common regions at 12q13-14, 12p, and 6p21 [91]. Parosteal osteosarcoma, another low-grade osteosarcoma that arises on the surface of bone, reportedly shows ring chromosomes with co-amplification of *SAS*, *CDK4*, and *MDM2* genes with a minimal common region at 12q13-15 [92–94]. As stated previously, osteosarcomas may arise secondary to a pre-existing condition (Paget disease) or after radiation therapy. In tumors forming after radiation therapy, chromosomal losses are more often observed than gains [95]; 1p is lost in 57 % of post-radiation osteosarcomas, in contrast to conventional osteosarcomas that demonstrate only a 3 % loss [95, 96]. Osteosarcomas arising secondary to Paget disease of the bone show a frequent loss of heterozygosity at 18q [97]. A unique and rare tumorous lesion with aggressive growth known as bizarre parosteal osteochondromatous proliferation (Nora's lesion) is an exception to the rule, in that this neoplasm possesses a unique recurrent aberration t(1;17)(q32;q21) not found in most bone-forming tumors [98].

Fibrogenic tumors do not produce a mineralized matrix, but form collagen. Desmoplastic fibroma of the bone is a rare, benign neoplasm composed of spindle cells with minimal atypia and abundant collagen [1]. The histomorphology is identical to fibromatosis of the soft tissue. Like its soft tissue counterpart, a subset of desmoplastic fibromas of the bone show trisomies of 8 and 20 [99].

Ewing's sarcoma, as previously mentioned above, demonstrates the same aberrations as seen in its soft tissue counterpart.

Giant cell tumor of the bone is a locally aggressive neoplasm. Histology reveals sheets of neoplastic ovoid mononuclear cells interspersed with uniformly distributed large osteoclast-like giant cells [1]. A frequent chromosomal aberration detected in giant cell tumor of the bone is a reduction in the length of telomeres. Most commonly affected telomeres are 11p, 13p, 14p, 15p, 19q, 20q, and 21p [100–105].

Chordoma is a neoplasm that arises from notochordal remnants. It usually occurs in the sacrum or clivus. Typically, chordomas display a lobular growth pattern and contain neoplastic cells with bubbly cytoplasm known as "physaliphorous cells" [1]. In 9 of 16 cases of chordomas, hypodiploidy was identified. Chromosomes 3, 4, 10, and 13 were frequently lost [68, 106]. A tumor suppressor gene is believed to exist on distal 1p due to loss of heterozygosity at band 1p36 [107].

Admantinoma, a malignant biphasic tumor characterized by an epithelial and osteofibrous component, may derive from their osteofibrous dysplasia-like counterparts. Osteofibrous dysplasia and adamantinoma show similar cytogenetic alterations such as trisomies of chromosomes 7, 8, 12, 19, and 21 [108–111].

As described in soft tissue tumors, there are also tumors of undefined histogenesis that occur in the bone. Primary aneurysmal bone cyst, a cystic lesion of bone composed of blood-filled spaces separated by connective tissue septa with

fibroblasts, giant cells, and reactive woven bone, has been shown to harbor a recurrent translocation t(16:17) in several cases [1, 112]. However, rearrangements are absent in secondary aneurysmal bone cysts [113]. Over the years, additional translocations have been identified, all of which result in oncogenic activation of the USP6 gene on chromosome 17p13 [114–116]. Fibrous dysplasia, a benign medullary fibro-osseous lesion, may involve one (monostotic) or more bones (polyostotic) [1]. It is characterized by activating mutations in the GNAS1 gene located on chromosome 20q12–q13.3, encoding the alpha subunit of the stimulatory G protein [117].

13.4 Therapy for Soft Tissue Tumors

Benign tumors are generally treated with surgical excision. Malignant tumors (sarcomas) can be treated with combination therapy, including surgery, chemotherapy, targeted therapy, radiation therapy, hormonal therapy, and immunotherapy. Surgery achieves local control and removes tumor burden. Radiation therapy is applicable when the surgical margin is positive or used neoadjuvant to facilitate subsequent resection. Cytotoxic chemotherapy is used for metastatic disease, unresectable tumors, or sensitive histological types. Sensitivity to chemotherapy is variable among sarcomas, and, at this time, histology appears to be the best predictor of response. Chemotherapy-sensitive tumors include Ewing's sarcoma, rhabdomyosarcoma, myxoid/round cell liposarcoma, synovial sarcoma, uterine leiomyosarcoma, desmoid tumor (fibromatosis), desmoplastic small-round-cell tumor, and angiosarcoma. Resistant histologic types include ASPS, clear cell sarcoma, and extraskeletal myxoid chondrosarcoma. Dedifferentiated liposarcomas, epithelioid sarcomas, and perivascular epithelioid cell tumors are minimally sensitive to cytotoxic chemotherapy, whereas fibrosarcoma, high-grade undifferentiated sarcoma, malignant peripheral nerve sheath tumor, solitary fibrous tumor/hemangiopericytoma, and hemangioendothelioma exhibit intermediate sensitivity to chemotherapy. Although histology may be the best predictor of response, histologic grade is the greatest predictor of recurrence [118]. Unfortunately, there are many more high-grade sarcoma subtypes resistant to anthracycline-based therapies compared to chemotherapy-sensitive subtypes. Furthermore, most clinical trials from the 1970s to present often "lump" multiple histologic subtypes together to increase the total sample size at the expense of diluting the overall effect of the chemotherapeutic agent. Thus, endpoints such as response rate or survival are often modest in benefit, resulting in controversy among many sarcoma specialists. Clearly, there is a need to improve our understanding of the molecular pathology so that the survival of patients afflicted with these rare diseases can be improved.

An excellent example of molecular-based therapy involves GISTs. This rare mesenchymal tumor is postulated to arise from the interstitial cell of Cajal and commonly involves the stomach and small intestine of adults (peak age of 60), with less than 10 % of cases in individuals under age 40 years. They may occur in

extra-gastrointestinal sites, including the retroperitoneum, mesentery, and omentum. Ninety percent of GISTs harbor a mutation within the KIT gene and/or PDGFRA gene (80 % and 10 %, respectively) [119]. Recently, V600E BRAF mutation was identified in adult patients with GIST lacking KIT and PDGFRA mutations [120, 121]. GIST mutational analysis is important in the evaluation of patients with GIST tumors to analyze prognosis, to make decisions regarding adjuvant treatment, to predict treatment response, and to select appropriate dosage. Different types of mutations involving different exons result in varying clinical prognosis, histologic appearance, and treatment decisions for patients. Internal tandem repeats involving exon 11 are associated with gastric GISTs and an indolent course, whereas deletions within exon 11 are associated with a shorter survival, higher risk of recurrence, and an aggressive course. Exon 9 mutations are associated with small intestinal GISTs and a clinically aggressive course. PDGFRA missense mutations in exon 18, exon 14, or exon 12 occur in GISTs involving the stomach, which have an epithelioid or mixed epithelioid/spindle cell histology [3, 122, 123]. Historically, treatment of advanced GIST is revolved around surgery, with few effective systemic therapeutic options; however, the introduction of imatinib mesylate (ST1571; Gleevec), a selective tryosine kinase inhibitor that targets KIT and PDGFRA, has changed the course of this disease.

Imatinib is an oral FDA-approved drug for use as an adjuvant therapy following complete gross resection of tumor and in the management of unresectable and metastatic disease. GIST tumors with exon 11 mutations are the most responsive to imatinib therapy and GISTs with exon 9 mutations may benefit from an increase dose (800 mg). Patients with wild-type GIST and wild-type PDGFRA rarely show a favorable or sustained response to treatment. In this situation, other less common gene mutations are likely involved, such as in the succinyl dehydrogenase gene, noted in patients with an inherited form of GIST that is often accompanied by the development of paragangliomas, and pulmonary hamartomas [124]. Other forms of wild-type KIT and wild-type PDGFRA GIST include tumors with activation the Ras pathway in NF1 patients or in tumors with insulin-like growth factor 1 receptor (IGF1R) amplification. Unfortunately, there are cases where patients with exon 9 and PDGFRA mutations have primary resistance to imatinib likely due to the structure of the ATP-binding loop, which results in decreased affinity for the drug [125]. Secondary resistance occurs and generally is due to additional mutations involving exon 11. Sunitinib malate (SU11248; Sutent) is a multi-targeted tyrosine kinase inhibitor that also inhibits vascular endothelial growth factor receptors (VEGFRs). It is used to treat patients with primary or secondary resistance to imatinib. Patients with exon 9 mutations or wild-type genotyping demonstrate a good response, although the overall response rate is noted to be less than 10 % [126]. As with imatinib, sunitinib resistance may evolve and thus represents an area of active investigation. Novel therapeutic strategies that target different aspects of the intracellular signaling pathway involving KIT and PDGFRA are being investigated, and potential options include the following: heat-shock protein (HSP)-90 inhibitor, everolimus, nilotinib, sorafenib, dasatinib, regorafenib, masitinib, PI3-kinase/mTOR dual inhibitors, and PI3-kinase inhibitors [127]. In a phase II efficacy study of sorafenib,

partial response (13 %) and stable disease (58 %) were reported in 24 patients with resistance to imatinib or sunitinib. A multicenter phase II study of Masitinib revealed preliminary observations of objective partial response (50 %), stable disease (47 %), and primary refractory (3 %) with overall disease rate of 97 %. Phase III studies are underway comparing nilotinib and masitinib as single agents with imatinib in the first-line setting. The HSP-90 inhibitor inhibits HSP-90, a chaperone protein that aids in protein folding to stabilize KIT from degradation, to prevent stabilization of KIT, and to lead to its degradation [127].

Pigmented villonodular synovitis (PVNS; also known as diffuse-type giant cell tumor) is a potentially aggressive proliferative lesion involving synovial tissues of joints, tendon sheath, and bursa. The extra-articular form presents with an infiltrative soft tissue mass with or without involvement of the adjacent joint. PVNS affects young adults under age 40 years with a slight female predominance and occurs around the knee region a majority of the time [1, 128].

Translocation t(1;2)(p13;q35) [CSF1-COL6A3 fusion transcript] is found in a minority of cells in 63–77 % of patients with PVNS. This translocation leads to overexpression of CSF1, which plays a major role in the pathogenesis of PVNS. The mononuclear-histiocyte-like neoplastic cells and their proliferation is driven by expression of CSF1. Besides the autocrine stimulation, a paracrine effect is present with the recruitment of adjacent histocytes and oseoclast-like giant cells harboring the CSF1 receptor [128]. Treatment entails partial or complete excision of synovium and involved bones, and aggressive cases may be followed by low-dose radiotherapy; however, recurrences commonly occur and patients can undergo multiple surgeries. The possibility of targeted therapy with CSF1 inhibitors as an adjunct to surgery and radiation therapy may provide another avenue for patients to avoid multiple surgeries. CSF1 inhibitors include imatinib, sunitinib, and nilotinib. In a small case series and a case report, imatinib demonstrated clinical and radiologic responses in patients with PVNS. Prospective clinical trials are underway testing nilotinib [129–131].

Synovial sarcoma makes up about 5–10 % of soft tissue sarcomas affecting young adults, more commonly males. This neoplasm is unrelated to synovium; 80 % of cases occur in the deep soft tissue of the extremities, especially around the knee. Histologic variants identified in synovial sarcoma are biphasic type, monophasic type, calcifying type, and poorly differentiated (high grade) type. The translocation t(X;18)(p11.2;q11.2) is common to synovial sarcoma involving the SYT gene and either SSX1, SSX2 or SSX4 [1]. Synovial sarcoma has a survival rate ranging between 36 % and 76 % in 5 years and 20 % and 63 % in 10 years. The clinical course demonstrates a high rate of local recurrence and metastatic disease. About one fourth of patients have metastatic disease at initial diagnosis either to lung, lymph nodes, or bone. Unlike other sarcomas, this particular soft tissue sarcoma is known for its responsiveness to ifosfamide-based therapies. In at least one series, the overall response rate in patients treated with doxorubicin/ifosfamide was 58.6 %, and a separate study revealed a superior disease-specific survival for doxorubicin/ifosfamide-treated synovial sarcoma patients compared to those without this chemotherapy regimen [132, 133]. Unfortunately, the mechanisms by which

ifosfamide-based regimens affect synovial sarcoma cells are poorly understood, primarily because ifosfamide requires hepatic activation. The VEGFR inhibitor, cediranib, inhibits tyrosine kinase activity of VEGFR-1 through VEGFR-3. A pediatric phase I study revealed objective responses in synovial sarcoma, Ewing's sarcoma, and osteosarcoma. mTOR inhibitors appear to be useful in possibly treating soft tissue neoplasms, including synovial sarcoma. Akt and mTOR are noted to be constitutively active in some synovial sarcomas, and their activation has correlated with metastasis. Bcl-2 inhibitors are another option to be studied in the treatment of synovial sarcoma. Bcl-2 is up-regulated in the common expression profile of synovial sarcoma and may be a major mechanism of chemoresistance. In vitro studies have proven successful at slowing growth and inducing apoptosis in cell lines in conjunction with cytotoxic agents [134–138].

WDLPS/ALT is one of the most common sarcomas of adulthood (age 40–60 years), commonly affecting the deep soft tissues of the proximal extremities and retroperitoneum. There are four subtypes: adipocytic, sclerosing, inflammatory, and spindle cell. Wide local excision is the choice of treatment. Although 15–53 % of these recur locally, this type of liposarcoma does not metastasize. The 5- and 10-year survival rates are 70–85 % and 50 %, respectively [1, 139, 140]. Dedifferentiation may occur especially in retroperitoneal lesions, which may metastasize. Dedifferentiated liposarcoma is composed of a well-differentiated liposarcoma and a non-lipomatous component. Ninety percent of dedifferentiated liposarcomas arise de novo, while the remaining 10 % occur in recurrences. Retroperitoneal location is a common site, making complete excision almost impossible. It has a metastatic rate between 10 % and 20 %, with an overall mortality of 50–75 %.

Both WDLPS/ALT and dedifferentiated liposarcoma demonstrate amplification of MDM2 and CDK4 on chromosome 12q13-15 due to ring and giant marker chromosomes [1, 141–143]. Besides surgery, potential molecularly targeted therapeutic entities include MDM2 and CDK4 inhibitors. MDM2 inhibitors aim to reactivate p53 and thus allow it to actively induce cell death in response to appropriate stressors. MDM2 antagonists have been shown to activate wild-type p53 and induce cell cycle arrest and apoptosis in cancer cell lines. Phase I trials with MDM2/p53 inhibitors (nutlin, RG7112) have shown signs of response in patients, with an induction of p53, p21, cell cycle arrest, and apoptosis. Phase I trials with CDK4 inhibitors are underway. Peroxisome proliferator-activated receptor-gamma (PPARγ) forms a heterodimeric complex with the retinoid X receptor. This complex regulates transcription of adipocyte-specific genes by binding sites on DNA. Agonist ligands for the PPARγ receptor have been shown to induce terminal differentiation of normal preadipocytes in human liposarcoma cells in vitro. Phase II trial with Troglitazone showed histologic/biochemical differentiation in three patients with high-grade liposarcoma; however, in a study of 12 patients treated with Rosiglitazone, which belongs to the same class of drugs (thiazolidinediones) as Troglitazone, results were not as promising, with median progression-free survival of 5.5 months.

Leiomyosarcomas exhibit smooth muscle differentiation and arise in the uterus, gastrointestinal tract, and in the lining of blood vessels. These tumors exhibit complex cytogenetics and no specific oncogenic event has been identified. This may be

further complicated by the fact that this phenotype may be organ-specific [144]. Mutations in the p53 gene have been identified, resulting in decreased protein expression. Other molecular events associated with decreased protein expression include the inactivation of DAP kinase, p16, and RASSF1A. As with dedifferentiated liposarcomas, overexpression of CDK4 and MDM2 has been observed [144]. The most notable molecular aberration of therapeutic interest is the inactivation of PTEN. Mouse models with homozygous deletion of PTEN have been observed to develop leiomyosarcomas within the abdomen [145]. Furthermore, inhibition of the mTOR pathway by everolimus appeared to decrease tumor growth and improve survival of the affected mice. Standard cytotoxic chemotherapy is utilized for the treatment of metastatic disease with variable response. As with other sarcomas, better therapeutics are needed.

13.5 Therapy for Bone Tumors

Ewing's sarcoma/PNET is a small blue round cell sarcoma with varying degrees of neuroectodermal differentiation commonly occurring in individuals younger than 30 years, with a male predilection. Ewing's sarcoma/PNET often arises in the diaphysis/metaphyseal-diaphyseal portion of long bones, pelvis, and ribs. However, this sarcoma may occur in soft tissue, although rarely in visceral organs [1, 140]. Several recurrent translocations have been identified in Ewing's sarcoma/PNET. The translocation t(11;22)(q24;q12) EWSR1-FLI-1 fusion and t(21;22)(q22;q12) EWSR1-ERG fusion are found in Ewing's sarcoma/PNET in 85 % and 5–10 % of cases, respectively. Translocations not involving the EWS gene occur such as t(16;21)(p11;q24) [1, 146]. Treatment involves chemotherapy along with adjunctive surgical resection with or without radiotherapy for local control. With the available treatment, survival for localized disease is 65–70 %; however, for those with metastatic disease at the time of diagnosis, a survival of between 25 % and 30 % has been reported [134]. Other possible therapies could include a more molecularly targeted approach. The IGF1R signaling pathway has been implicated in the cancer biology of several tumors, including Ewing's sarcoma. Ewing's sarcoma clinical studies using IGF1R inhibitors (antibody-based therapy) are underway: Ganitumab (AMG479), Figitumumab (CP-751), and Cixutumumab (ADVL0821). mTOR inhibitors (rapamycin) have demonstrated the ability to arrest ES cell proliferation and downregulate levels of EWS/FLI-1 proteins. Finally, in studies of combined IGF1R and mTOR inhibition, response in patients was demonstrated [134, 147, 148]. Other therapeutic avenues include immunotherapy; in one reported case, complete remission of residual tumor was reported following autologous SCT and pulsed vaccination with dendritic cells. NK cell therapy is also being studied. Chemotherapy and radiation-induced tissue injury on malignant tissue may increase expression of NK-activating ligands, thus sensitizing tumor cells to NK killing. With NK cell infusion following chemoradiation, the NK cells should migrate to the site of disease and kill tumor tissue. Toxicity has been shown against ES cells in

Table 13.3 Promising targeted agents

Hallmarks	Mechanism of inhibition	Targeted agents	Sarcomas of therapeutic interest
Sustaining proliferation	AKT inhibitors	MK2206, Perifosine	Leiomyosarcoma (LMS), Gastrointestinal stromal tumor (GIST)
	EGFR inhibitors	Lapatinib	Chordoma
	ER inhibitors	Anastrozole, Letrozole	Endometrial stromal sarcoma (ESS)
	IGF1R inhibitors	IMC-A12, SCH 717454	Ewing's Sarcoma, Solitary Fibrous Tumor/ Hemangiopericytoma (SFT/HPC)
	KIT inhibitors	Imatinib, Nilotinib, Sunitinib	GIST
	mTOR inhibitors	Everolimus, Rapamycin	LMS
	PDGFR inhibitors	Axitinib, imatinib, Pazopanib, Sorafenib	Chordomas, Desmoid, DFSP
	PI3K inhibitors	BEZ235, LY249002	GIST, LMS, Myxoid liposarcoma
Evading growth suppressors	CDK inhibitors	Dinacliclib, Flavopiridol, PD0332991	Dedifferentiated Liposarcoma, Osteosarcoma
	y-secretase inhibitors	R04929097	Liposarcoma, Osteosarcoma
	MDM2 inhibitors	R05045337	Dedifferentiated Liposarcoma
	Notch inhibitors	GDC0449	Liposarcoma, Osteosarcoma
	Weel inhibitors	MK-1775	Osteosarcoma
Avoiding immune destruction	Anti-CTLA-4 antibody	Ipilimumab	N/A
Replicative immortality	Telomerase inhibitor	GRN163L	Liposarcoma, Malignant fibrous histiocytoma
Tumor-promoting inflammation	COX2 inhibition	Celecoxib	Epithelioid hemangioendothelioma (EHE)
Activating invasion & metastasis	c-MET inhibitors	Cabozantinib, Crizotinib	Alveolar soft part sarcoma (ASPS)
Inducing angiogenesis	VEGF inhibitors	Bevacizumab	Angiosarcoma, HPC/SFT
	VEGFR inhibitors	Pazopanib, Sunitinib, Sorafenib, Vandetanib	Angiosarcoma, ASPS, EHE, HPC/SFT
Genomic instability & mutation	HDAC inhibitors	Vorinostat, Panobinostat	ESS, Conventional Chondrosarcoma, LMS
	PARP inhibitors	Olaparib	Ewing's sarcoma
Resisting cell death	BH3 mimetics	ABT-263, ABT-737	GIST, Osteosarcoma
	TRAIL antibodies	Apo2L/TRAIL	Conventional chondrosarcoma
Deregulating cellular energetics	IHD1/2 inhibition	N/A	Conventional chondrosarocoma

vitro and in vivo (mice) [149, 150]. Lastly, angiogenesis inhibitors that bind to vascular endothelial growth factor, inhibiting the proliferation of endothelial cells and the formation of new blood vessels, could reduce interstitial pressure in tumors, aiding in the effective delivery of chemotherapy and improving the oxygenation of tumors, leading to better radiation effectiveness. Vascular endothelial growth factor is upregulated in Ewing's sarcoma/PNET. The Children's Oncology Group completed a clinical trial (COG-AEWS0521) of vincristine, cyclophosphamide, and topotecan with or without bevacizumab in patients with their first episode of recurrent ES [151]. Preliminary results have yet to be published [151].

13.6 Targeted Therapy for Bone and Soft Tissue Tumors

It is obvious that understanding the molecular pathology of sarcoma is an arduous task, given the number of histologic subtypes and the unique biology of each entity. Therefore, it is no surprise to assume that one therapy does not work for all. Targeted therapies offer patients a chance of better response for their disease process, especially when chemotherapy may not be effective. Although the scope of this chapter is the molecular pathology of sarcomas and some of these points have already been discussed, a summary of promising targeted agents have been framed in the context of the recently revised hallmarks of cancer [152] (see Table 13.3). Clearly, we need to further refine our diagnostic skills and improve upon our therapeutic approach to best optimize the care of our sarcoma patients.

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Chapter 14
Molecular Pathology and Diagnostics of Pancreatic Endocrine Neoplasms

Aejaz Nasir, Jonathan R. Strosberg, Larry K. Kvols, Jalil Muhammad, Nelly A. Nasir, Omie Mills, and Domenico Coppola

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A. Nasir, M.D., M.Phil., FCAP (✉)
Diagnostic & Experimental Pathology & Tailored Therapeutics Program,
Eli Lilly & Company, Indianapolis, IN 46285, USA
e-mail: nasirae@lilly.com

J.R. Strosberg • L.K. Kvols
GI Tumor Program, Neuroendocrine Research Division,
H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

J. Muhammad
School of Medicine, George Mason University, Falls Church, VA, USA

N.A. Nasir
Department of Pathology & Laboratory Medicine, Sir Mortimer Jewish General Hospital,
McGill University, Montreal, Canada

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Abstract Pancreatic Endocrine Neoplasms (PENs) are a group of rare tumors thought to arise from the endocrine cells of the pancreas. These tumors may be functional (hormone producing), or non functional. They have a wide range of presenting symptoms. Recent research efforts have shown the complex biology of these tumors, and have started to uncover the molecular alterations responsible for the genesis of these neoplasms. In this chapter we give an overview of the molecular tests available to detect such alterations, and of their diagnostic and prognostic significance.

Keywords Pancreatic endocrine neoplasms • Pancreatic endocrine tumors/carcinomas • Classifications of PENS • Molecular genetic • Islets of Langerhans

Abbreviations

AP1	Activator protein-1
bFGF	Basic fibroblast growth factor
CLP-PET	Clinically localized pancreatic endocrine tumors
CPGs	Candidate progression genes
EFR1	Estrogen receptor gene
FHIT	Fragile histidine triad
hMLH1	Human MutL homologue
IGF	Insulin-like growth factor
IGFBP-3	Insulin-like growth factor binding protein 3
IHC	Immunohistochemistry
LOH	Loss of heterozygosity
MAG	Metastasis-associated gene
MECC	Monohormonal endocrine cell clusters
MEN1	Multiple endocrine neoplasia type 1
MP	Metastatic primary
mTOR	Mammalian target of rapamycin
NF1	Neuofibromatosis type 1

O. Mills
Department of Pathology & Cell Biology, College of Medicine,
University of South Florida, Tampa, FL, USA

D. Coppola
Department of Anatomic Pathology, H. Lee Moffitt Cancer Center
and Research Institute, Tampa, FL, USA

GI Tumor Program, Neuroendocrine Research Division,
H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

Department of Pathology & Cell Biology, College of Medicine,
University of South Florida, Tampa, FL, USA

NGF	Nerve growth factor
PECAs	Pancreatic endocrine carcinomas
PENS	Pancreatic endocrine neoplasm
PETs	Pancreatic endocrine tumors
PRAD-1	Parathyroid adenoma-related protein
SDHD	Succinate dehydrogenase subunit D
SST	Somatostatin
SSTRs	G-protein-coupled transmembrane receptors
TGF	Transforming growth factor
TSC1, TSC2	Tuberous sclerosis genes 1 & 2
VHL	von Hippel-Lindau genes

14.1 Introduction

The endocrine pancreas is composed of groups of endocrine cells known as islets of Langerhans, which are scattered throughout the organ and in the vicinity of pancreatic ducts [1–3]. The pancreatic islet cell population consists of alpha (α) cells, beta (β) cells, pancreatic polypeptide (PP) cells and delta (δ) cells [4]. Pancreatic endocrine neoplasms (PENs) are characterized by diverse clinical presentation and outcome and complex biology. PENs comprise about 2 % of all pancreatic neoplasms [5]. Every year, about 2,500 new PENs are diagnosed [6, 7]. The incidence of PENs is <1 per 100,000 person-years, although autopsy incidence ranges from 0.8 % to 10 % as they often remain clinically silent and undiagnosed [8]. PENs are associated with a better prognosis than exocrine pancreatic cancers, with an overall 5-year patient survival rate of 35–60 % [9]. There has been no strong association between environmental risk and the development of PENs. A recent study found no association with first-hand tobacco exposure or alcohol use [8].

14.2 Functional and Pathologic Classification of PENs

PENs are categorized as functional or nonfunctional. Functional tumors secrete polypeptide hormones such as insulin, gastrin, glucagon, somatostatin, vasoactive intestinal peptide, adrenal corticotrophic hormone, parathyroid hormone-related peptide, growth hormone, calcitonin, melanocyte-stimulating hormone, vasopressin, and norephinephrine, and elicit a clinically recognizable, hormone-related syndrome. Nonfunctional tumors secrete biologically inactive peptides and do not produce hormone-associated symptoms [10]. Approximately half of all PENs are nonfunctional [7].

Pathologic classification of PENs based on tumor size, proliferative activity (Ki-67 index), angioinvasion, invasion of adjacent organs, the presence or absence of distant metastases, hormone activity and clinical syndromes has been shown

to be of some use in predicting the clinical behavior of these neoplasms [7, 11]. However, a better understanding of the molecular diagnostic aspects of these unpredictable neoplasms will contribute to improved diagnostic and therapeutic strategies and better patient outcome.

Current molecular techniques have shown that the biology of PENs is complex, without a particular biologic pathway driving these clinically challenging neoplasms. These tumors appear to develop in stages with multiple sequential mutations that contribute to ultimate progression to malignancy [12]. Several factors interact in complex ways to influence development, differentiation, secretion and the interaction of PENs with the tumor microenvironment [13]. Major events involved in pancreatic endocrine neoplasm initiation, progression and distant metastasis include basic fibroblast growth factor (bFGF), fragile histidine triad (FHIT), multiple endocrine neoplasia type 1 (MEN1), neurofibromatosis type 1 (NF1, neurofibromin), nerve growth factor (NGF), parathyroid adenoma–related protein (PRAD-1), transforming growth factor (TGF), tuberous sclerosis genes 1 and 2 (TSC1, TSC2), vascular endothelial growth factor (VEGF) and von Hippel-Lindau (VHL) genes.

14.3 Hereditary Syndromes Associated with PENs

The multiple endocrine neoplasia syndromes associated with PENs include multiple endocrine neoplasia type 1 (MEN1 gene), von Hippel-Lindau disease (vHL gene), neurofibromatosis (NF-1 gene), and tuberous sclerosis (TSC1 and TSC2 genes).

14.3.1 Multiple Endocrine Neoplasia Syndrome, Type 1

Multiple endocrine neoplasia syndrome, type 1 (MEN-1) is an autosomal dominant familial syndrome first described by Moldawer et al. [14] and Wermer [15]. Clinically, it is characterized by parathyroid adenomas, pancreatic endocrine neoplasms, and pituitary hyperplasia or neoplasia [16]. Other rarer neoplasms include bronchial and thymic carcinoids, adrenocortical tumors and cutaneous lipomas and collagenomas [17]. While hyperparathyroidism is the most common endocrine manifestation of MEN-1, PENs are the second most common endocrine manifestation and occur in about 60 % or more of MEN-1 patients. The majority of PENs in MEN1 are non-functional. However, most common functional entero-pancreatic endocrine tumors in MEN-1 patients are gastrinomas [18, 19], which often occur in the wall of the duodenum and in peri-pancreatic lymph nodes, and also insulinomas. Clustering of subvariants of MEN-1 such as insulinomas [20, 21] and aggressive gastrinomas [22] within small MEN-1 families suggest specific MEN-1 mutations may correlate with specific clinical variants. PENs in MEN-1 syndrome are invariably multifocal and may be widely dispersed in the pancreas and duodenum [18].

Consequently, the role of surgical management becomes particularly challenging and controversial [23, 24]. PENs in MEN-1 may also present as multiple clinically silent entero-pancreatic macroadenomas, which may be found at surgery or at autopsy in almost 100 % of MEN-1 patients older than 40 years [20, 25, 74]. Approximately 80 % of MEN1 cases are familial, whereas 20 % appear to be associated with new mutations based on negative familial history [26].

The MEN1 gene is a tumor suppressor gene [25, 27] mapped to a specific region on the long arm of chromosome 11 (11q13). The gene encodes a nuclear protein called menin, which interacts with several other proteins including junD, a member of the activator protein-1 (AP1) transcription factor family, SMAD1, SMAD3, SMAD5, PEM, NM23, nuclear factor kB and runx2 [28–35]. Mutations in MEN1 gene are the principal genetic abnormality in MEN1 syndrome, seen in most PENs in MEN1 patients and in about one third of sporadic PENs, as an early event in the neoplastic process.

The tumorigenesis in MEN-1 patients is thought to be a two-step inactivation of the MEN1 gene, in which both copies of the MEN1 gene must be inactivated in order for tumorigenesis to occur. A "two-hit" hypothesis has been proposed whereby germline inactivation of one allele is followed by somatic inactivation of the second allele in a predisposed cell, leading to clonal proliferation [36]. Virtually all first hits at the MEN1 gene are small mutations involving one to several bases [37, 38]. Most MEN1 gene mutations occur in the locus of exon 2 [39]. However, hundreds of unique germline and somatic mutations, broadly distributed across the MEN1 open reading frame, have been found [16, 38, 40]. Most of the first-hit mutations predict premature truncation of the menin protein, while other mutations predict missense mutations or replacement of one to three amino acids, all of which result in inactivation or absence of menin protein. The second step in MEN-1 tumorigenesis always occurs in somatic tissue, usually as a postnatal event. The second-hit mutations are usually large chromosomal or subchromosomal rearrangements with a resultant deletion that includes the remaining normal MEN1 gene.

14.3.2 Loss of Heterozygosity at 11q13

Loss of heterozygosity (LOH) is used mainly to show loss of the normal copy of the MEN1 gene. In MEN-1 patients, LOH at 11q13 was found in almost 100 % of gastrinomas and other pancreatic endocrine tumors, as well as non-pancreatic endocrine neoplasia [41, 42]. Some sporadic endocrine tumors of the type found in MEN-1 show frequent LOH at 11q13. An underlying mutation at 11q13 has been traced to the MEN1 gene in about 50 % of sporadic MEN-1 like tumors with 11q13 LOH. Somatic mutations of the MEN1 gene occur in about 20 % of sporadic solitary PENs [26, 30, 43]. Therefore, MEN1 gene mutations are among the most common mutations in sporadic PENs. Among functional PENs, MEN1 gene mutations occur in about 25 % gastrinomas, 10–20 % insulinomas and 50 % VIPomas [44–47].

14.3.3 Events Following Inactivation of MEN1 Gene

After MEN1 gene inactivation, other unknown genes or undetected mutations in MEN1 gene may contribute to MEN-1 tumor development. Studies suggest that the tumorigenic pathway of MEN-1 overlaps and interacts with other homeostatic cell pathways [48–51]. Multifocal microadenoma is an early monoclonal or oligoclonal islet cell lesion in MEN-1, which may represent a hyperplastic precursor stage to subsequent neoplastic development [52]. Monohormonal endocrine cell clusters (MECCs) develop most frequently within normal pancreatic islets but also in exocrine pancreatic ducts and hyperplastic islets through 11q13 LOH. MECCs progress to microadenomas (MA) with disruption of the normal islet structure and transformation into neoplastic process. Evidence in support of microadenomas in MEN1 patients includes hyperplastic foci of gastrin cells seen by light microscopy in the duodenum of gastrinoma specimens from MEN-1 but not from sporadic gastrinomas [53]. Furthermore, in the heterozygous knockout of the MEN1 gene in mice, giant hyperplastic islets precede the development of insulinoma, suggesting that subtle islet hyperplasia may be an unrecognized precursor lesion in MEN-1 of humans despite the presence of one normal MEN1 allele. One could speculate that hyperplasia is an expression of MEN1 heterozygosity [54, 55].Further studies are needed to link these findings with an as yet undiscovered genetic basis of tumor development.

14.3.4 Von Hippel-Lindau Syndrome

Von Hippel-Lindau syndrome (VHL) is an autosomal dominant disorder characterized by hemangioblastomas of the central nervous system, renal cell carcinomas, retinal angiomas, visceral cysts, pheochromocytomas and PENs, which occur in10–20 % of VHL patients [56–59]. The VHL gene, mapped to chromosome 3p25.3 [60], is a tumor suppressor gene that has an inhibitory effect on transcription elongation and facilitates the proteasome-mediated degradation of the hypoxia-inducible factor 1 (HIF-1) protein [16, 61]. The alpha subunit of HIF-1 is highly sensitive to tissue oxygen levels. In the presence of normal oxygen levels, it is bound by the VHL protein complex and covalently linked to ubiquitin in order to be targeted for degradation. In the absence of the VHL protein, HIF-1 alpha levels increase, leading to overproduction of hypoxia-associated cytokines, including erythropoietin, vascular endothelial growth factor (VEGF), and platelet-derived growth factor [5, 62–67]. These cytokines have been implicated in tumor growth. However, the precise mechanism of tumorigenesis is unknown. Other factors contributing to tumor pathogenesis may include matrix metalloproteinases such as MMP1 [68, 69]. VEGF inhibition, with resultant inhibition of angiogenesis, is currently under investigation as a potential therapeutic strategy for PENs [70, 71].

The majority of patients present with a germline mutation of the gene from the affected parent and a normal copy of the gene from the unaffected parent. Tumor develops when both alleles are inactivated, usually as the result of a deletion [5]. In one study 12.3 % of 155 patients with VHL went on to develop PENs [72]. Majority of these neoplasms tend to be nonfunctional, and are frequently composed of cells with foamy, clear cytoplasm, similar to clear cells typical of renal cell carcinomas in VHL [41, 73]. LOH of the VHL gene was found in 100 % of PENs (6 of 6 tumors) analyzed by PCR-single strand conformational polymorphism and fluorescent in situ hybridization. All the tumors in this study were nonfunctional [41]. These findings support a role for VHL gene mutation in the formation of VHL-associated PENs. In patients with sporadic PENs, no mutations were found in the VHL gene, although allelic loss on chromosome 3p was found in 33 % of 43 patients with sporadic PENs [74].

14.3.5 Neurofibromatosis Type 1

Neurofibromatosis type 1 (NF-1) is a neurocutaneous syndrome characterized by neurofibromas, Lisch nodules on the iris, dermal café-au-lait spots, as well as a variety of endocrine neoplasms, including somatostatin-producing neuroendocrine tumors of the duodenal wall, pheochromocytomas, hyperparathyroidism, hypothalamic or optic nerve tumors [16] and rarely somatostatinomas of the pancreas [5, 75, 76]. NF-1 is caused by a mutation of the NF-1 gene, a tumor suppressor gene located on chromosome 17q11 that code for the protein neurofibromin. Mutations cause the premature truncation of neurofibromin. The precise role of the NF-1 gene in the development of PENs still remains to be elucidated.

14.3.6 Tuberous Sclerosis

Tuberous sclerosis is a rare autosomal dominant syndrome associated with the development of hamartomas and benign tumors in multiple organs, including skin, brain, and kidney. Two gene mutations have been described: TSC1 on 9q34 encoding hamartin [77] and TSC2 on 16p13.3 encoding tuberin, with identification of the tuberous sclerosis gene on chromosome 16 [78]. Together, these proteins function as a tumor suppressor gene complex and control the activity of mammalian target of rapamycin (mTOR) [16]. A complex of hamartin and tuberin is thought to regulate cell-cycle progression, possibly through upregulation of the mTOR cell-signaling pathway [79, 80]. mTOR is an intracellular protein that is key in the control of cell growth, protein synthesis, and autophagy [71] and is involved in the regulation of β-catenin stability and activity [5]. One to five percent of patients with tuberous sclerosis can develop PENs that demonstrate LOH on 16p13.3 or lack of tuberin immunoreactivity [5, 81–83]. Based on these findings, the RADIANT 1 (RAD In

Advanced Neuroendocrine Tumors) trial, a phase II study, focused on the evaluation of everolimus, an mTOR inhibitor, in patients with advanced PENs who have failed cytotoxic chemotherapy [71].

14.4 Molecular Genetic Analyses in Sporadic PENs

The majorities of PENs are sporadic and lack any association with germline mutations. The genetic aberrations implicated in sporadic PENs are poorly understood. Oncogenes and tumor suppressor genes that are mutated in common human malignancies (p53, APC, Rb, K-ras) do not appear to be associated with PENs [84–86]. Both within genetic syndromes characterized by PENs and in sporadic PENs, genomic studies have identified specific molecular patterns that characterize individual PENs. In the case of MEN1 associated insulinoma, these molecular abnormalities include gain of function in dlk1, PCNA, TERT, ASK and NFkB [93, 257, 262, 283], and loss of function of menin, p27, p18, JunD and FANCD2 [87–89]. Sporadic insulinomas are characterized by gain in function of cyclD/CDK4, Akt1, Abl, TGFa/EGFR and Bcl2 [193, 194, 263, 263, 264, 265, 266, 277, 280] and loss of function of several proteins, including PTEN, p16, p15 and RKIP [168, 190, 260, 261, 270, 271]. Several of these genes and markers have also been studied in other PENs with variable findings.

14.5 Comparative Genomic Hybridization Studies in PENs

Several studies have used comparative genomic hybridization (CGH) to identify chromosomal aberrations in PENs. One of the most consistent chromosomal alteration is loss of 11q, which harbors the MEN1 gene [90]. Zikusoka et al. [81] compared six studies using comparative genomic hybridization to detect gains and losses in various chromosomes in PENs. The most frequent gains were on chromosomes 7 and 20. The most frequent losses were on chromosomes 2, 6q, 21q, and Y. Chromosomal aberrations associated most frequently with malignant behavior and metastasis included gains of chromosomes 7, 14q, 4, and Xq, as well as losses of chromosomes 6p, 3p, 6q, and 21q [91–93]. Male patients with malignant insulinomas have been reported to show loss of Y chromosome and gain of Xp [75, 91]. Similarly, PENs in females have been shown to exhibit loss of X chromosome [75]. Furthermore, loss of sex chromosome has been associated with metastatic disease, local invasion and poor patient survival [75]. Other chromosomal aberrations identified in PENs include gains of chromosomes 19, 5, 14p, 12q, 17, 20q, 15, 18, 9q, and 17p, as well as losses of chromosomes 1p, 6, 11q, 3q, 11p, and Xq [91, 92, 94–97]. Overall, nonfunctional PENs contained more genetic aberrations than functional PENs [91], metastases had a higher average number of chromosomal aberrations than matched primaries [94] and 11q losses and 7q gains were reported in multiple studies, supporting their role in the development of PENs.

14.6 Loss of Heterozygosity (LOH) Studies in PENs

LOH analysis is a powerful molecular tool used to identify tumor suppressor gene loci that are involved in the formation and progression of neoplasms, including PENs. An LOH frequency greater than 35 % at a specific chromosomal locus exceeds the rates of random genomic instability and strongly suggests a relevant tumor suppressor gene at that locus [98, 99]. A technical advantage with LOH is that it can detect even smaller chromosomal deletions that could be missed by CGH. Several chromosomal aberrations have been identified in sporadic PENs. Using LOH, most common abnormalities are found in chromosomes 1, 3 and 6. LOH at chromosome 1 was found in 34 % of PETs and was found to be more common in tumors with hepatic metastasis [100, 101]. Chen et al. [102] identified chromosome 1 LOH on 1q31–32 and 1q21–23 in almost half of the gastrinomas studied and found an association with aggressive growth, liver metastasis and post-surgical recurrence of liver metastasis, suggesting a worse prognosis for patients with chromosome 1 aberrations. Overall, 56 % of 273 metatsatic PENs analyzed in several different studies were found to show various chromosomal aberrations, especially in chromosomes 1, 3, 6, X and Y, as opposed to 39 % of 264 non-metastatic PENs [75, 91, 98, 100, 102–109]. These data point toward a potential association between chromosomal aberrations and progression of PENs.

Chromosome 3 is the location of the vHL gene (3p25.3) [60], which has been associated with vHL syndrome-associated PENs. Recent studies have shown LOH at loci proximal to vHL locus [103] and LOH at 3p14.2–3p21 loci occur more often in malignant insulinomas than in benign insulinomas. Barghorn et al. [104] found highly statistically significant increased frequency of LOH at 3p25.3–p23 in malignant as compared to benign PENs, and in metastasizing as compared to non-metastasizing PENs. Additionally, a strong correlation was found between the loss of alleles on chromosome 3p and clinically metastatic PENs. These findings suggest a tumor suppressor gene at 3p25.3–p23 that may be associated with sporadic PEN development and that losses of larger centromeric regions are associated with metastatic progression. In another study, LOH at 3q was found in half of sporadic PENs with hepatic metastases, while PENs without hepatic metastasis showed no LOH at this location [105]. Microsatellite markers demonstrate the smallest common deleted region at 3q27-qter, the region of p51 (a member of the p53 tumor suppressor family) [110]. These findings are suggestive of a late event in the molecular pathogenesis of PENs, consistent with advanced stage of tumor development [81].

LOH detected chromosomal loss at 6q in 6 % of sporadic PENs overall and in 100 % of insulinomas, suggesting a chromosomal aberration specific to insulinomas [91]. Additional findings were smallest regions of allelic deletions at 6q22 (50 %) and 6q23–24 (41.2–56.3 %). Also, more chromosome 6 aberrations were detected by FISH in metastatic relative to benign PENs [106]. Thus, chromosome 6 alterations may play a specific role in the molecular pathogenesis and progression of insulinomas. LOH at chromosomal arm 9p, the home of p16, is a frequent finding in PENs. However, Moore et al. [111] found a p16 mutation in only one insulinoma

out of 41 PENs, none of which showed methylation. Current studies seem to indicate that p16 inactivation by promoter methylation may be restricted to functional gastrinomas. Rare homozygous p16 gene deletions have also been reported in PENs [107]. Using CGH, Speel et al. [93] found 9q gain to be the most common gain in insulinomas (50 %).

The tumor suppressor gene, PTEN, is located at chromosome 10q23 [112] performed a mutation analysis of the entire coding region of PTEN in 33 PENs and found only one tumor with a somatic mutation in exon 6. However, 10q23 region LOH was detected in more than half of malignant and in none of 7 benign PENs. All samples with LOH at 10q23 were malignant PENs. This suggests that allelic loss of this region could be associated with malignant behavior. In non-neoplastic pancreatic islet cells, immunohistochemical expression of PTEN protein is localized to the nucleus. PTEN expression was lost in the single malignant PEN with two structural hits; however, all of the PENs with LOH remained PTEN-immunoreactive but PTEN was localized predominately in the cytoplasm and cell membrane in 23 of 24 (96 %) PENs. No increase in malignant behavior is associated with this shifting of PTEN from the nucleus but is associated with the neoplastic state in general. Based on the above findings [112] it was proposed that inappropriate compartmentalization of PTEN (cytoplasmic as opposed to nuclear localization of PTEN) could be an initiating event in the genesis of PENs, whereas physical loss of 10q leads to progressive malignancy.

Chromosome 11q13, discussed previously, is associated with *MEN1* and the development of most MEN1-associated PENs as well as some sporadic PENs. Chromosome 11p13–15 was studied in a comparative genomic hybridization investigation of 25 PENs from 23 patients. 11p13–15 loss was found in 24 % of cases, likely representing uncharacterized tumor suppressor genes in this region [94]. Chromosome 11q23 harbors the tumor suppressor gene *succinate dehydrogenase subunit D (SDHD)* [113]. A number of studies have shown significant allelic loss of 11q extending to 11q23, or distal to 11q13, and have thus postulated that a previously unrecognized tumor suppressor in this region plays a role in PEN development [47, 91, 108]. Chromosome 12p12 is the location of the K-ras gene, which is commonly mutated in pancreatic ductal adenocarcinomas but is found only rarely in PENs [111], supporting the idea that exocrine and endocrine pancreatic neoplasms involve different genetic pathways. Chromosome 17p13 is home to TP53, which plays a significant role in the tumorigenesis of pancreatic ductal carcinomas, but not PENs. A study by Moore et al. [114] supported previous suggestions that the presence of a tumor suppressor gene other than TP53 on chromosomal arm 17p is involved in the molecular pathogenesis of nonfunctional PENs.

Chromosome 18q21 mutations may play a role in the molecular pathogenesis of nonfunctional PENs, whereas select functional tumors lack this change [115]. 18q21 is the location of the *DPC4/Smad4 gene*, a cell cycle regulator [116]. However, in another series of PENs, these chromosomal aberrations were not detected in any of the 19 nonfunctional PENs analyzed [117]. Chromosome 22 was studied in gastrinomas, insulinomas, VIPomas, and nonfunctional PENs, and LOH was found

on chromosome 22q in 22 of 23 tumors [109]. Another study found LOH in 57 % of insulinomas at 22q12.1–q12.2 [118]. This site is the location of the hSNF5/INI1 gene, implicated in medulloblastoma and other pediatric central nervous system tumors [119]. Further studies could not find an alteration in this gene suggesting it is not the cause of tumor development [81, 118]. X chromosome losses were seen in patients with functional and nonfunctional PENs and were associated with shorter patient survival [8] and clinically aggressive behavior [75, 98, 120]. Y chromosome losses were found frequently in PENs from males (36 %) and were associated with metastasis, local invasion, and high proliferation rates [75].

14.7 Cell Cycle Regulars in Sporadic PENs

Regulation of the cell cycle keeps cell death (apoptosis) in balance with cell growth (proliferation). Loss of cell cycle regulation is one of the hallmarks of neoplasia. Understanding the regulatory mechanisms of the cell cycle are complex, as multiple, often repetitive pathways may be involved. A number of studies have shown that common cell cycle regulators are involved in the molecular pathogenesis of PENs.

P27KIP1 is a cell kinase inhibitor that opposes cell cycle progression and is located on chromosome 12p12–p13.1 [121]. A study by Guo et al. [122] found overexpression of P27KIP in sporadic PENs. An elevation of P27KIP1 expression was found to be inversely related to Ki-67 in a study of 109 gastroenteropancreatic NETs, suggesting that P27KIP1 may inhibit proliferation in these tumors [123].

Loss of p16INK4/p14ARF, a tumor suppressor gene located on 9p21 [124] l, leads to tumorigenesis as a result of deregulation of p53 and cyclin-dependent kinase/retinoblastoma pathways [125]. Reports found inactivating p16INK4 gene alterations (such as homozygous deletion and methylation at the 5′CpG islands of promoter regions) in 92 % of gastrinomas and nonfunctional PENs. Loss of expression of genes in the 9p21 region was found in 57 % of nonfunctional PENs, 30 % of insulinomas, and 22 % of gastrinomas. This study also found CpG promoter methylation of the p16 gene [126].

Cyclin D1, on chromosome 11q13 [127, 128], plays an important role in cell cycle regulation. Nuclear expression of Cyclin D1 was found to be increased in almost half of the PENs [129]. Sporadic PENs were specifically studied and Cyclin D1 overexpression was found in 65 % (20 out of 31) of the PENs studied compared to normal pancreatic tissue [130]. Pathways associated with Cyclin D1, specifically the P38/mitogen-activated protein kinase and Akt/PKB pathways, were activated in PENs, whereas down-regulation of the extracellular signal-regulated kinase pathway was also found with overexpression of Cyclin D1 [131–133].

Other cell cycle regulators have been studied with controversial results, including the retinoblastoma tumor suppressor gene initially found to be deleted in insulinomas [134, 135] but without further confirmation [74, 136, 137] and, as mentioned previously DPC4/Smad4 on chromosome 18q21 [116].

Cell cycle regulators studied and found not to be important contributors to PEN development to date include P53 located on chromosome 17p13.1 [114, 138–140], β-catenin located on chromosome 3p21 [141, 142], phospholipase CB3 located on chromosome 11q13 [143], and retinoic acid receptor β located on chromosome 3p24 [144, 145].

14.8 Other Molecular Genetic Abnormalities in Sporadic PENs

PI3K-Akt/PKB pathway participates in the mediation of β-cell mass up-regulation [146–148]. This pathway activates downstream messengers and transcription factors such as PDX-1, Ngn-3, Isl-1, NeuroD/Beta2, and Nkx2.2, known to act during pancreatic embryogenesis. Glucose and insulin-like growth factor (IGF) induce activation of the PI3K-Akt/PKB pathway and promote in vitro proliferation of insulinoma cells [147]. A persistent stimulus that promotes proliferation is seen in other tumors, including the persistence of achlorhydria inducing gastrinomas. Mouse studies found that up-regulation of the PI3K-Akt/PKB pathway is not sufficient for neoplastic transformation [146].

K-RAS2, on chromosome 12p12.1 [149], is an important oncogene that transduces cell growth signals, mutations of which lead to growth factor-independent stimulation of cell proliferation [150]. K-RAS2 mutations were not detected in PENs in a study by Yashiro et al. [151]. Another study found strong K-RAS2 immunoreactivity and mutations in 4 of 6 insulinomas studied [152].

Somatostatin (SST) and G-protein-coupled transmembrane receptors (SSTRs) seem to play a role as regulators of islet cell proliferation [1]. Loss of SST/SSTR signaling may contribute to the genesis of PENs. In support of this theory, MEN-1 studies showed decreased expression of SST and islet amyloid polypeptide in MEN syndrome type 1 [153], providing a link between loss of menin, suppression of SST and islet amyloid polypeptide expression and oncogenesis.

The dominant effect of the protooncogene c-Myc is apoptosis of the islet cells. However, increased expression of c-Myc has been demonstrated in glucose-induced hyperplasia of pancreatic islets. Thus, the effects of this gene depend on the environmental effects and the influences of other genes and proteins. Pelengaris and Khan [154] proposed a model of accumulating mutations leading to progression from hyperplasia to neoplasia. c-Myc is thought to be an early event in hyperplastic islets. c-Myc expression was found to be increased in hyperplastic islets and benign and malignant insulinomas.

RIP-Tag2 oncogene expression in transgenic mice leads to islet cell hyperplasia and neoplasia. Tag oncoprotein inactivates tumor suppressor proteins p53 and pRb. Decreased apoptosis is also seen due to overexpression of antiapoptotic protein Bcl-XL and Bcl-2. These antiapoptotic proteins counteract the effects of proapoptotic c-Myc [154, 155].

HER-2 neu, found on chromosome 11q21 [156], is a well-known oncogene that is overexpressed in some cases of breast carcinoma and is associated with increased

malignant behavior, proliferation, and metastasis [157]. HER-2 neu has been studied in gastrinomas, where its overexpression was found in a minority of these tumors, and was associated with liver metastasis [158].

The CpG island methylation of the estrogen receptor gene (ESR1), located on chromosome 6q24 [159], has also been described in breast carcinoma and has significant therapeutic implications, indicating tamoxifen resistance [160]. Estrogen receptor gene methylation was found in 64 % of PENs in one study [144].

14.9 Molecular Biologic Aspects of PENs

Human MutL homologue 1 (hMLH1), found on chromosome 3p21.3 [161], is a mismatch repair gene. One study found hMLH1 to be hypermethylated in 23 % of PENs with evidence of microsatellite instability [162]. Promoter hypermethylation (gene silencing) was associated with an improved 5-year survival (100 % vs. 56 %).

Telomerase, on chromosome 5p15.33 [163], is an enzyme that maintains the chromosomal telomere. Telomere degradation is a normal part of the cell cycle, but aberrations of telomerase can lead to tumorigenesis [81]. Telomerase activity may predict an unfavorable outcome in PENs [8, 164].

Thrombomodulin, an endothelial anticoagulant, when overexpressed, reduces cellular proliferation and promotes cellular adhesion in vitro, while expression of thrombomodulin in vivo is inversely correlated with metastatic spread [165, 166].

E-cadherin functions to promote cell-cell adhesion. Loss of E-cadherin is associated with invasion and metastasis in many malignancies. Chetty et al. [13] found aberrant E-cadherin expression in more than 50 % of PENs, which strongly correlated with lymph node and liver metastasis. In addition, nuclear E-cadherin was seen in 18/57 cases when stained with antibodies detecting the cytoplasmic fragment of E-cadherin. This is a previously undescribed staining pattern in PENs.

Cell signaling pathways influence tumor growth and hormonal activity. Neuroendocrine cells can express the insulin-like growth factor (IGF) and its receptor (IGFR) [167]. Cell line studies indicate that IGF-1 can act in autocrine and paracrine fashion to inhibit apoptosis and stimulate secretion of chromogranins, possibly by activating the P13K-AKT pathway. VEGF is also expressed by neuroendocrine tumors, and elevated levels of VEGF have been associated with tumor progression [168, 169].

14.10 Gene Expression Profiling Studies in PENs

Pancreatic endocrine tumors/carcinomas (PETs/PECAs) are clinically challenging neoplasms. Based on the presence or absence of metastases and/or gross invasion of adjacent organs, they are classified as pancreatic endocrine carcinomas (PECAs) or pancreatic endocrine tumors (PETs) [WHO-2004] [10]. In the presence of negative metastatic work-up at the time of first tissue diagnosis, it is difficult to predict

which of these tumors will become metastatic ('malignant') or remain localized to the pancreas ('benign'). Standard histologic criteria, including grade, tumor size and Ki-67 index, often fail to predict malignant behavior [170]. Patients who develop metastases are rarely cured and 5-year survival rates diminish significantly. Therefore, discovery of molecular markers of prognosis is vitally important in order to quantify the risk of metastatic recurrence in patients with resected primary tumors.

The broad heterogeneity that characterizes neuroendocrine tumors has always posed problems regarding their correct classification [171]. In recent years, several refined histopathologic classification, grading and staging schemes have been proposed for PETs and, to some extent, validated to categorize these heterogeneous neoplasms into various diagnostic and prognostic subgroups [10, 11, 172–179]. However, due primarily to the diverse biology of these rare neoplasms, there is no single internationally acceptable prognostic scheme that would allow accurate determination of prognosis in a particular case.

In order to identify newer diagnostic, prognostic markers and therapeutic targets, lately there has been an increasing application of high-throughput technologies, including gene expression profiling, to the study of pancreatic endocrine tumors [153, 180–185]. Genes that have been associated with malignant behavior in human PENs include VEGF-C [186], met proto-oncogene, insulin-like growth factor binding protein 3 (IGFBP-3) [187], MEN-1 gene [188], c-N-ras , HER-2/neu [158] and c-myc, TGF-alpha, c-K-ras and p53 [152]. Most of these studies were conducted on small numbers of specimens and have not been fully validated. In a recent study [182], gene expression profiles of primary PECAs and their hepatic metastases were found to be similar, suggesting that most genetic abnormalities accumulate at the level of primary PETs. Therefore, identification of a set of genes and/or their protein products in the primary tumor tissue in clinically localized pancreatic endocrine tumors (CLP-PET), may have significant prognostic and therapeutic implications. Clinical utility of a metastasis-associated gene (MAG) signature as a predictor of future metastases has been shown in breast [189] and prostate [190] cancers and in medulloblastoma [191]. However, molecular markers of progression in primary pancreatic endocrine tumors are largely unknown.

Gene expression profiling using microarray analysis has identified a number of genes typical of PENs [153, 182, 186, 187, 192]. Similarly, histologically normal pancreatic islets were compared with PENs, showing overexpression of 66 genes, especially IGFBP3 (a growth factor), fibronectin (a cell migration/adhesion molecule), and oncogenes like MLLT10/AF10. One hundred and nineteen genes were under-expressed, including p21CIP1 (a cell cycle regulator), JunD (a transcription factor), and NME3 (a metastasis suppressor gene). Another gene expression study compared normal islet cells and three neuroendocrine tumor cell lines; 667 genes were up-regulated, and 323 were down-regulated [182, 192].

Recently, we selected pathologically well-characterized subsets of frozen PEN tissues and adjacent histologically normal pancreatic islets, and used a comprehensive genome-wide expression profiling approach and discovered a novel set of candidate progression genes (CPGs) that were differentially expressed in surgically resected metastatic primary (MP)-PECAs relative to clinically localized primary

(CLP)-PETs [193–195]. Many of these genes, including RUNX1T, palladin, insulin receptor, CD24 antigen and NRCAM had not previously been associated with progression in PENs. We have successfully validated two of our leading candidate progression genes (RUNX1T1, palladin) at the protein level using immunohistochemistry (IHC) on larger independent test sets of MP-PECAs and CLP-PETs [196, 197]. We are now focused to advance our discovery of novel molecular markers (CPGs) to the next step of larger scale clinical validation, using the roadmaps proposed in recent literature with regard to the discovery and validation of gene-based biomarkers. Although gene expression profiling studies have identified newer candidate genes that are providing newer insights into the molecular pathology and biology of PENs, comparison among these published studies continues to be a challenge due to variations in study designs, patient populations investigated, and tumor samples analyzed.

14.11 Summary

A large number of studies have contributed significantly to our current understanding of molecular pathology and diagnosis of pancreatic endocrine neoplasms. PENs exhibit a diverse spectrum of molecular genetic aberrations similar to their broad clinical presentation. Nonfunctional PENs exhibit more molecular aberrations than functional PENs. Also, malignant behavior seems to be associated with increasing genetic aberrations, suggesting specific genes may be associated with metastases in PENs. Genome wide studies have identified several genetic abnormalities in PENs, some of which predict malignant behavior in various subtypes of PENs and adverse clinical outcome while others provide molecular evidence in support of earlier stages of neoplastic transformation of PENs. Comparative genomic hybridization studies found that most frequent chromosomal gains were on chromosomes 7 and 20. The most frequent losses were on chromosomes 2, 6q, 21q, and Y. The chromosome aberrations most frequently associated with metastasis included gains of chromosomes 7, 14q, 4, and Xq, as well as losses of chromosomes 6p, 3p, 6q, and 21q. LOH analyses have identified multiple tumor suppressor gene loci that contribute to the pathogenesis of PENs. Genetic syndromes, which include PENs as one of their components, allow for the identification of genes associated with the genesis of PENs, including MEN1, vHL, NF-1, TSC1 and TSC2. Gene expression profiling using microarray analyses has identified a large number of genes differentially expressed in PENs when compared with normal islets, suggesting their involvement in initiation or progression of PENs. These include IGFBP3, fibronectin, oncogene MLLT10/AF10, p21C1P1, JunD, NME3, RUNX1T1, paladin and p21. While these studies are providing critical scientific evidence to refine our understanding of the molecular complexity of these neoplasms, pooling of large data sets and comparison among these molecular studies is challenging because of variations in study designs, patient populations, and tumor samples (fresh-frozen versus archival) and variation in the technical platforms used. A number of interesting lines of investigation are lending credence to various hypotheses regarding the molecular pathogenesis,

progression and therapeutic responsiveness of PENs. Some the recent studies have focused on cathepsins, CD44, NESP-55, hMLH1, telomerase, thrombomodulin, and E-cadherin expression/activity. Advanced molecular testing, is currently making it more feasible to pursue newer lines of genetic studies to unravel an increasing number of chromosomal aberrations associated with PENs. Multiple molecular alterations, involving cell migratory, cell cycle and angiogenic functions have been found to promote PEN development, growth, invasion, and metastases. As a result of these studies, phase III trials of novel therapies targeting cell regulators such as mTOR, VEGF and other targets have been completed. Focused investigation of various mediators/mechanisms implicated in the molecular pathogenesis and progression of PENs will contribute to novel diagnostic, therapeutic and preventive strategies. These efforts need to be integrated with the development and validation of robust assays for prognostic and predictive biomarkers that can be used in the clinic. All of these efforts will ultimately translate into more efficacious, safer and cost-effective therapeutic and monitoring options for patients with PENs.

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Chapter 15
Molecular Pathology and Diagnostics of Childhood Tumors

Enid Gilbert-Barness and Kristen Eisbrenner

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E. Gilbert-Barness (✉)
Department of Pathology and Cell Biology, Pediatrics and Obstetrics and Gynecology, University of South Florida College of Medicine at Tampa General Hospital, Tampa, FL, USA
e-mail: egilbert@tgh.org

K. Eisbrenner
University of South Florida College of Medicine at Tampa General Hospital, Tampa, FL, USA

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Abstract Neoplasms in the fetus and infant are discussed in reference to types, incidence, clinical features, behavior, and response to treatment. Tumors that occur predominately in the first year of life have certain features of embryonic growth, including Wilms' tumors, neuroblastomas, embryonic sarcomas, yolk sac tumors of the testis, hepatoblastomas, and medulloblastomas of the brain, as well as sacrococcygeal tumors that have an overgrowth of embryonic components. Chromosome translocations, tumor suppressor genes, and some syndromes and congenital malformations associated with childhood tumors are discussed.

Keywords Childhood tumors • Molecular • Genetic • Chromosome abnormalities

Abbreviations

ARMS	Alveolar rhabdomyosarcoma
BWS	Beckwith-Wiedemann syndrome
CHEK2	Checkpoint kinase 2
CN	Cystic nephroma
CNS	Central nervous system
ERMS	Embryonal rhabdomyosarcoma
EWS	Ewing sarcoma
FAP	Familial adenomatous polyposis
FISH	Fluorescent in situ hybridization
GIST	Gastrointestinal stromal tumors
HPV	Human papilloma virus
IGF2	Insulin-like growth factor II
MEN1	Multiple endocrine neoplasia type 1
MEN2	Multiple endocrine neoplasia type 2
NF1	Neurofibromatosis type 1
NF2	Neurofibromatosis type 2
PDGF	Platelet-derived growth factor
PNET	Primitive neuroectodermal tumor
PTCH	Patch
RT-PCR	Reverse transcription-polymerase chain reaction
SHH	Sonic hedgehog
SMO	Smoothened
VHL	Von Hippel-Lindau
WAGR syndrome	Wilms' tumor, aniridia, genitourinary abnormalities and mental retardation
WT-1	Wilms' tumor-1 gene product

15.1 Introduction

Neoplasms in the fetus and infant are not entirely the same as those in the adult; differences include the types, incidence, clinical features, behavior, and response to treatment [1–13].

Continued growth without maturation of certain cells that are normally present during embryonic development, but that usually mature after birth, is responsible for many malignant congenital tumors. Wilms' tumors are derived from the metanephric blastema that has lost its propensity for differentiation but not for growth. Neuroblastomas of the adrenal gland occur from cells that arise in the neural crest and wander into the gland early in embryonic life. Normally, these cells differentiate into chromaffin tissue, but occasionally the transformation fails to take place, and a proliferation without differentiation produces a neuroblastoma.

Other tumors that occur predominantly in the first year of life and have certain features of embryonic growth include embryonic sarcomas, yolk sac tumors of the testis, hepatoblastomas, and medulloblastomas of the brain. Some teratomas of the sacrococcygeal and retroperitoneal regions can be included with those having an overgrowth of embryonic components that fail to mature. The cells of many sarcomas resemble immature fibroblasts and are also a result of failure of maturation coupled with an excessive stimulus for growth.

15.2 Incidence

The most common malignancies noted at birth are, in order, neuroblastoma, leukemia, sarcomas, and brain tumors [1, 11, 13–15]. Leukemia-lymphoma, brain tumors, neuroblastoma, and soft tissue sarcomas, in decreasing order of occurrence, are the more common neoplasms noted in older children and adolescents under 15 years of age [16].

15.3 Mechanisms in Carcinogenesis

Cancer is an uncontrolled growth of cells resulting from an alteration in cell DNA that produces a deregulation of normal growth [17]. A malignant cell becomes independent of normal regulatory control, and multiplies, producing a clone of cancer cells that subsequently develops into a neoplasm. There are several mechanisms, occurring as single or multiple events, by which the DNA of a normal cell becomes transformed into a cancer cell; this process is termed carcinogenesis. The known mechanisms include point mutations in DNA (the replacement of a single base pair within a gene by an incorrect one), gene deletions (loss of a large amount of DNA resulting in loss of all or part of the gene), and chromosomal translocations with gene rearrangements (the broken ends of the DNA from two different chromosomes may be joined incorrectly, resulting in parts of each chromosome being exchanged) [17].

15.4 Chromosome Translocations

Lejeune's recognition of trisomy 21 in Down syndrome predicted the many steps at which altered gene expression can produce abnormal tissue growth (neoplasia) or development (congenital anomalies). The similarities between molecular embryology and molecular oncology are strengthened by anomaly patterns (syndromes) that include cancer predisposition (e.g., Beckwith-Wiedemann syndrome [BWS], neurofibromatosis-1),

Table 15.1 Examples of chromosomal abnormalities associated with childhood tumors

Chromosomal defect	Childhood tumor
1p del, 1p32-p36 del, double minutes	Neuroblastoma
11p13 del, trisomy 18	Wilms' tumor
13q14 del	Retinoblastoma
	Osteosarcoma
Trisomy 21 (Down syndrome)	Leukemia
Monosomy 7	Leukemia and myelodysplasia syndrome
t(11q23), t(1;22), t(9;11)	Leukemia
t(1:22) (p13;q13)	Leukemia
Gonadal dysgenesis (46,XY; 45X/46,XY)	Gonadoblastoma, Germinoma
Klinefelter síndrome (XXY)	Teratoma
t(11;22)(q24;q12) t(21;22)	Ewing sarcoma/PNET
t(11;22)(p13;q12)	Intra-abdominal desmoplastic small round cell tumor
t(2;13)	Alveolar rhabdomyosarcoma
del 1p	Astrocytoma

From: Isaacs [18]

the dual roles of many proto-oncogene/tumor suppressor genes as developmental genes (e.g., PAX/C-KIT genes in Waardenburg syndrome/Piebald trait), and the stepwise progression from primary mutation, environmental factor, and epigenetic change to multifactorial (e.g., HOX genes in human leukemias and limb defects). Chromosomal abnormalities associated with neoplasia are shown in Table 15.1.

The number of chromosomal rearrangements and gene mutations associated with tumors is now so great that almost every cancer patient undergoes some sort of molecular or cytogenetic testing. The same progression from chromosome anomaly to causative breakpoint to cancer/tumor suppressor gene (Table 15.2) can now be followed for most developmental anomalies, and epigenesis is a central factor in both neoplasia and development [20, 21].

Chromosome analysis is now merging with DNA chips to provide telomere or array analyses, capable of defining subtle deletion/duplication of any chromosome segment by its altered fluorescent pattern.

15.5 Controlling Gene Expression

Increasing gene expression occurs by increasing the number of DNA copies of that gene (gene amplification). Human folate-resistant cell cultures are seen in aggressive neuroblastomas that amplify N-Myc. The new controlling mechanisms offer novel means for understanding and manipulating developmental gene regulation.

Table 15.2 Tumor suppressor genes involved in human neoplasms

Gene	Chromosomal location	Neoplasms associated with somatic mutations	Neoplasms associated with inherited (germ-line) mutations
WT-1	11p13	Wilms' tumor	Wilms' tumor
p53	17p13.1	Most human cancers	Li-Fraumeni syndrome: carcinomas of breast and adrenal cortex; sarcomas; leukemias; brain tumors
APC	5q21	Carcinomas of colon, stomach and pancreas	Familial adenomatous polyposis coli; carcinomas of colon
DCC	18q21	Carcinomas of colon and stomach	Unknown
VHL	3p25	Renal cell carcinoma	von Hippel-Lindau disease: retinal and cerebellar hemangioblastomas; renal cell carcinomas; angiomas and cysts of many visceral organs
NF-1	17q11	Schwannomas	Neurofibromatosis type 1; neural tumors
NF-2	22q12	Schwannomas and meningiomas	Neurofibromatosis type 2; central (acoustic) schwannomas; meningiomas
Rb	13q14	Retinoblastoma; osteo- sarcoma; carcinomas of breast, bladder, prostate, and lung	Retinoblastoma; osteosarcoma

From: Isaacs [19]

The relevance of new, non-classical pathways that control gene expression is reinforced by progressive DNA expansions/contractions and a myriad of possible gene rearrangements characteristic of most cancers.

15.6 DNA Methylation

Methylation of DNA contributes to cell differentiation, since the changes in DNA structure and expression will be transmitted to all daughter cells of a progenitor cell. Methylation of promoters probably plays a major role in cellular differentiation during embryogenesis: cells eliminate the transcription of unwanted genes by methylating their promoters.

Methylation inhibits (and occasionally activates) a very large number of genes, including some proto-oncogenes [22]. In cancers, there is often a decreased expression of DNA methyltransferase which leads to a nonspecific demethylation of 5-methylcytosine. This demethylation can reactivate the expression of proto-oncogenes, which thus act as unregulated growth-promoting agents (e.g., oncogenes, telomerases). Reactivated expression of genes involved in cell migration may lead to the emergence of more aggressive clones (clonal evolution). Abnormal methylation (hence inactivation) of anti-oncogenes similarly plays a major role in cancer development.

15.7 Zinc-Fingers

Transcription factors are those with a zinc-finger motif; zinc molecules cause these proteins to have a tertiary structure resembling fingers. The "fingers" bind the promoters of the genes under their control, to activate or inhibit them. Zinc finger proteins have multiple domains, allowing them to coordinate multiple different expression pathways. WT-1 (the Wilms' tumor-1 gene product), the fly developmental gene hunchback, and certain hormone and retinoic acid receptors belong to the zinc-finger class of transcription factors.

15.8 Loss of Imprinting and Cancer

Genomic imprinting was discovered in the 1980s and is now recognized to be very important clinically [23–25]. Imprinting is characterized by the differential activation of alleles according to their parental origin, associated with different patterns of DNA methylation.

Mammals are diploid organisms, meaning that all somatic cells possess two copies of the genome. Each autosomal gene is therefore represented by two copies, or alleles, with one copy inherited from each parent at fertilization. For the vast majority of autosomal genes, expression may occur from either allele. However, a small proportion (<1 %) of genes are imprinted, meaning that expression occurs from only one allele. Imprinting, therefore, is defined as the parental allele-specific expression of a very limited set of genes. This is an epigenetic phenomenon whereby the DNA of the two alleles of a gene is differentially modified so that only one parental allele is normally expressed [26]. The expressed allele is dependent on its parental origin. For example, the gene encoding insulin-like growth factor II (IGF2) is only expressed from the allele inherited from the father. Similarly, human triploidy with two paternal genomes produces fetal and placental tissue (hydatidiform mole), whereas triploidy with two maternal genomes biases toward fetal tissues only (ovarian teratomas). This regulation on an epigenetic marking of parental alleles occurs during gametogenesis. Mis-regulation of imprinted gene expression or loss of imprinting refers to loss of monoallelic gene regulation and concomitant biallelic expression.

In Beckwith-Wiedemann syndrome (BWS), partial moles, complete moles, and many cancers, this equilibrium is altered, leading to loss of imprinting and IGF2 over-expression. This has been documented in Wilms' tumor, Ewing sarcoma, rhabdomyosarcoma, adrenocortical tumors, hepatoblastoma, hepatocellular carcinoma, and pheochromocytoma. Conversely, the incidence of these tumors is greatly increased in BWS, and placentas of BWS fetuses can show mole-like changes. IGF2 is also overexpressed in choriocarcinoma, leukemias, and germ cell tumors, as well as in bladder, breast, cervical, esophageal, gastric, colorectal, pulmonary, ovarian, prostatic, renal cell and other carcinomas and tumors.

15.9 Intercellular Signaling in Development: Sonic Hedgehog

Embryonic cellular signaling pathways can be extremely complex, with core molecules that influence the development of multiple organs. A prototype is the sonic hedgehog (SHH; a member of the hedgehog gene family). In the absence of SHH protein, the Patch (PTCH) plasma membrane protein inhibits smoothened (SMO) protein. Binding of SHH with PTCH lifts this inhibition, allowing SMO to activate the SHH cascade, including GL12, GL13, CBP, and SUFU molecules [27, 28]. Mutations involving this cascade can result in a wide range of diseases, including tremors, holoprosencephaly, Greig cephalopolysyndactyly, Pallister-Hall syndrome, Rubinstein-Taybi syndrome, the nevoid basal cell carcinoma syndrome, basal cell carcinoma (syndromic and sporadic forms), medulloblastoma, meningiomas, primitive neuroectodermal tumors, breast adenocarcinomas, trichoepitheliomas, squamous cell carcinomas, esophageal carcinomas, fetal rhabdomyomas, and rhabdomyosarcomas [27].

15.9.1 Examples: Sequential Gene Expression in Growth and Neoplasia

Molecular analysis has defined many growth-related molecules through their alteration in tumors [27, 29–35]. The definition of molecular changes that fulfill Knudson's two-hit or two-stage hypothesis has also been reviewed [36]. Although Knudson's explanation involved one abnormal RB1 allele from the germline (predisposition or first hit), followed by somatic RB1 gene mutations in susceptible tissue (second hit in retina), epigenetic changes can also be placed on this pathway to neoplasia. This is reflected in the fact that most germline RB1 mutations originate on the paternal chromosome, implying a role for genomic imprinting/DNA methylation. Characterization of the RB1 gene as a cell cycle regulatory element places it within cell proliferation/cell death pathways.

15.10 Handling of Pediatric Tumor Specimens

Many pediatric tumors require immunostaining, molecular investigation, and electron microscopy for a definitive diagnosis and prognosis. All diagnostic pediatric tumor specimens should be submitted to the laboratory as fresh tissue immediately following biopsy when possible. The specimen should be divided into samples for formalin fixation and paraffin embedding, glutaraldehyde fixation for electron microscopy, snap freezing or storage in RNA protection medium for molecular studies such as reverse transcription-polymerase chain reaction (RT-PCR), with

Table 15.3 Staging system for Wilms' tumor cancer oncology group

Stage I	Limited to kidney and completely resected, renal capsule intact
	Fine needles aspiration or percutaneous core needle biopsy does not upstage the tumor
	The presence of necrotic tumor or chemotherapy-induced change in the renal sinus and/or within perirenal fat should not be regarded as a reason for upstaging a tumor providing it is completely excised and does not reach the resection margins
Stage II	Tumor infiltrates beyond kidney but is completely resected
	(a) The tumor extends beyond the kidney or penetrates through the renal capsule and/or fibrous pseudocapsule into perirenal fat but is completely resected (resection margins clear)
	(b) Tumor infiltrates the renal sinus and/or blood and/or lymphatic vessels outside the renal parenchyma but is completely resected
	(c) Tumor infiltrates adjacent organs or ureter but is completely resected
Stage III	Gross or microscopic residual tumor confined to abdomen
	(a) Incomplete excision of the tumor which extends beyond resection margins (gross or microscopic tumor remains postoperatively)
	(b) Any abdominal lymph nodes are involved
	(c) Tumor rupture before or intraoperatively
	(d) The tumor has penetrated through the peritoneal surface
	(e) Tumor implants are found on the peritoneal surface
	(f) Tumor thrombi present at resection margins of vessel or ureter transected or removed piecemeal by surgeon
	(g) The tumor has been surgically biopsied (wedge biopsy) prior to preoperative chemotherapy or surgery
Stage IV	Hematogenous metastasis or lymph node metastasis outside the abdominal and pelvic region
Stage V	Bilateral renal tumors at diagnosis. Each side should be sub-staged according to the above classification

imprints ("dabs") fixed for fluorescent in situ hybridization (FISH) in methanol, cytogenetic studies, or frozen for storage and subsequent analysis if required. Many molecular techniques such as FISH and RT-PCR may also be performed on routinely fixed paraffin-embedded material.

15.10.1 Wilms' Tumor (Nephroblastoma)

Wilms' tumor (Table 15.3) is a malignant embryonal neoplasm derived from nephrogenic blastemal cells. Several lines of differentiation are commonly seen and often replicate the histology of developing kidneys. Approximately 10 % of patients with Wilms' tumor also have congenital abnormalities and malformation syndromes. The most common malformations are hemihypertrophy and genitourinary anomalies. The common syndromes associated with Wilms' tumor include BWS, WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities and mental retardation),

and Denys-Drash syndrome (mesangial sclerosis, pseudohermaphroditism and nephroblastoma) [37–41]. Abnormalities involving the Wilms' tumor locus, 11p13, are consistently found in the tumors of patients with WAGR and Denys-Drash syndrome. The 11p13 Wilms' tumor locus encodes two coordinately regulated zinc-finger transcripts, WT-1 and WIT-1. These genes are highly expressed in the developing urogenital system [42–44]. The WT-1 protein binds to several sites on promoters of an IGF2 gene as well as to a promoter of the platelet-derived growth factor (PDGF) A-chain gene [45, 46]. The gene controls mesenchymal-epithelial transition during renal development. Furthermore, WT-1 expression induces transcription of one of the seven proapoptotic genes, *Bak,* and blocks cellular proliferation and DNA synthesis [47]. Interference with these normal regulatory influences of WT-1 may be an important factor in the genesis of nephroblastoma, which typically express high levels of IGF2 [48] and may also overproduce PDGF [49]. The expression patterns of WT-1 and Pax-2 in the metanephros overlap to a considerable extent; however, expression of WT-1 peaks as that of Pax-2 is decreasing. Therefore, it is possible that WT-1 represses Pax-2, and Pax-2 expression fails to down-regulate in the epithelial component of Wilms' tumor and in nephroblastomatosis, the putative precursor of nephroblastoma [50]. Despite the strong association of WT1 mutations with Wilms' tumor predisposition, WT-1 is mutated in only a minority of sporadic Wilms' tumors [51]. This low prevalence of WT-1 abnormalities in sporadic Wilms' tumor led to the recognition of other genes involved in pathogenesis. Evidence supporting this is provided by the linkage of familial BWS to a locus at chromosome 11p15, designated WT-2 [52, 53]. Approximately 1 % of the patients with Wilms' tumor have a positive family history for the same neoplasm. Most of the pedigrees suggest autosomal dominant transmission with variable penetrance and expressivity. This suggests that genetic loci other than WT-1 and WT-2 are responsible for the pathogenesis of many familial as well as sporadic Wilms' tumors [54, 55].

In gross examination, the most important factors to document are capsular invasion and hilar and sinus involvement along with adequate sampling of the tumor. Histology blocks should include at least one block per centimeter of the largest tumor diameter, tumor-kidney interface, capsule, hilar area, macroscopically normal kidney (at least two), and other areas of pathological significance. Some cases may be markedly cystic; in these, the tumor must be carefully examined for the presence of nodular solid areas. Particular attention should be paid to assessment of the capsule, hilar vessels, including the cut end, and renal sinus.

A triphasic Wilms' tumor includes epithelial, stromal, and blastemal elements microscopically. Relative proportions of the epithelial components may vary widely such that one is markedly dominant (Fig. 15.1). The stromal component may vary widely from areas of stellate cells within a myxoid background to spindle cells recapitulating primitive mesenchyme. Smooth and striated muscle are the most common differentiated stromal cell types. Occasionally, a wide range of other stromal components may be present, including cartilage, osteoid, and even glial tissues.

Post-chemotherapy features include necrosis, fibrosis, and foamy and hemosiderin-laden macrophages. Rapidly dividing cells, such as those of the blastemal or primitive epithelial components, tend to respond to chemotherapy, whereas the stromal

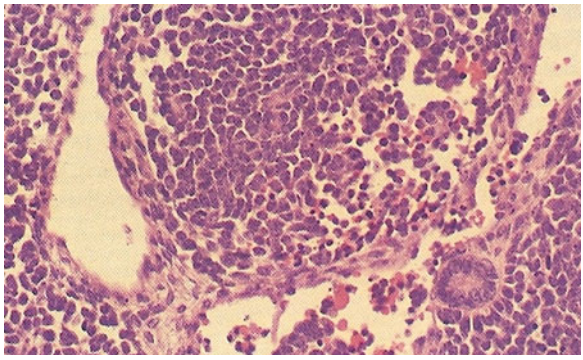

Fig. 15.1 Nephroblastemal elements in Wilms' tumor (From Gilbert-Barness [56])

elements will not. Thus, tumors with significant stromal differentiation may not reduce much in size during chemotherapy. Also, stromal components may appear to be more prominent in a post-chemotherapy specimen. The tumor that remains predominantly viable following chemotherapy and in which the main component is blastema is associated with an adverse prognosis.

15.10.2 Unfavorable Histology in Wilms' Tumor (Anaplasia)

Nuclear anaplasia in the context of Wilms' tumor has very specific criteria required for its definition, such as nuclear hyperchromasia, nuclear enlargement in which affected nuclei are at least three times the size of adjacent non-anaplastic nuclei, and abnormal multipolar mitoses. Using such criteria, anaplasia is present in approximately 5–10 % of Wilms' tumors, occurring more commonly in those diagnosed in later childhood and in black patients. Anaplasia may either be focal or diffuse. Focally, one or a few, well-circumscribed, localized regions of anaplasia are found within the primary tumor that is completely excised, with the majority of tumor containing no significant nuclear atypia. Diffuse anaplasia occurs in multiple fields or outside the primary tumor and is associated with a poor response to chemotherapy. Anaplasia is of major clinical significance when present in tumors, which are potentially incompletely excised. Focal anaplasia or anaplasia in stage I cases may be of importance. Anaplasia is identified as an adverse prognostic factor. Transcription profiling indicates that a cluster of genes separates anaplastic from non-anaplastic Wilms' tumor.

Immunohistochemical staining is often extremely useful in the diagnosis of Wilms' tumor from a needle core biopsy. A core biopsy may simply demonstrate small round blue cells, which could represent the blastema of Wilms' tumor or several other embryonal tumors of infancy and childhood. The most useful immunohistochemical marker is WT-1. Areas of stromal differentiation, and well-differentiated epithelial structures, may not express nuclear WT-1. Cytokeratin staining may help with the identification or confirmation of epithelial differentiation. CD56 is also useful,

since primitive renal blastema is diffusely and strongly CD56 positive. Nuclear p53 is expressed in 75 % of anaplastic nephroblastomas. At present, there are no diagnostic or prognostic markers useful for the evaluation of Wilms' tumor.

15.10.3 Nephrogenic Rests and Nephroblastomatosis

Nephrogenic rests are classically divided into perilobar and intralobar types. Perilobar rests are more common and are found in subcapsular peripheral locations with well-demarcated margins on low power. Intralobar rests present anywhere within the kidney and often have a more irregular and intermixed margin. The clinical relevance is their potential increased risk for subsequent development of Wilms' tumor. Wilms' tumors in association with intralobar rests develop at a younger age on average. The risk appears highest for intralobar rests in patients under 1 year of age. When a Wilms' tumor develops within a nephrogenic rest, there is a superimposed nodular growth of the proliferating component and formation of a fibrous pseudocapsule around the tumor with the compressed rest at the periphery.

15.10.4 Cystic Nephroma and Cystic Partially Differentiated Nephroblastoma

Cystic nephroma (CN) is predominantly seen in children under 5 years of age. Its characteristic imaging appearance is of a well-circumscribed mass distinct from the surrounding kidney and predominantly cystic with thin septa. In CN, the septa contain no solid nodules and only mature elements. In cystic partially differentiated nephroblastoma, the septa also contain embryonal elements that do not form solid nodules, thus conforming to the outlines of the septa. If classical components of Wilms' tumor are present, forming nodular structures that distort the septal contours, the lesion should be classified as cystic Wilms' tumor. Both CN and cystic partially differentiated nephroblastoma are adequately treated by resection alone with an excellent prognosis and no chemotherapy required. Predominantly, cystic Wilms' tumor, although almost always stage I and therefore with a correspondingly good prognosis, should be treated as an appropriately staged Wilms' tumor. There is an association between CN and pleuropulmonary blastoma [57].

The classical pattern of CN demonstrates nests of ovoid cells separated by fibrovascular septa. Cord cells may be ovoid, epithelioid, or spindled with bland nuclei without prominent nucleoli. Septa demonstrate a marked regularly branching "chicken wire" pattern of capillaries. A wide variety of other patterns have been described. These include myxoid, sclerosing, cellular epithelioid, palisaded, spindled, storiform, and anaplastic variants. Most cases are composed of a mixture of such patterns.

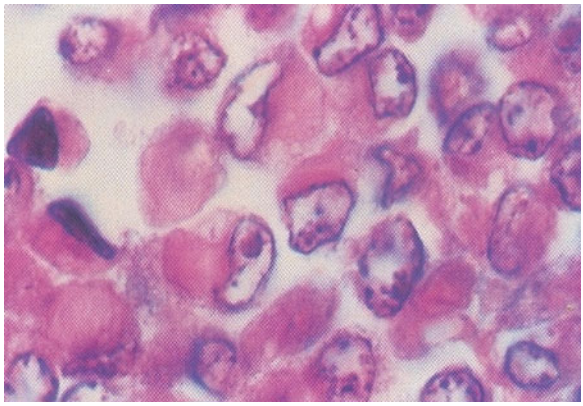

Fig. 15.2 Rhabdoid tumor cells with variable presence of intracytoplasmic inclusions (From Gilbert-Barness [56])

15.10.5 Clear Cell Carcinoma of the Kidney

Clear cell carcinoma of the kidney comprises 3 % of malignant pediatric renal tumors. The average age at diagnosis is 3 years. There are no specific diagnostically or prognostically useful genetic features. Metastatic disease is present in 5 % of cases and up to 60 % of those have bone metastases. The tumor is solid, cystic, and cream to tan in color. Immunohistochemical stains vimentin and bcl2 are usually positive. Cytokeratin, WT1, and CD99 staining is negative [57]. The vimentin staining is unlike the dot-like pattern characteristic of renal rhabdoid tumors.

15.10.6 Renal Rhabdoid Tumor

Rhabdoid tumors are rare, comprising 2 % of pediatric renal malignancies. These tumors are predominantly shown in young children, with average age at diagnosis of 1 year. This tumor is exceptionally rare in children over 5 years of age.

In patients with renal rhabdoid tumors, genetic abnormalities of the *hsNF5/INI1* tumor suppressor gene on chromosome 22 are characteristic. The gene is important for chromatin remodeling, and inactivation is tumorigenic. Mutations, deletions, and chromosome losses inactivating INI1 vary widely; therefore, a single readily available RT-PCR-based assay is not available.

Patients with renal rhabdoid tumors have an increased risk of malignant embryonal tumors of the posterior fossa; approximately 10–20 % develop a posterior fossa tumor. Rhabdoid tumors are aggressive, and the majority of patients (up to 80 %) present with metastatic disease. Grossly, rhabdoid tumors have large, solid and cystic areas with extensive areas of hemorrhage and necrosis. They are poorly defined and locally infiltrative. Microscopically, rhabdoid tumors contain eccentric nuclei and eosinophilic intracytoplasmic inclusions (Fig. 15.2). Vimentin immunostaining

demonstrates characteristic strong dot-like perinuclear cytoplasmic staining in a significant proportion of cells. INI1 immunostaining is characteristically absent in the nucleus of rhabdoid tumor cells. INI1 nuclear staining is positive in the nucleus of almost all other pediatric tumors, although, renal medullary carcinoma and some cells of synovial sarcoma may also be INI1 negative.

15.10.7 Congenital Mesoblastic Nephroma

Congenital mesoblastic nephroma is a low-grade spindle cell neoplasm affecting the renal medulla. Representing 2–5 % of pediatric tumors, congenital mesoblastic nephroma is also the most common congenital renal neoplasm. A vast majority of cases present in the first year of life (90 %). Some patients may develop hypertension. Congenital mesoblastic nephroma demonstrates a t(12;15)(p13;q25) ETV6-NTRK3 translocation, also found in congenital/infantile fibrosarcoma and secretory carcinoma of the breast. RT-PCR may be useful diagnostically [58]. Overall, 95 % of patients have an event-free survival; however, prognosis is worse with stage III with cellular congenital mesoblastic nephroma in patients 3 months or older. Grossly, the tumor has a cystic and solid whorled fibrous appearance resembling a fibroid. The cellular subtype may appear softer and more hemorrhagic.

Microscopic subtypes are classical, cellular, and mixtures and may be identified on the basis of molecular characterization and morphological features. The cellular subtype represents 40–60 % of cases and is morphologically indistinguishable from an infantile fibrosarcoma. There is increased cellularity with mitoses and focal necrosis. Mixed subtypes comprise less than 10 % of cases. The presence of incomplete excision is the main cause of local recurrence. The majority of cases with recurrence have been the cellular subtype with an abnormal gene fusion product present.

15.10.8 Renal Cell Carcinoma in Childhood

Renal cell carcinoma is uncommon in childhood and comprises 2–5 % of pediatric renal tumors. It usually occurs in children 10–15 years of age. Genetic changes are similar to adult cases. A range of familial and syndromic conditions may be associated with renal cell carcinoma development such as Von Hippel-Lindau (VHL) syndrome, hereditary papillary renal carcinoma syndrome, hereditary leiomyomatosis and renal cell cancer, Birt-Hogg-Dubé syndrome, and tuberous sclerosis. Twenty percent have metastases at diagnosis. The tumor may have a predominant clear cell, cystic papillary, or chromophobe pattern. Renal cell carcinoma is cytokeratin positive in all tumors and CD10 positive in greater than 90 %. EMA, vimentin, and CEA staining may also be positive.

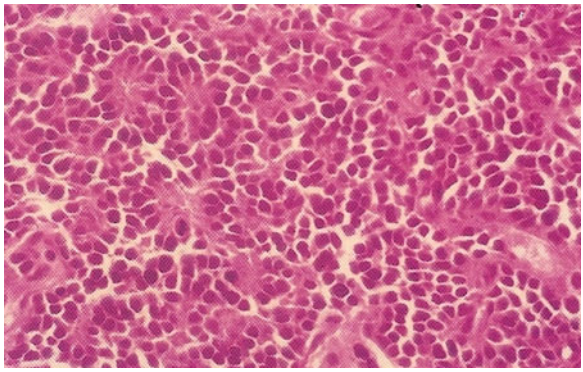

Fig. 15.3 Homer-Wright rosettes in neuroblastoma (From Gilbert-Barness [56])

15.10.9 Neuroblastoma

The incidence of neuroblastoma is 1 per 100,000 [59]. Seventy percent of patients have metastatic disease at presentation [60]. Age, clinical stage, molecular findings, and an undifferentiated subtype all influence overall survival [61]. The International Neuroblastoma Pathology Classification system [62] is as follows:

1. Neuroblastoma, undifferentiated: No ganglion cell differentiation of neuroblasts, no definite neurofibrillary stroma on light microscopy, sheets of small round to ovoid cells.
2. Neuroblastoma, poorly differentiated: Nests of neuroblasts within neurofibrillary background stroma, usually identifiable on routine light microscopy, < 5 % of neuroblasts show ganglion cell differentiation (Fig. 15.3).
3. Neuroblastoma, differentiating: > 5 % of neuroblasts show ganglion cell differentiation, nuclei have a prominent nucleolus. The cell has abundant eosinophilic/amphophilic cytoplasm, and the cell diameter must be more than twice nuclear diameter.
4. Ganglioneuroblastoma, intermixed: Well-defined nests of neuroblasts within neurofibrillary background stroma that are scattered throughout the lesion. Usually presents differentiating neuroblasts within the nests of cells (Fig. 15.4). No discreet identifiable nodules are present. Less than 50 % of the lesion is represented by neuroblastoma cells. Greater than 50 % of the lesion is represented by Schwannian stroma and mature ganglion cells.
5. Ganglioneuroblastoma, nodular: There is a macroscopic, hemorrhagic nodule within a larger bland lesion. The surrounding areas are stroma-rich or stroma dominant areas with mature or maturing ganglion cells; the well-demarcated nodule has Schwannian stroma, poor neuroblastomatous tissue; may be multiple, nodules morphologically those of neuroblastoma. Outcome of ganglioneuroblastoma, nodular is based on prognostic features of the neuroblastoma component [64], including all molecular/biological markers.

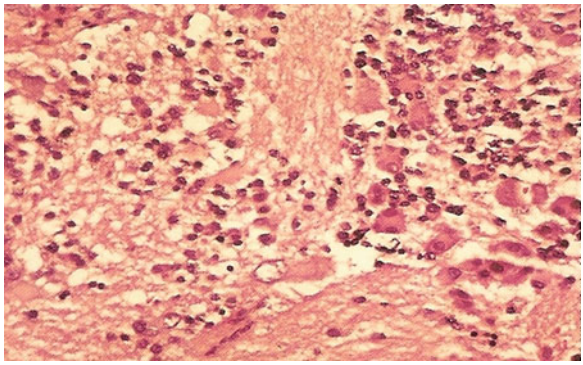

Fig. 15.4 Neuroblasts and variable ganglion cell differentiation in ganglioneuroblastoma (From Gilbert-Barness [63])

Table 15.4 Neuroblastoma prognostic categories

Mitosis karyorrhexis index (MKI)	
Low MKI	Fewer than 100 mitotic and karyorrhectic cells/5,000 tumor cells, or less than 2 % of tumor consisting of mitotic and karyorrhectic cells
Intermediate MKI	100–200 mitotic and karyorrhectic cells/5,000 tumor cells, or 2–4 % of tumor consisting of mitotic and karyorrhectic cells
High MKI	More than 200 mitotic and karyorrhectic cells/5,000 tumor cells, or greater than 4 % of tumor consisting of mitotic and karyorrhectic cells
International neuroblastoma staging system	
Stage 1	Localized tumor with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumor microscopically (nodes attached to and removed with the primary tumor may be positive)
Stage 2A	Localized tumor with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumor microscopically
Stage 2B	Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor; enlarged contralateral lymph nodes must be negative microscopically
Stage 3	Unresectable unilateral tumor infiltrating across the midline, with or without regional lymph node involvement or localized unilateral tumor with contralateral regional lymph node involvement; or localized unilateral tumor with contralateral regional lymph node involvement, or midline tumor with bilateral extension by infiltration (unresectable) or by lymph node involvement. The midline is defined as the vertebral column. Tumors originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column
Stage 4	Any primary tumor with dissemination to distant lymph nodes, bone marrow, liver, skin, and/or other organs (except as defined for stage 4A)
Stage 4S	Localized primary tumor (as defined for stage 1, 2A, or 2B), with dissemination limited to skin, liver, and/or bone marrow (limited to infants less than 1 year of age). Marrow involvement should be minimal (less than 10 % of total nucleated cells identified as malignant by bone biopsy or by bone marrow aspirate). More extensive bone marrow involvement would be considered stage 4 disease. The results of the MBG scan (if performed) [57] should be negative for disease in the bone marrow

Molecular studies are mandatory for risk assessment and management in neuroblastic tumors, and it is important for optimal specimen handling to allow FISH and other molecular studies. Current biological markers associated with adverse prognosis are N-MYC amplification, DNA diploidy, 1p deletion and 17q gain. Mitosis karyorrhexis index is calculated per 5,000 neuroblasts, usually within 6–8 high-power fields in cellular tumors in a 3 μm-thick evenly stained section. The mitosis karyorrhexis index is not carried out on small needle biopsy specimens or post-treatment specimens.

Specimens are assigned to 1 of 3 prognostic categories (see Table 15.4). On immunohistochemical staining CD56 is positive and also highlights neurofibrillary stroma. NB84 is positive. CD44 is positive and associated with a better prognosis. Schwannian stroma is S100 positive.

15.10.10 Rhabdomyosarcoma

This is a malignant mesenchymal tumor with skeletal muscle differentiation. In childhood, there are two main types: embryonal (ERMS) and alveolar (ARMS). The pleomorphic type affects adults.

15.10.10.1 Embryonal Rhabdomyosarcoma

This is the most common pediatric soft tissue sarcoma. Most develop in children less than 10 years old, 50 % in those less than 5 years old, and 5 % in those less than 1 year old. 11p15 loss is reported; however, it is not diagnostically useful, and there is no characteristic fusion transcript. Despite skeletal muscle differentiation, only 5 % arise in the skeletal muscle of extremities. Fifty percent develop in the head and neck, whereas 30 % are genitourinary including paratesticular. The remainder are from a wide range of sites including bile ducts and retroperitoneum.

Microscopically there are sheets of immature round to ovoid to spindle mesenchymal cells (rhabdomyoblasts). Cellularity is variable within a loose stroma. The degree of morphological skeletal muscle differentiation varies and includes strap cells, tadpole cells, and spider cells (Fig. 15.5). Differentiation is especially prominent post-chemotherapy. The spindle cell variant consists predominantly of spindle cells with fascicles, whorls, or a storiform pattern. The Botryoid variant consists of ERMS affecting the wall beneath an epithelial surface in vagina, bladder, or bile ducts. There is an intraluminal polypoid growth pattern macroscopically. The microscopic cambium layer is a layer of neoplastic cells immediately beneath the epithelium in loose myxoid stroma. The anaplastic/pleomorphic variant has nuclear enlargement, hyperchromasia, and anaplasia.

Immunohistochemical staining with vimentin is positive. Desmin positivity is cytoplasmic, whereas myogenin positivity is nuclear. All rhabdomyosarcomas show myogenin expression with more widespread expression in ARMS (usually greater

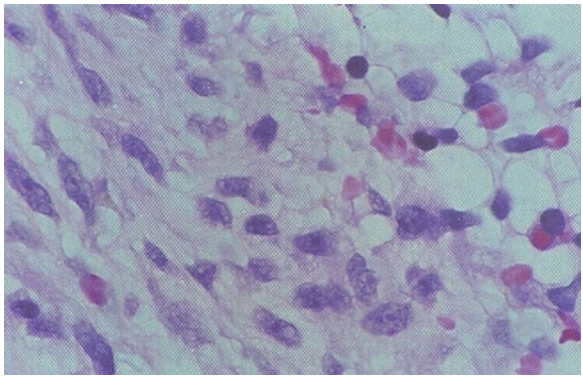

Fig. 15.5 Atypical cells with skeletal muscle differentiation in rhabdomyosarcoma (From Gilbert-Barness [56])

than 70 % positive) than in ERMS (usually less than 30 % positive). Myogenin may be very focal in a limited sample such as core biopsy. MyoD1 is positive in nuclei in cases with myogenin staining. Myoglobin is positive in well-differentiated cells. Other markers may highlight aberrant expression.

15.10.10.2 Alveolar Rhabdomyosarcoma

Clinically, this tumor is the more aggressive variant of rhabdomyosarcoma. Alveolar architecture is not always present. The characteristic gene fusion transcript is present in the majority of cases, detectable by molecular techniques including FISH with FKHR break-apart probes and RT-PCR for fusion transcript deletion. Approximately 60 % have the PAX3-FKHR translocation, 20 % have PAX7-FKHR translocation, and 20 % are fusion-negative [57]. Clinical features include a rapidly growing mass with almost any site involved, and 40 % are located in the extremities. ARMS may present with disseminated disease with or without an identifiable primary site. Bone marrow metastases occur. The classic alveolar pattern consists of fibrovascular septa, tumor cell nests that may be dyscohesive (Fig. 15.6). The solid variant has sheets of round tumor cells and no septae surrounding or stroma formation. The histological features show a limited correlation with PAX/FKHR fusion status. Only totally solid alveolar architecture is associated with absence of PAX/FKHR fusion products. No morphological features predict any particular fusion type.

15.10.11 Hepatoblastoma

Hepatoblastoma is the most common hepatic malignancy of childhood and comprises 1 % of pediatric malignancies. Ninety percent occur in the first 5 years of life and less than 5 % are congenital. Fifty-fold have increased risk of birth weight of

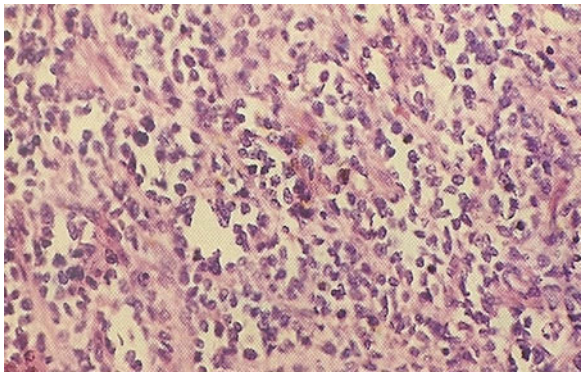

Fig. 15.6 Dyscohesion of cells resulting in the characteristic alveolar growth pattern of alveolar rhabdomyosarcoma (From Gilbert-Barness [56])

less than 1,000 g. There are rare predisposing syndromes in 5 % of cases such as BWS, trisomies, and familial adenomatous polyposis (FAP). Ten percent of sporadic hepatoblastomas have APC gene mutations.

In 80 % of cases, there is a single mass, whereas 20 % have multiple lesions. Anemia is present in 70 % and thrombocytosis in 50 %. Greater than 90 % of cases show elevated serum AFP concentration. Forty to 60 % are unresectable at diagnosis, but 85 % are resectable post-chemotherapy. Ten to 20 % of cases have lung metastases at diagnosis. The overall survival rate is 70 %.

Histopathologically, the tumor has a mixture of features that may be highly variable in both fetal and embryonal epithelial cells, stroma of connective tissue, osteoid, skeletal muscle or rare other epithelial types as well as teratoid features. Extramedullary hematopoiesis may be present. Immunohistochemical staining is especially useful other than to exclude other tumors.

15.10.12 Desmoplastic Small Round Cell Tumor

Desmoplastic small round cell tumor affects children and young adults, with more males affected than females. Characteristic and diagnostic is the t(11;22)(p13;q12), Ewing sarcoma (EWS)-WT1 fusion. The patient usually presents with disseminated abdominal disease. Presentation depends on the site; if aggressive, the patient has a poor prognosis. Histopathological features include infiltrative growth with desmoplastic spindle cell stroma that is variably vascular. Tumor cells are uniform, ovoid, and hyperchromatic with scant cytoplasm. Intracytoplasmic inclusions may be present. The cells are arranged in nests of variable shapes and sizes. Mitoses and necrosis may be present.

The tumor has polyphenotypic expression with CK, EMA, desmin, vimentin, and NSE staining. Anticarboxyterminus WTI is nuclear positive, and myogenin and MyoD1 are negative. Molecular techniques are required for confirmation of diagnosis.

15.10.13 Extrarenal Malignant Rhabdoid Tumor

This tumor occurs in infants and children. Congenital tumors are reported. There is an association with family history of a range of malignancies and posterior fossa tumors. Like renal rhabdoid tumors, there are abnormalities of the *hSNF5/INI1* gene on chromosome 22 [57]. Clinical features consist of mass lesions at almost any site. The tumor is aggressive and has a poor prognosis; however, this has improved with current chemotherapy regimes. Histopathological features include sheets of malignant cells with vesicular nuclei, prominent nucleoli, eosinophilic cytoplasm, mitoses, and necrosis. There is a variable extent of intracytoplasmic inclusions, which are often easier to identify at the periphery or in myxoid areas. The small cell variant has cells with scant cytoplasm. Some tumors may have a markedly myxoid stroma. On immunohistochemical staining, there is co-expression of CK and vimentin. Perinuclear "dot-like" expression highlights inclusions that are composed of intermediate filaments by electron microscopy. There is variable non-diagnostic expression of other antibodies. Negative INI1 nuclear expression is essentially diagnostic.

15.10.14 Ewing Sarcoma/Primitive Neuroectodermal Tumor

EWS and primitive neuroectodermal tumor (PNET) are primary malignant small round cell tumors of bone and soft tissue. Because they have a similar phenotype and share an identical chromosome translocation, they are viewed as the same tumor, differing from each other only in their degree of neural differentiation. Tumors that demonstrate neural differentiation by light microscopy, immunohistochemistry, or electron microscopy have been traditionally labeled PNET, and those that are undifferentiated by these analyses are diagnosed as EWS. These tumors account for approximately 6–10 % of primary malignant bone tumors. Most patients are 10–15 years old, and approximately 80 % are younger than 20 years. Boys are affected more frequently than girls, although the disease occurs much more often in girls than boys during the first 3 years of life. There is a striking predilection for whites; blacks are rarely afflicted.

In EWS/PNET, approximately 85 % have a t(11;22)(q24;q12) translocation, 5–10 % of cases have a t(21;22)(q22;q12) translocation, and in less than 1 % of tumors, a t(7;22)(p22;q12) translocation is present. The fusion gene (EWS-FLI1) generated from the translocation plays a vital role in the molecular genesis of this neoplasm [57]. The aberrant gene acts as a dominant oncogene, and the resultant chimeric proteins function as aberrant transcription factors that stimulate cell proliferation.

EWS/PNET can arise in any portion of the skeleton but usually develops in the diaphysis or metadiaphysis of long tubular bones and the flat bones of the pelvis. In young children, the pelvis, ribs, and proximal long bones are preferentially affected. The tumor typically presents as a painful enlarging mass, and the affected site is frequently tender, warm, and swollen. Systemic findings that mimic infection include fever, elevated sedimentation rate, anemia, and leukocytosis.

Arising in the medullary cavity, EWS/PNET usually transgresses the cortex and periosteum, producing a soft tissue mass. The tumor is tan-white or fish flesh-like in appearance and frequently contains areas of hemorrhage and necrosis. The cells grow in sheets or irregular islands with an organoid growth pattern within which Homer-Wright rosettes may be present. Occasionally, tumor cells are larger, have irregular nuclear contours, and prominent nucleoli. Mitoses are easily identified, and necrosis may be extensive. Special histochemical stains demonstrate glycogen in up to 75 % of tumors. In a minority of cases dense core neurosecretory granules are seen by electron microscopy.

EWS/PNET cells express vimentin, CD99 (MIC-2), FLI-1, C-kit and one or more neural markers including NSE, Leu-7, S100, neurofilament, and keratin in approximately 20 % of cases [57]. EWS/PNET is usually treated with a combination of chemotherapy and surgery. The chemotherapy is given preoperatively, and chemotherapy-induced necrosis of 90 % or greater is considered a good response. Radiotherapy is reserved for tumors that are surgically inaccessible, inadequately excised tumors, and patient palliation. Effective chemotherapy has dramatically improved the prognosis to a 75 % 5-year survival of which at least 50 % are long-term cures.

15.10.15 Testicular and Paratesticular Tumors

Most germ cell tumors are seminomas in young adults; however, in younger children, they are usually teratomas and yolk sac tumors.

Seminomas. Seminomas are composed of sheets of uniform round to ovoid cells with minimal pleomorphism; in most cases, they are associated with lymphoid infiltrates. PLAP and CD117 are positive in almost all cases [57].

Embryonal Carcinoma. Embryonal carcinoma is composed of sheets, papillae, and cysts with poorly differentiated cells containing overlapping polygonal, vesicular nuclei and prominent nucleoli. CD30, CK and focal PLAP stains are positive.

Yolk Sac Tumor. This is the most common malignant germ cell tumor in infancy. Ninety percent are associated with elevated serum AFP levels and contain ovoid vacuolated cells with scattered hyaline globules. Yolk sac tumors are mitotically active. There are numerous growth patterns, and most cases contain many of the patterns: microcystic/reticular, macrocytic, solid, alveolar, endodermal sinus (Schiller-Duval bodies), papillary, myxomatous, polyvesicular/vitelline, hepatoid, and enteric (Fig. 15.7). They are cytokeratin and AFP focally positive.

Choriocarcinoma. This tumor is very rare in childhood even as a component of mixed malignant germ cell tumor. Choriocarcinoma is positive for cytokeratin, inhibin, and hCG.

Teratoma. Teratoma occurs in infancy and is composed of tissues recapitulating all three germ layers. It is well-demarcated. Malignant areas may be present including focal PNET. It has a benign behavior in infancy.

Mixed Germ Cell Tumor. Normal immature fetal germ cells express PLAP and CD117. Infantile/congenital testicular tumors are most likely juvenile granulosa cell tumors rather than germ cell tumors.

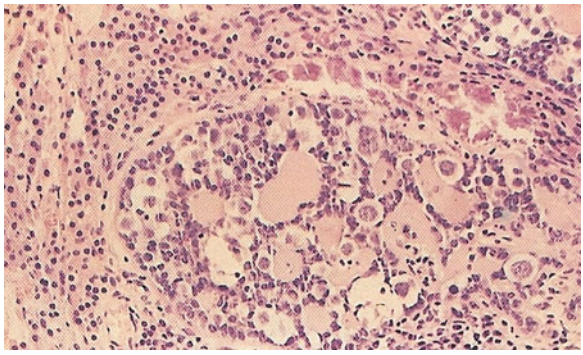

Fig. 15.7 Organoid structures in a yolk sac tumor (From Gilbert-Barness [56])

15.10.16 Testicular Sex-Cord Stromal Tumors

Leydig Cell Tumors. This comprises less than 5 % of pediatric testicular tumors. Leydig cell tumors occur in children between ages 3 and 9 years, presenting as a painless unilateral testicular enlargement. Endocrine features may be present, including gynecomastia and precocious puberty. The tumor is a well-circumscribed, yellow-brown nodule. Histopathologically, it is composed of diffuse sheets of large polygonal cells with eosinophilic cytoplasm; Reinke crystals are present in less than 40 %. The cells have regular nuclei with prominent nucleoli. Malignant behavior occurs in 10 % of tumors, particularly tumors greater than 5 cm in diameter and those containing cytological atypia, mitoses, necrosis and vascular invasion. The cells are vimentin and inhibin positive [57].

Sertoli Cell Tumors. Sertoli cell tumors are very rare in childhood. The histopathological appearance is sheets of bland ovoid cells with tubular formations. Malignancy is associated with pleomorphism, mitoses, and invasion. It is vimentin positive and variably inhibin positive.

Large Cell Calcifying Sertoli Cell Tumor. Large cell calcifying Sertoli cell tumor is a variant occurring in childhood. The average age at diagnosis is 16 years. It may be sporadic and is associated with Carney and Peutz-Jeghers syndromes. Twenty percent are malignant and 40 % are bilateral. It is composed of nests/cords of polygonal cells with eosinophilic cytoplasm with myxoid/hyaline stroma. Foci of calcification may be present.

Juvenile Granulosa Cell Tumor. Juvenile granulose cell tumors are the most frequent congenital testicular neoplasm. It presents as a testicular mass. Twenty percent are associated with ambiguous genitalia. It is composed of a cystic and solid mass with focal areas of hemorrhage. Cysts with inner granulosa-like cells and outer theca-like cells are present. There are intervening solid areas with plump ovoid-spindle cells. The tumor cells are vimentin, cytokeratin, and S100 negative. Inhibin is positive.

Gonadoblastoma. Usually, gonadoblastoma occurs in gonadal dysgenesis patients who have a Y chromosome present. This tumor has been rarely reported in

otherwise normal males. It is composed of Sertoli-like cells surrounding seminoma-like germ cells. Hyaline nodules and basement membrane-like material may be calcified.

Testicular Involvement. The testes may be involved in hematopoietic neoplasms, especially acute lymphoblastic leukemia.

15.10.17 Juvenile Laryngeal Papillomatosis

This is a human papilloma virus (HPV)-related lesion, especially types 6 and 11. HPV types 11 and 16 are associated with a more aggressive course. Presenting usually during the first 5 years of life, it affects around 1 % of children born to mothers with vaginal condyloma. Clinically, there are multiple laryngeal papillomatous lesions. Extralaryngeal spread also occurs in 30 % of patients, and bronchial/pulmonary involvement occurs in 5 %. There is rare esophageal involvement. The mortality rate is 5–15 %. Complications include infection, asphyxia, secondary pulmonary complications, and malignant transformation to squamous cell carcinoma (rare). HPV-11 is especially associated with more severe disease, including tracheal involvement and pulmonary disease. Papillomatous lesions are covered by non-keratinizing squamous epithelium. There may be koilocytosis and dyskeratosis. Usually pleomorphism is absent.

15.10.18 Thyroid Carcinoma

Children with thyroid carcinoma tend to present with more advanced disease than adults, with lymph node and lung metastases. They have higher recurrence rates but better overall survival.

*Papillary Carcinoma of Thyroid. T*his tumor presents as a single thyroid nodule/mass lesion. The mass is usually solid and unencapsulated, and it may appear multifocal. Lymphatic spread may occur but metastases rarely occur outside the lymph node. There is follicular architecture of cells with characteristic overlapping nuclei that are enlarged, oval, optically clear or ground glass, and have nuclear grooves. There are variable areas of papillary architecture with central vascular stromal cores; some nuclear features are present. Psammoma bodies with concentric lamination and calcification are present. There are several variants of papillary thyroid carcinoma: follicular, macrofollicular, oncocytic, clear cell, diffuse sclerosing, tall cell, columnar, solid, and microcarcinoma (less than 1 cm). Immunohistochemical staining shows CK19, cytokeratin, and TTF-1 positivity, whereas thyroglobulin and chromogranin are negative [57]. Five-year survival is greater than 95 %.

Follicular Carcinoma of Thyroid. This is mainly found in adults and is rare in children. It is a thyroid mass lesion. The tumor is usually solid and encapsulated or infiltrating with vascular and lymphatic spread. There is a follicular architecture without the characteristic grooved nuclei of papillary carcinoma. Variants include minimally

invasive, widely invasive, oncocytic, and clear cell. The immunohistochemical staining pattern is: cytokeratin, thyroglobulin, TTF-1, CK19, bcl2, beta-catenin positive, and chromogranin negative.

Medullary Thyroid Carcinoma. Children are affected when associated with multiple endocrine neoplasia types 2A (MEN2A), 2B (MEN2B), and familiar medullary thyroid carcinoma syndromes. It may even present in infancy in these syndromes. RET germline mutations are present. These patients may have other MEN2 features and have distant metastases. Calcitonin levels are high, and they may present with other unusual endocrine paraneoplastic features. Immunohistochemical staining includes positive CEA, with strong expression associated with a worse prognosis. Calcitonin, chromogranin, synaptophysin, and TTF1 stains are positive, and there are variably S100 positive sustentacular cells present peripherally.

Thyroid C-cell Hyperplasia. Thyroid C-cell hyperplasia is a precursor of medullary carcinoma of thyroid. It is calcitonin and chromogranin positive. This may be a difficult diagnosis, and some cases may represent microinvasive medullary carcinoma.

15.10.19 Adrenal Cortical Carcinoma

Clinical features include mass effect and excess hormone production, including androgen-virilization, glucocorticoid-related Cushing syndrome, and mineralocorticoid effect, resulting in hypertension. There are sheets and trabeculae of cells morphologically resembling normal adrenal cortex. Clear and eosinophilic cytoplasm exists with variable nuclear atypia and a high mitotic rate. The main features suggesting malignant behavior are diameter greater than 5 cm, fibrous bands, necrosis, capsular invasion, muscular invasion, and a high mitotic rate. Adrenal cortical carcinoma is alpha-inhibin positive and chromogranin negative. The tumor may be only weakly positive for cytokeratin, and EMA and CEA negative. It may be related to a germline point mutation of p53 even without a positive family history, acting in a tissue-specific manner. It may be associated with Li-Fraumeni or BWS. Overall, children have a better prognosis than adults.

15.10.20 Pheochromocytoma

This is a tumor of chromaffin cells from the adrenal medulla. Ninety percent are benign and 10 % are malignant. Ten percent are associated with neurofibromatosis type 1 (NF1), MEN2, VHL, and/or familial cases. Many abnormalities are reported, including RET defect, but currently this is non-diagnostic. Clinical features include catecholamine secretion effect, especially episodic hypertension. This tumor is solid with areas of hemorrhage/necrosis. The tumor has a nested architecture (zellballen) with surrounding sustentacular cells. The neoplastic cells show granular cytoplasm and variable nuclear pleomorphism. Scattered melanin containing cells

and ganglion cells may be present. Features of malignancy are metastatic disease, capsular invasion, vascular invasion, local extension into periadrenal tissue, necrosis, increased cellularity, pleomorphism, spindle cell phenotype, atypical and increased mitoses, loss of S100 positive sustentacular cells, and an increased proliferation index (MIB-1). Chromogranin is positive, and EMA is negative.

15.10.21 Other Predisposing Conditions Associated with Pheochromocytoma and Other Endocrine Tumors

15.10.21.1 Multiple Endocrine Neoplasia Type 2

Autosomal dominant activating germline mutations of the RET gene at 10q11.2 cause this syndrome. Specific codon mutations within RET correlate with disease phenotype and severity [65]. MEN2 pheochromocytomas may be preceded by diffuse medullary adrenal hyperplasia. Nodules are less than 1 cm in diameter and are more commonly bilateral and multiple. Less than 5 % of MEN2 pheochromocytomas are malignant. Parathyroid hyperplasia occurs when all glands become enlarged and hypercellular with mainly cytologically bland chief cells, histologically identical to sporadic cases. MEN2 is rarely associated with Hirschsprung disease. In view of the risk of medullary carcinoma, prophylactic thyroidectomy is performed in children less than 6 years of age in MEN2a and less than 2 years of age in MEN2b. Screening for pheochromocytoma should start at the age of 6 years.

15.10.21.2 Multiple Endocrine Neoplasia Type 1

This is an autosomal dominant inherited disorder due to mutation of the MEN1 gene, which codes for a protein called menin. Presentation occurs in late adolescence to adulthood, and 50 % have hyperparathyroidism by 20 years of age. Neuroendocrine tumors develop in parathyroid, pancreas, pituitary, adrenal, and other locations. Gastrinoma/Zollinger-Ellison syndrome occurs in about 50 % of cases.

15.10.22 Breast Carcinoma

Breast carcinoma is rare in children and may affect both females and males. The secretory subtype is most common, and it may be solid and tubular. The ovoid neoplastic cells have pale eosinophilic granular cytoplasm and intracytoplasmic lumina. Secretory breast carcinoma is associated with a balanced translocation, t(12;15), creating a ETV6-NTRK3 gene fusion [57]. This same translocation is also seen in congenital fibrosarcoma and congenital cellular mesoblastic nephroma [66]. Prognosis is good in children.

15.10.23 Juvenile Papillomatosis

This is a mass lesion in the breast that usually occurs in young adults but is also reported in children and rarely in infant boys [57]. There is an increased risk of familial breast cancer. The unencapsulated macrocystic lesion with fibrous stroma consists of multiple cysts, dilated ducts with apocrine metaplasia, duct ectasia, sclerosing adenosis-like changes, epithelial hyperplasia, and microcalcifications.

15.10.24 Phyllodes Tumor

This is a biphasic lesion similar to fibroadenoma that occurs only rarely in children. Most are benign, but local recurrence and metastases have been reported. There is a 2 % mortality, and 10–20 % have local recurrence [67]. Microscopically, epithelial structures are surrounded by stromal overgrowth. The stroma is more cellular than in fibroadenoma. Frankly malignant mesenchymal change may occur. The epithelium forms broad leaf-like areas with epithelial hyperplasia/metaplasia. Malignant behavior is based on the degree of hypercellularity, pleomorphism, mitotic count, and stromal overgrowth. Local relapse is associated with positive margins and fibroproliferation in surrounding tissue and necrosis [68].

15.10.25 Osteosarcoma

Osteosarcoma is a malignant mesenchymal tumor producing osteoid. All ages are affected, with a peak incidence between ages 10 and 20 years. Males are more often affected than females. Osteosarcoma usually presents in the metaphysis of the long bones, and pathological fractures occur in 5–10 %. Aggressive behavior results in metastases especially to the lungs and bones. There is a 60–80 % survival rate. Response to preoperative chemotherapy is the best predictor of outcome. After chemotherapy, necrosis greater than 90 % indicates a 80–90 % survival rate. A minority of patients have predisposing syndromes, mainly in older patients, including retinoblastoma syndrome, Li-Fraumeni syndrome, Paget disease of the bone, fibrous dysplasia (McCune-Albright syndrome), and previous radiotherapy. Children with a high dose of radiotherapy are at the highest risk for osteosarcoma to develop in adulthood.

Subtypes of osteosarcoma include osteoblastic, fibroblastic, and chondroblastic types. CD99 staining is positive, diffuse, and cytoplasmic. Osteopontin/osteonectin staining is positive. It may be focally CK positive or SMA positive. Response to chemotherapy is of vital importance. The operative specimen must have formal mapping in order to estimate the response to chemotherapy.

15.10.25.1 Telangiectatic Osteosarcoma

Pathological fractures occur more commonly than in the classical type. A destructive, lytic lesion usually involves a long bone. Cystic blood-filled spaces are separated by thin septa, which contain the malignant osteoid-producing cells [57].

15.10.25.2 Low-Grade Central Osteosarcoma

This is a well-differentiated intramedullary tumor. The long bones are preferentially affected, especially the femur. The tumor has a firm, solid, gray-white, fibroblastic stroma with focal osteoid production. The stroma contains malignant cells that are less pleomorphic, and it appears less cellular. There are bony spicules with intervening spindle cell stroma.

15.10.25.3 Parosteal Osteosarcoma

This tumor is usually seen in young adults. It affects females more than males. It is low-grade with good survival. Long bones are affected, especially the femur. It arises on the bone surface with well-formed trabeculae.

15.10.26 Malignant Skin Tumors in Childhood

15.10.26.1 Basal Cell Carcinoma

Basal cell carcinoma (BCC) is extremely rare in children. It occurs in association with xeroderma pigmentosum, nevus sebaceous, multiple nevoid BCC (Gorlin syndrome), and other predisposing genodermatoses, as well as in previous radiotherapy fields. The histopathological features consist of asymmetric aggregates of basaloid cells with peripheral palisading. Some aggregates of cells are separated from the stroma by retraction clefts. The stroma may be edematous and contain mucin. Every pattern of BCC may be found in children.

15.10.26.2 Squamous Cell Carcinoma

This is rare in children and occurs in association with xeroderma pigmentosum, pre-existing dermatoses, dystrophic epidermolysis bullosa, old burn scars, dyskeratosis, congenital (Fanconi) anemia, congenital immune defects, and immunosuppressive treatment. The most common sites are the face, hands, and forearms. Squamous cell carcinoma presents as a superficial erythematous nodule with ulceration covered by crusts. Histopathological features consist of irregular nests of epidermal cells that

proliferate downwards and invade the dermis. There is dyskeratosis and keratin pearls with varying degrees of differentiation. Prognosis depends on the extent of invasion and the degree of differentiation.

15.10.26.3 Dermatofibrosarcoma Protuberans

This is an infiltrative fibrohistiocytic tumor that is locally aggressive. There is a t(17;22) translocation of the COL1A1 and PDGF receptor genes. Dermatofibrosarcoma protuberans is closely related to giant cell fibroblastoma. Spindled-shaped fibrohistiocytic cells are arranged in short interweaving fascicles. Mitoses are rare. Immunostaining for CD34 is positive, and some cells are positive for Factor XIIIa. CD34 is only focally positive in a minority of dermatofibrosarcoma protuberans cases.

15.10.27 Pediatric Pulmonary Tumors

15.10.27.1 Inflammatory Myofibroblastic Tumor

This tumor comprises 80 % of benign lung tumors in children. It is most common in the second decade but can occur in the first year of life. Cough, fever, hemoptysis, and chest pain are the usual symptoms. The recurrence rate is approximately 14 %. It is usually a solitary lesion in the lung periphery that is firm and white on cut surface and may be gritty. The lesion contains two main components: a proliferation of spindle cells in fascicles and a variable admixed lymphoplasmacytic infiltrate. Central scarring is common. There may also be multinucleated giant cells, granulomas, ossification, calcification, and vascular invasion. Spindle cells express vimentin and smooth muscle actin. ALK-1 is positive in 40 % of cases. Those with extension beyond the lung may recur in approximately 50 % of cases.

15.10.27.2 Pleuropulmonary Blastoma

This is a malignant tumor of early childhood. It is associated with trisomy 8 in some cases, but this is nonspecific. It may be multifocal and may be associated with neoplasia in other family members. Particularly common is the association with cystic nephroma. It presents with chest pain, cough, fever, respiratory distress, and pneumothorax. It is classified into types I, II and III based on the macroscopic and microscopic features [57].

Type I. This is the least common. It occurs in children under 2 years of age with median age of 9 months. The muscle cells stain with vimentin, desmin and myogenin.

Type II. This accounts for about 45 % of cases. It occurs in slightly older children than type I, with median age of 31 months. It is cystic either grossly or microscopically

with solid areas. Solid areas are composed of malignant connective tissue elements, rhabdomyosarcoma, blastema, and/or spindle cell sarcoma. The cystic lining is benign.

Type III. This type is present in 40 % of cases, in a slightly older group than type II, with median age of 42 months. It is a solid tumor with no cysts. It is large and attached to the pleura and invades the lung. It may obliterate a lobe or an entire lung, and it is hemorrhagic and friable. The histological appearance is similar to the solid areas of type II. It may contain immature or frankly malignant cartilage, and it may metastasize to the brain, bone, liver or other sites.

15.10.27.3 Primary Pulmonary Carcinoid

This lesion is second in frequency to pleuropulmonary blastoma as a primary malignant lung tumor of childhood. WHO classifies carcinoids into typical and atypical based on mitotic count and presence of necrosis [57]. It is intrabronchial or peripheral.

15.10.27.4 Mucoepidermoid Tumor

Symptoms are related to bronchial obstruction, such as recurrent pneumonia, pyrexia, hemoptysis, or cough. It is centrally located in the proximal bronchial tree. Less than 5 % metastasize to local nodes, and it generally follows an indolent course. Mucoepidermoid tumors are usually an endobronchial tumor with a yellow color with extension through the bronchial wall. Low-grade tumors predominate in children and have solid and cystic areas; solid areas consist mainly of epithelium [57]. Necrosis is uncommon, and there are infrequent mitotic figures. Non-keratinizing squamous epithelium may be present. The stroma may show edema, fibrosis, hyalinization, and calcification.

15.10.27.5 Teratoma

In the lung, teratomas are most common in the left upper lobe. Symptoms include chest pain, hemoptysis, and cough. Chest x-rays show cysts with calcification. Teratomas are cystic and contain all three germinal layers. Large bronchi may be involved [57]. Most are mature and benign.

15.10.27.6 Cardiac Teratoma

Nearly two-thirds of cases present in infants, usually within the pericardial cavity and attached to the heart near the great arteries. The tumor may be intramural. The presentation may be cardiac failure, pericardial effusion, or cardiac tamponade. These tumors are rarely malignant.

15.10.27.7 Askin Tumor/Small Round Cell Tumor of the Thoracopulmonary Region

This is identical to a soft tissue PNET at other sites. Usually presenting in the second decade, it is more common in females with a ratio of 3:1. The tumor arises in the chest wall with variable involvement of the pleura and lung. Grossly, tumors up to 15 cm in diameter are round or multinodular and non-encapsulated. The cut surface is grayish-white with foci of necrosis and hemorrhage. Both the histological features and molecular characteristics are similar to PNET at other sites [57].

15.10.27.8 Bronchogenic Carcinoma

This is rare in children but has been reported. Histological appearances are similar to adult types.

15.10.27.9 Pulmonary Metastatic Disease

For some tumors such as adrenocortical carcinoma, alveolar soft part sarcoma, and osteosarcoma, surgical removal of the metastatic tumor is necessary for patient survival. In Wilms' tumor and EWS/PNET, which are radiation sensitive, the value of surgical removal of the metastatic tumor is not proven. In the case of neuroblastoma, differentiated thyroid cancer and rhabdomyosarcoma, secondary tumors in the lung are rarely removed. Histopathological features are identical to the primary site. They may show superimposed post-chemotherapy changes.

15.10.28 Familial Adenomatous Polyposis

FAP includes greater than 100 polyps throughout the colon. There is an increased risk of hepatoblastoma and endocrine tumors. Adenomas usually develop in teens, but FAP may also present in childhood with desmoid/Gardner fibromatoses or congenital hypertrophy of retinal pigment epithelium. Colorectal carcinoma develops at an average age of 40 years and rarely in childhood. Prophylactic colectomy should be performed. Other soft tissue lesions may occur, including juvenile nasopharyngeal angiofibroma and rhabdomyosarcoma. In the stomach, cystic polyps occur in one-fourth to three-fourths of cases. Histologically there is a mixture of normal mucosa and cystic glands lined by mucus-secreting epithelium, parietal cells, and chief cells. These lesions in the stomach are generally regarded as hamartomas with a very low risk of malignancy. However, gastric adenomas do occur in about 10 % of cases of FAP, but malignant transformation is uncommon. Duodenal adenomas are much more common, and these can develop into the usual adult type carcinoma.

15.10.29 Burkitt's Lymphoma

This is one of the most common malignant tumors of the intestine in children. It is either endemic and associated with EBV infection or sporadic and not associated with EBV. Symptoms are associated with bowel obstruction, perforation, or intussusception. Burkitt's lymphoma is very sensitive to chemotherapy. Grossly, there is an ulcerating mass in the ileocecal area with infiltration of the bowel wall. There are sheets of monotonous lymphoid cells microscopically with a starry-sky pattern. The mitotic count is high, and greater than 95 % of cells are positive with Ki67 staining (proliferation index). Antigens expressed include CD19, CD20, CD22, and CD79a. Many cells express CD10 [57]. The tumor cells dissect among the smooth muscle cells of the muscularis propria without destroying them.

15.10.30 Mesenchymal Gastrointestinal Tumors

These are infiltrating lesions composed of bland spindle cells with no significant atypia. Leiomyomas are desmin and caldesman positive, although CD34 and CD117 are negative.

15.10.31 Gastrointestinal Stromal Tumors

Gastrointestinal stromal tumors (GIST) consist of spindle and epithelioid cells and are associated with KIT gene mutations and PDGF receptor-alpha activating mutations. They are treated with imatinib. Different mutations/mechanisms of tyrosine kinase activation occur in childhood cases; they may have no detectable common mutations. CD117 stains are positive with strong diffuse membranous and cytoplasmic staining. CD34 stains are positive in greater than 70 % of cases. Desmin is negative, with one-third of cases focally positive for smooth muscle actin. Familial cases have been reported. The carney triad is composed of gastric GIST, pulmonary chondroma, and extra-adrenal paraganglioma with increased risk of association with NF1.

Gastrointestinal autonomic nerve tumors (GANT) are morphologically spindle and epithelioid tumors which present similar to GIST and are now known to be a GIST variant.

15.10.32 Pancreatoblastoma

This is the most common malignant pediatric pancreatic tumor but is rare nonetheless. The patient is usually less than 10 years of age with a median age of 2–4 years. One-third of pancreatoblastomas occur in adults. Congenital cases have been reported [69]. Most cases are sporadic, and some are associated with BWS,

especially congenital cases. Pancreatoblastoma may present as an extracolonic manifestation of FAP. There is an increased risk in Asians. Allelic loss on chromosome 11p occurs in 90 %. Molecular alterations in the APC/beta-catenin pathway occur in 70 % of patients [57]. Clinical features include a non-specific abdominal mass and a solitary solid pancreatic lesion in the head or tail of the pancreas. Metastases have occurred by presentation in 20–35 % of cases, with one-third of patients having increased alphafetoprotein levels. The prognosis is good if localized but poor if metastases are present. Histological features include a solid or cystic mass that is well circumscribed with hemorrhage and necrosis. It has a nested and organoid pattern with epithelial islands separated by fibrous bands. Epithelial components are nests of polygonal cells with bland nuclei and granular cytoplasm plus squamoid nests of variable size. Areas with ductal differentiation are rare. The stroma consists of fibrous tissue that may be hypercellular with rare foci of metaplastic bone or cartilage. There may be primitive mesenchymal areas. Immunohistochemical staining shows a variable expression of trypsin, chymotrypsin, lipase, chromogranin, synaptophysin, CEA, and cytokeratin. Nuclear and cytoplasmic beta-catenin expression is present in 80 % of cases, especially in the squamoid areas [57].

15.10.33 Brain Tumors

Gangliocytomas are rare tumors that have been described in patients with neurofibromatosis and Peutz-Jeghers syndrome. Seventy percent of these tumors are in the temporal lobe. Gangliocytoma consists of dysplastic neurons with loss of cytological organization, including perimembranous Nissl substance, cytomegaly, abnormal clustering, abnormal localization, and multinucleation. There are intermixed reactive glial cells and perivascular reticulin deposition.

Ganglioglioma consists of dysplastic neurons and neoplastic glial cells, which resemble a diffuse or pilocytic astrocytoma; this tumor rarely contains a clear cell (oligodendroglial-like) component. Rosenthal fibers, eosinophilic granular bodies, and perivascular lymphocyte cuffing may be present. The stroma is often microcystic and myxoid but may be fibrillary and calcified. Necrosis is usually absent. Microvascular proliferation may be seen. The ganglion cells stain with a range of routine neuronal markers. Coarse synaptophysin and strong chromogranin staining are in contrast to the weak staining of most native neurons. The glial component stains with GFAP. The tumor and peritumoral parenchyma stains with CD34 in approximately 70 % of cases. Ki67 is positive but stains less than 3 % of cells.

15.10.33.1 Desmoplastic Infantile Astrocytoma and Ganglioglioma

These tumors usually occur in the first 1–2 years of life, but occasional cases may occur later. They are WHO grade I. Seizures or neurological deficit may occur.

It has a very good prognosis. Desmoplastic infantile ganglioglioma will contain dysplastic ganglion cells. The poorly differentiated neuroepithelial component contains small round cells. The desmoplastic component stains positive for vimentin and GFAP. The small cell component stains positive for GFAP and MAP2. Neuronal markers are negative. It may express desmin. Cytokeratin and EMA are negative. Ki67 may be increased, but there is poor correlation with the clinical outcome.

15.10.33.2 Dysembryoplastic Neuroepithelial Tumor

This tumor is classified as WHO grade I. The age at presentation is highly variable, but typically it occurs in teenagers and young adults. There is a relatively uniform population of small round cells. A metachromatic stroma with scattered ganglion cells with calcification may be seen. Oligodendrocyte-like cells are small round cells with finely granular chromatin and indistinct cytoplasm. Floating neurons are present in pools of mucinous material. Calcification and hemosiderin may be present. The mucinous material is positive for Alcian blue. Oligodendrocyte-like cells are typically GFAP negative and S100 positive. Floating neurons will stain with standard neuronal markers (for example, NeuN, neurofilament). CD34 may be positive and Ki67 proliferation index is low.

15.10.33.3 Central and Extraventricular Neurocytoma

Neurocytomas are WHO grade I. Most show no genetic predisposition. Clinical features include occurrence at any age, but these tumors are uncommon in childhood. Extraventricular neurocytomas have a similar morphology but can occur anywhere in the central nervous system (CNS). Raised intracranial pressure is the commonest clinical finding.

Central Neurocytoma. Histological features include uniform round nuclei with salt and pepper chromatin, and they are usually cytologically bland. There is patchy fibrillary stroma with neuropil islands. Other patterns may include oligodendroglioma-like pattern, rosettes resembling pineocytomatous rosettes, Homer-Wright rosettes, perivascular pseudorosettes, ganglion cell differentiation, and arborizing vessels resembling a neuroendocrine tumor. Calcification is present.

Atypical Neurocytoma. Atypical features include increased mitotic activity, microvascular proliferation, and necrosis. Histological atypia is poorly predictive of clinical behavior. Ki67 is greater than 2–3 %.

On immunohistochemical staining, synaptophysin is positive. In some cases, NeuN is positive. Usually GFAP is negative. Ki67 labeling indices usually are very low. Values greater than 2–3 % are associated with a worse prognosis. By electron microscopy the cellular processes are rich in microtubules, dense core and clear vesicles and synapses.

15.10.34 Primitive/Embryonal Tumors

15.10.34.1 Medulloblastoma

This is the commonest malignant brain tumor in children [70, 71]. It is defined as an embryonal tumor of the cerebellum. Medulloblastoma may be associated with Gorlin (basal cell nevus) syndrome and adenomatosis polyposis coli (Turcot syndrome). These can present at any age from birth until the fifth decade; the peak age of presentation is 7 years. Symptoms are due to raised intracranial pressure and cerebellar dysfunction. Three-fourths of cases arise in the midline of the cerebellum (vermis), with the remainder arising in the cerebellar hemispheres. Metastatic spread in the craniospinal axis may occur. Histological features include a dense cellular tumor with either a diffuse or nodular architecture. Typically, the small cells have dense hyperchromatic and rounded or wedge-shaped (carrot-shaped) nuclei. Mitotic activity is usually present, but it is not unusual for mitotic figures to be very difficult to identify; apoptoses and coagulative necrosis are frequent. Homer-Wright rosettes show a circular arrangement of tumor cells surrounding a small acellular region of neuropil, lacking a central lumen. Flexner-Wintersteiner rosettes occur occasionally.

Nodular (desmoplastic) Medulloblastoma. This tumor has a biphasic nodular pattern with intra-nodular areas consisting of lower cellularity and more differentiated tumor cells set in neuropil. Inter-nodular areas are densely cellular, consisting of reticulin-rich, poorly differentiated cells with little intervening neuropil. Tumors showing a greater extent of nodularity carry a better prognosis, particularly in infants. Anaplastic medulloblastoma shows cellular anaplasia.

Melanotic Medulloblastma. This tumor is very rare and is characterized by the presence of melanotic differentiation in an otherwise unremarkable medulloblastoma. Reticulin stains highlight nodularity and desmoplasia in nodular medulloblastoma. The immunohistochemical stain INI-1 is positive distinguishing it from atypical teratoid/rhabdoid tumor. Synaptophysin positivity is usually present but is occasionally weak and patchy. GFAP is mostly negative or only positive in trapped reactive cells. True glial differentiation may be seen, particularly in nodular medulloblastomas. The Ki67 (proliferation index) is usually high (greater than 20 %). In older age groups, staining for lymphocyte markers and cytokeratin may be helpful in the exclusion of lymphoma and metastatic small cell carcinoma respectively. Nuclear staining for β-catenin is a good prognostic factor. Staging of medulloblastoma is of great prognostic value and depends predominantly on the surgical and radiological findings.

On molecular investigation, a number of chromosomal abnormalities may occur. In particular, C-myc and N-myc amplification are associated with a poor prognosis.

15.10.34.2 Supratentorial PNET/CNS-PNET

CNS-PNET should be distinguished from the unrelated EWS/PNET. The mean age of presentation is 5 years. The most common site is the cerebral hemispheres; in rare cases, it occurs in the spinal cord. Prognosis is generally poor.

Variable features include Homer-Wright rosettes, streams and palisading cells; calcification is relatively common along with microvascular proliferation and fibrous septae. Immunohistochemical staining is typically positive for neuronal markers including synaptophysin. Glial differentiation is common. GFAP staining is frequently seen. Ki67 labeling indices are generally high, and INI-1 staining is positive. Evidence of muscular and melanocytic differentiation may be seen. On electron microscopy, the tumor is very poorly differentiated. Presence of dense core vesicles should be regarded as diagnostic of cerebral neuroblastoma.

15.10.35 Atypical Teratoid/Rhabdoid Tumor

This is a malignant tumor of the CNS associated with mutations in the INI-1/hSNF5 gene. It may be sporadic or associated with the rhabdoid tumor syndrome. Germline mutations occur in the INI-1 gene on chromosome 22q11.2. For the majority of cases, occurrence is in children less than 6 years old, with most are under age 3. It is slightly more common above the tentorium, but no site is exempt. The prognosis is poor and most die within 2 years.

Typical rhabdoid cells show eccentric nuclei, vesicular chromatin, prominent nucleoli, and cytoplasmic eosinophilic globules. Evidence of mesenchymal and epithelial differentiation may be seen, and they may show papillary structures or resemble poorly differentiated carcinoma. Other non-specific features of a malignant tumor are frequent with high mitotic rate, necrosis, and hemorrhage. Immunohistochemical staining reveals the diagnostic feature of loss of the nuclear expression of INI-1. Lack of expression is also described in choroid plexus carcinoma and supratentorial PNET. INI-1 negative tumors in the brain are associated with a poor prognosis. Other immunostaining is highly variable, including EMA, vimentin, SMA, GFAP, neurofilament, and cytokeratin. Paranuclear staining for vimentin or other intermediate filaments is useful for the diagnosis. On electron microscopy, whorled intermediate filaments indent the nucleus in typical rhabdoid cells.

15.10.36 Medulloepithelioma

This tumor is WHO grade IV. Most occur before the age of 5 years, with 50 % of cases shown in children who are less than 2 years old. The most common cerebral site is periventricular, but these may also occur in peripheral nerve spinal cord and orbit. A large tumor and raised intracranial pressure are typical. Survival is usually less than 1 year. Histological features include pseudostratified epithelium resembling primitive neural tube. The architecture is papillary, trabecular, and tubular. The luminal surface may contain small blebs but should not be ciliated. The inner membrane sits along a primitive basement membrane. Mitotic figures

are generally toward the luminal aspect in hyperchromatic anaplastic cells. On immunohistochemical staining, nestin and vimentin are positive in the neuroepithelial areas. Focal staining may also be seen with neurofilament, CK, and EMA in the neuroepithelial areas. Typically, neuroepithelial areas are GFAP and S100 negative. A Ki67 index is variable. On electron microscopy, prominent lateral cell junctions are present in the basal lamina on the outer surface of the neuroepithelium.

15.10.37 Ependymoblastoma

This tumor is WHO grade IV. Ependymoblastomas usually occur in young children and neonates. In addition, it is usually supratentorial, but it may occur at other sites. The prognosis is poor, and death usually occurs within 1 year of diagnosis. Histological features include ependymoblastic rosettes, comprised of multilayered neuroectodermal cells with a small central lumen. The tumor contains primitive neuroectodermal cells with a malignant cytology. On immunohistochemical staining, GFAP is positive; it is also usually positive with S100, vimentin, and cytokeratin. Neurofilament stains may be positive. On electron microscopy, rosette cells are joined by junctional complexes; abortive cilia and a few basal bodies may be seen near the lumen.

15.10.38 Pineocytoma

This is a low-grade intrinsic tumor of the pineal gland. It is WHO grade I with an excellent prognosis. Histological features include an architecture with moderate cellularity in sheets or ill-defined lobules. Pinecytomatous rosettes are relatively large compared with Homer Wright rosettes. Pineocytoma has low mitotic activity, and the Ki67 index is low. Silver stains (e.g., Bielschowsky) may demonstrate club-like protrusions at the end of cellular processes. A range of neuronal markers are positive but most consistent is synaptophysin. Retinal antigens (rhodopsin, retinal S antigen) may be positive. Neurofilaments may highlight the clubbed ends of the cell processes. Occasionally, glial differentiation is present, evidenced by GFAP positivity.

15.10.39 Pineoblastoma

This is a primitive malignant tumor of the pineal gland resembling a CNS-PNET. It is WHO grade IV. When pineoblastoma occurs in familial retinoblastoma

Table 15.5 Histologic types of meningioma WHO grade I

Meningothelial Meningioma	Meningothelial meningioma has lobules and nests of cells that are separated by thin strands of collagen. Uniform arachnoid cells and rare psammoma bodies or whorls are seen
Fibrous (Fibroblastic) Meningioma	This is a spindle cell tumor with rich collagenous matrix
Psammomatous Meningioma	This type typically occurs in middle-aged women, frequently in the thoracic cord. Psammoma bodies are the predominant feature, and they may contain confluent calcification
Angiomatous Meningioma	Vessels predominate over tumor cells
Secretory Meningioma	These have characteristic pseudo-psammoma bodies with focal epithelial differentiation forming intracellular lumina contain eosinophilic inclusions, which are PAS and CEA positive. Serum CEA may even be raised. Mast cells may be prominent in this variant
Lymphoplasmacytic-Rich Meningioma	This type is characterized by chronic inflammation that obscures the tumor. There is an occasional association with hematological disorders, including iron refractory anemia and hypergammaglobulinemia
Metaplastic Meningioma	This is characterized by prominent focal or diffuse mesenchymal differentiation with cartilage, bone, lipid, or skeletal muscle formation

(trilateral retinoblastoma), it carries a worse prognosis. Diffuse rosettes including Homer Wright and Flexner-Wintersteiner are present. Ironically, they do not have pineocytomatous rosettes.

15.10.40 Meningioma

Meningiomas that are WHO grade I include most morphological variants. WHO grade II types have defined cytological features (atypical meningioma) or are of a specific subtype, including chordoid and clear cell meningioma. WHO grade III meningioma has cytological features of malignancy and include rhabdoid and papillary subtypes. There is an increased frequency of neurofibromatosis type 2. The rare malignant cases may have metastasis. General histological features include a whorled architecture and pseudo-syncytial sheets. Arachnoidal cells are oval with relatively uniform nuclei, small indistinct nucleoli, and nuclear pseudo-inclusions. Histologic types of meningioma WHO grade I are shown in Table 15.5; types of meningioma grade II are shown in Table 15.6.

Meningioma staging for brain invasion is defined by breach of the pia mater and presence of individual tumor cells invading into the brain parenchyma. It must be distinguished from prominent extensions of tumor that may extend into the brain. Brain invasion is associated with an increased risk of recurrence. It may be easier to assess by GFAP immunostaining.

Table 15.6 Types of meningioma grade II

Chordoid Meningioma	**Chordoid Meningioma** is a tumor that resembles a chordoma. These are associated rarely with Castleman disease
Clear Cell Meningioma	**Clear Cell Meningioma** is a rare tumor, which occurs relatively more frequently in children and young adults. The cerebellopontine angle and the cauda equina are the most common sites. The clear cells are due to glycogen accumulation. Collagen deposition is often very prominent
Atypical Meningioma	**Atypical Meningioma** is any meningioma with either 4–20 mitotic figures per 10 high-power fields, a patternless sheet-like growth, high nuclear-cytoplasmic ratio, prominent nucleoli, or foci of spontaneous/geographic necrosis
Papillary Meningioma	**Papillary Meningioma** is rare and relatively more frequent in children. It has a pseudo-papillary pattern and a poor prognosis
Rhabdoid Meningioma	**Rhabdoid Meningioma** contains rhabdoid cells, which have eccentric nuclei, prominent nucleoli, and eosinophilic cytoplasmic inclusions. It has a high mitotic count and may be papillary. This variant carries a poor prognosis. It is not associated with INI-1 loss, unlike atypical teratoid rhabdoid tumor
Anaplastic (Malignant) Meningioma	**Anaplastic (Malignant) Meningioma** is any meningioma with severe anaplasia and is akin to poorly differentiated carcinoma or sarcoma. The mitotic count is greater than or equal to 20 per 10 high power fields. Anaplastic meningioma has a poor prognosis

15.10.41 Retinoblastoma

Retinoblastoma is the most common primary intraocular neoplasm of children. Children usually present with leukocoria and/or strabismus. Of all retinoblastoma cases, approximately 60–70 % are unilateral and 30–40 % are bilateral. In unilateral cases, typically one single tumor is present in the affected eye. In bilateral cases, multifocal tumors in both eyes are usual. The median age at presentation is approximately 12 months in children who have bilateral retinoblastoma and 24 months in those with unilateral disease. The tumor has a strong tendency to invade the optic nerve and brain and to metastasize widely. The growth pattern in retinoblastoma is endophytic, exophytic, or diffusely infiltrating. Endophytic growth is growth predominantly toward the vitreous. Exophytic growth is growth toward the subretinal space and leads to retinal detachment. The diffuse infiltrating variety exhibits a spread of the tumor diffusely throughout the retina with infiltration of adjacent structures. Spontaneously arrested retinoblastoma may be seen in unaffected family members. Most commonly it is asymptomatic and is seen clinically as a translucent retinal mass with calcification.

Second non-ocular cancers are common in patients with germline mutations. The incidence of second cancers is approximately 1 % per year of life. The most common second cancers are osteogenic sarcomas of the skull and long bones, other sarcomas, cutaneous melanomas, Hodgkin lymphomas, and breast carcinomas.

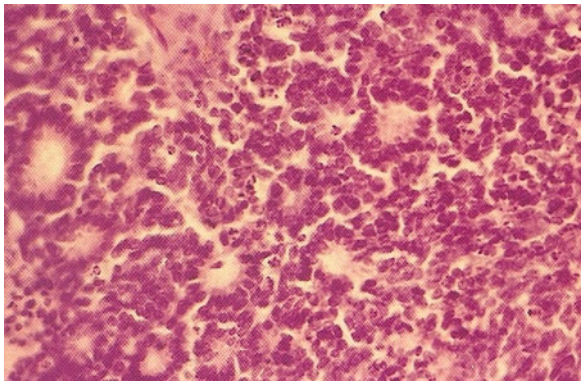

Fig. 15.8 Flexner-Wintersteiner rosettes of retinoblastoma with nearly empty central lumens (From Gilbert-Barness [56])

Trilateral retinoblastoma (pinealoblastoma) is a primitive neuroectodermal neoplasm occurring in the pineal region. Histologically, the tumor is composed of small, round, undifferentiated retinoblastoma cells with hyperchromatic nuclei and scant cytoplasm. Necrosis and calcification are often present. Several types of cellular patterns are seen. Flexner-Wintersteiner rosettes are specific for retinoblastoma but are not always seen (Fig. 15.8) Homer-Wright rosettes are indistinguishable from those found in neuroblastomas. Pseudorosettes are seen as viable tumor cells that cluster around blood vessels. Fleurettes are flower-like groupings of tumor cells that clearly show a differentiation into photoreceptor elements. Extensive necrosis is often present with calcification.

Retinoblastoma has been shown to result from loss or inactivation of both alleles present at the time of fertilization, but spontaneous mutations occur in which both the alleles become deleted or inactive in at least one immature retinal cell. Overall, only 6–7 % of newly diagnosed retinoblastoma patients have a family history of the disease; the vast majority of retinoblastoma cases are sporadic.

Retinoblastoma results from the effects of two independent mutations that cause a defective regulatory or suppressor gene at the chromosomal 13q14 locus. Tumors develop when both suppressor genes, one on each homologous chromosome, are inactivated. Hereditary retinoblastomas arise from a single mutagenic event (somatic mutation) in a cell that previously carried an inherited mutation (germinal mutation). Sporadic cases (non-heritable) require two somatic (postzygotic) mutations.

Retinoblastoma is inherited as an autosomal recessive disease at the cellular level, but clinically it behaves as if it has an autosomal dominant pattern of inheritance with almost complete penetrance.

15.11 Tumor-Associated Syndromes

Tumor-associated syndromes are listed in Tables 15.7 and 15.8.

Table 15.7 Some syndromes and congenital malformations associated with childhood tumors

Malformation or syndrome	Neoplasm
Beckwith-Wiedemann syndrome hemihypertrophy	Wilms' tumor
	Adrenocortical adenoma
	Adrenocortical carcinoma
	Hepatoblastoma
	Pancreatoblastoma
Aniridia	Wilms' tumor
Genitourinary system anomalies and Perlman syndrome	Nephroblastomatosis
	Wilms' tumor
Hirschsprung disease	Neuroblastoma
Poland syndrome	Leukemia
Drash syndrome	Wilms' tumor
Tuberous sclerosis	Cardiac rhabdomyoma
	Angiomyolipoma
	Astrocytoma
Multiple endocrine neoplasia (MEN2)	Thyroid medullary carcinoma
	Pheochromocytoma
	Submucosal neuromas
Nevoid basal cell carcinoma (Gorlin) syndrome	Basal cell carcinoma
	Medulloblastoma
	Ovarian fibroma
Wiskott-Aldrich syndrome	Lymphoma
Bloom syndrome	Leukemia

From: Isaacs [19]

Table 15.8 Some syndromes that Predispose to solid tumors

Syndromes	Chromosomal locus	Gene	Tumor
Neurofibromatosis type 1	17q11	*NF1*	Rhabdomyosarcoma
Beckwith-Wiedemann	11p15.5	Unknown	Wilms' tumor
			Hepatoblastoma
WAGR Denys-Drash	11p13	*WT1*	Wilms' tumor
Adenomatous polyposis coli	5q21	*APC*	Hepatoblastoma
Hereditary retinoblastoma	13q14	*RB1*	Retinoblastoma
			Osteosarcoma
Li-Fraumeni	17p13.1	*p53*	Osteosarcoma
	22q12.1		Rhabdomyosarcoma

From: Isaacs [72]

15.11.1 Beckwith-Wiedemann Syndrome

BWS and its related tumors are an excellent example of loss of imprinting. BWS is a clinically heterogeneous disorder of growth regulation manifesting as somatic overgrowth, congenital malformations, and tumor predisposition. The majority of

cases are sporadic; however, a small number of pedigrees with autosomal dominant inheritance demonstrate linkage to 11p15.5 [73, 74]. Genomic imprinting in the phenotype was suggested by the preferential loss of maternal alleles in BWS and related tumors [75]. BWS is associated with abnormal transcription and regulation of genes associated with cell cycle and growth control in the imprinted domain on chromosome 11p15.5. The cluster of genes on chromosome 11p15.5 contains at least 12 imprinted genes. The chromosome 11p15.5 region has been divided into two distinct domains that are thought to be regulated by two imprinting centers separated by a non-imprinted region [76–81].

15.11.2 Central Nervous SystemTumor Syndromes

Although genetic factors account for only a minority of childhood CNS tumors, the incidences of particular types of CNS tumors are greatly increased in some tumor syndromes. All of these CNS tumor syndromes share autosomal dominant inheritance.

15.11.2.1 Neurofibromatosis Type 1

NF1 has an incidence of 1:4000. The responsible gene is located on chromosome 17q12 [82]. NF1 is frequently associated with optic pathway gliomas in children [83]. Histologically, the vast majority of these tumors are pilocytic astrocytomas, although diffuse astrocytoma and glioblastomas are also described.

15.11.2.2 Neurofibromatosis Type 2

Neurofibromatosis type 2 (NF2) is more commonly associated with adult CNS tumors than with pediatric CNS malignancies. The responsible gene is located on chromosome 22q12.2, encoding a product known as Merlin or schwannomin that functions as a tumor suppressor gene. Several types of mutations occur in the gene, leading to formation of a truncated product. A study by Evans et al. [84], showed that 18 % of patients with NF2 (61/334) presented in the pediatric (0–15 years) age group. Of these, 26 presented with vestibular schwannoma, 19 with meningioma, and 7 with spinal tumors.

15.11.2.3 Tuberous Sclerosis

Tuberous sclerosis is characterized by hamartomas and benign neoplastic lesions in the CNS and other organs. There is a locus heterogeneity in tuberous sclerosis with disease determining genes on chromosomes 9 and 16. The mutant genes occur in

small regions of telomeric chromosome bands at 9q34.3, designated as TSC1 (encodes for protein hamartin) and at 16p13.3, designated as TSC2 (encodes for protein tuberin) [85, 86]. Hamartin complexes with tuberin to regulate the cell cycle. Tuberin participates in normal brain development and cardiomyocyte terminal differentiation [87]. The most common CNS neoplasm in tuberous sclerosis is the subependymal giant cell astrocytoma arising from the wall of the lateral ventricles. Other tumors associated with tuberous sclerosis include facial angiofibromas, cardiac rhabdomyoma, retinal nodular hamartomas, lymphangiomyomatosis, renal angiomyolipoma, and hamartomatous rectal polyps. The giant cell astrocytoma rarely becomes malignant (glioblastoma).

15.11.2.4 Von Hippel-Lindau Disease

This disease is caused by the mutation of the VHL gene. It is characterized by angiomatosis retinae and hemangioblastomas of the CNS and retina. Hemangioblastomas may also involve the face, adrenals, lungs, and liver. Other pathological lesions are renal cell carcinoma, pheochromocytoma, and visceral cysts [6].

15.11.2.5 Turcot Syndrome

This is a heterogenous group of disorders; both autosomal dominant and recessive inheritance have been described. This syndrome is characterized by the coexistence of colorectal neoplasms with either medulloblastoma (predominantly) or glioblastoma. Some cases are variants of FAP syndrome, whereas others are variants of the hereditary non-polyposis colorectal carcinoma syndrome [88]. At least two defined clinical groupings can be seen within Turcot syndrome. In the first, medulloblastoma is associated with FAP. In these cases, there is mutation in the APC gene on chromosome 5q21. The gene encodes a 300 kDa protein that is ubiquitously expressed and modifies the interaction between the beta-catenin protein and E-cadherin cell adhesion molecule. In the second clinical group, glioblastoma is seen in patients without FAP, some of whom have hereditary non-polyposis colorectal carcinoma and mutations in DNA mismatch repair genes.

15.11.2.6 Gorlin Syndrome (Nevoid Basal Cell Carcinoma Syndrome)

This syndrome is characterized by nevoid basal cell carcinomas and jaw keratocysts, palmar and plantar pits, skeletal abnormalities, ectopic calcifications, and ovarian fibromas. The characteristic associated CNS tumor is medulloblastoma and tends to be the desmoplastic variant [89, 90]. The affected gene is PTCH, which lies on chromosome 9q31.

15.11.2.7 Cowden Syndrome

This is a rare syndrome associated with hamartomatous and neoplastic lesions such as dysplastic gangliocytoma of the cerebellum (Lhermitte-Duclos disease), verrucous skin lesions, trichilemmomas, oral mucosa fibromas, hamartomatous colon polyps, thyroid neoplasms, and breast cancer. The affected gene is PTEN/MMAC1, located on chromosome 10q23. The gene product may be involved in cell growth and differentiation [91].

15.11.2.8 Li-Fraumeni Syndrome

Li-Fraumeni syndrome is characterized by the presence of bone or soft tissue sarcomas presenting before the age of 45 years, with presence of other cancers in first-degree relatives presenting before age 45 years. The pediatric brain tumors associated with Li-Fraumeni syndrome are medulloblastoma/PNET, choroid plexus tumor, and ependymoma. There are two genetic determinants of Li-Fraumeni syndrome. The first is a germline mutation of the p53 gene at chromosome 17P13.1. This gene plays a key role in cell cycle control and apoptosis. The second gene is CHEK2 (checkpoint kinase 2) on chromosome 22q. It has been shown that the gene product of CHEK2 directly phosphorylates p53, indicating its involvement in p53 regulation of DNA damage [92].

15.12 Conclusions

Neoplasms in the fetus and infant very frequently are of embryonal origin from cells that fail to mature and differentiate. Wilms tumors are derived from the metanephric blastema that has lost its propensity for differentiation. Neuroblastomas arise from the sympathetic nervous system in the neural crest and wander usually into the adrenal gland early in embryonic life. Other tumors occur predominantly in the first year of life and have certain features of embryonic growth and include embryonic sarcomas, yolk sac tumors of the testis, and heptaoblastomas. They are characterized by various chromosomal alternations. The growth, prognostic features, and clinical course have been addressed.

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Chapter 16
Molecular Pathology and Diagnostics of Cutaneous Malignancy

Sudeep Gaudi, Patricia M. McNab, Timothy W. McCardle, and Jane L. Messina

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Abstract Advances and discoveries in molecular genetics over the past decade have literally changed the face of diagnosis and treatment decisions for patients with melanoma and non-melanoma skin cancer. These include genetic analysis of skin biopsies as a diagnostic aid, genotyping of tumors from patients with malignant melanoma for personalized treatment of metastatic disease, and discovery of

S. Gaudi • P.M. McNab • T.W. McCardle • J.L. Messina, M.D. (✉)
H. Lee Moffitt Cancer Center and Research Institute,
12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: jane.messina@moffitt.org

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a new virus thought to play a role in the pathogenesis of Merkel cell carcinoma. Initiated by the discovery of a familial cancer gene in patients with familial melanoma and atypical nevi over three decades ago, molecular pathology techniques now play a central role in genetic testing for melanoma risk, in evaluation of melanocytic proliferations with uncertain biologic potential, and in stratifying patients with metastatic melanoma for treatment with targeted therapeutic agents, driving the search for better markers of prognosis and response to therapy. Targeted therapy is now available for treatment of unresectable or metastatic basal cell carcinoma. It is likely that continual improvements in techniques for analyzing blood and fresh and archival tumor tissue will uncover the pathogenesis of skin tumors related and unrelated to sun exposure, spawn the development of vaccinations or chemopreventive agents for these tumors, and enable physicians to customize treatment based on unique patient and tumor characteristics. This chapter will highlight relevant information concerning the molecular pathogenesis of melanoma, basal and squamous cell carcinoma, and Merkel cell carcinoma. Sections on molecular techniques available for diagnosis, prognosis, and treatment selection, emphasizing melanoma where this is most highly developed, are included

Keywords Nonmelanoma skin cancer • Molecular genetics of cutaneous malignancy • Melanoma prognosis • Prognostic biomarkers • Merkel cell carcinoma pathogenesis

Abbreviations

AE	Aloe-emodin
ASIP	Agouti signaling protein
BCC	Basal cell carcinoma
CGH	Comparative genomic hybridization
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FISH	Fluorescent in situ hybridization
GST	Glutathione S-transferase
GWASs	Genome-wide association studies
Hh	Hedgehog
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 receptor
McPyV	Merkel cell polyomavirus
MEK	MAPK extracellular signal-regulated kinase
PCR	Polymerase chain reaction
SCC	Squamous cell carcinoma
SNP	Single nucleotide polymorphism
UV	Ultraviolet

16.1 Introduction

An estimated 76,250 new cases of cutaneous melanoma will have been diagnosed in the United States in 2012 [1], and this disease will claim the lives of approximately 9,000 American men and women this same year [1]. In contrast, greater than two million new cases of nonmelanoma skin cancer are anticipated to occur in the United States in 2012, while deaths from these malignancies are projected to be less than 1,000 [2]. Given the disproportionate mortality of melanoma compared to other skin cancers, detection of early-stage disease constitutes a major effort in all diagnostic endeavors. Many of the strides made in the molecular biologic analysis of melanoma have been aimed at increasing diagnostic accuracy. This is an important goal, especially in light of the number of skin biopsies performed to rule out melanoma—estimated to be more than one to two million in the United States in 2008 alone [3]. While histopathologic examination of suspicious pigmented lesions remains the "gold standard" for diagnosis, and the vast majority of malignancies can be readily distinguished from melanocytic nevi on the basis of histopathology, cases not so easily discernable can wreak havoc. The consequences of both false negative and false positive diagnoses are considerable: false positive diagnosis may subject the patient to undue mental anguish, financial burden, difficulties in attaining health insurance, and disfiguring surgical as well as toxic medical interventions. False negative diagnosis of melanoma, in addition to delaying appropriate treatment, is the single most common cause of medical malpractice lawsuits against a pathologist [4]. There have been numerous studies focusing on diagnostic discrepancy rates in melanocytic lesions [5–16]. In one illustrative example, experienced dermatopathologists considered experts in their field rendered opposing histopathologic diagnoses in as many as 25 % of cases reviewed [14].

Current molecular diagnostic tests, employed for the purpose of increased diagnostic accuracy and selection of targeted therapy, are informed by the earliest research into the molecular pathogenesis of melanoma. Therefore, this section will highlight current knowledge into the molecular basis for the disease, followed by discussion of established and emerging molecular techniques that may assist in the diagnosis and treatment of melanoma.

16.2 Familial Melanoma

Familial melanoma accounts for only 10 % of melanoma cases [17] but formed the basis for the earliest studies on genetic abnormalities in this disease, specifically mutations in p14/ARF, p16/INK4a, and CDK4. The best-characterized high-penetrance susceptibility gene predisposing to melanoma is CDKN2A, located on chromosome 9p21 [18–23]. This gene encodes two distinct tumor suppressor proteins (p14/ARF and p16/INK4a), implicated in the oncogenesis of melanoma.

The former hinders neoplasia through its indirect effect on p53 by binding to and inhibiting the function of human homolog of murine Mdm2 (HDM2), a protein that tags other proteins for ubiquitination. In mutated form, p14/ARF cannot bind to and suppress HDM2, allowing it to mark p53 for ubiquitination. With less p53 available to identify damaged DNA, genomic instability results, predisposing the afflicted individual to the development of melanoma. p16/INK4a functions in concert with retinoblastoma protein, a tumor suppressor protein, to regulate the G_1 phase of the cell cycle. In familial melanoma patients, compromised expression of p16 allows for uninhibited phosphorylation of retinoblastoma protein. This leads to transcription of genes encoding cyclin E, a protein that is required for the initiation of DNA replication in the S phase. CDK4 is another high penetrance but low-frequency melanoma susceptibility gene identified in linkage studies [19–22]. Located on chromosome 12q14, CDK4 encodes cyclin-dependent kinase 4 protein, a constituent of the CDK4/6 complex discussed above. Germline mutations that activate this gene occur at codon 24 (Arg24Cys and Arg24His) and render the CDK4/6 complex resistant to p16 inhibition. Thus, mutations in the p15/INK4a and CDKN2A genes produce a similar effect.

Additional genes involved in cases of familial melanoma have been identified through the use of genome-wide association studies (GWASs). While linkage studies have allowed the identification of the aforementioned high-penetrance, low-frequency genetic variants implicated in familial melanoma, GWASs have enabled high-powered searches for low-penetrance, high-frequency melanoma susceptibility genes. The melanoma genetics consortium, GenoMEL, performed the largest of these studies with the purpose of identifying common genetic variants contributing to melanoma risk across populations of European ancestry residing at varying altitudes [24]. This study found the strongest association in a single nucleotide polymorphism (SNP) that is a likely variant of the MC1R (melanocortin 1 receptor) gene, located on chromosome 16q24. This SNP was also discovered to be associated with hair color and skin pigmentation in a separate GWAS [25]. The link between pigmentation phenotype and melanoma susceptibility has long been established by epidemiological studies [26]. Thus, the relationship between allelic variants of the MC1R gene and both pigmentation phenotype and melanoma risk is not unexpected. The GenoMEL study also corroborated other previously suggested melanoma associations—locus 11q14-21 encompassing tyrosinase and locus 20q11.22, near ASIP (agouti signaling protein)—as well as two SNPs, for which prior associations with melanoma were not made. These latter two variants were located on chromosome 9p21, respectively, within the methylthioadenosine phosphorylase gene, which flanks CDK2NA, and ANRIL, an antisense noncoding mRNA that overlaps with the promoter of the p14ARF transcript of the CDK2NA gene and the transcribed sequence of CDK2NB.

A separate GWAS of nevus count using cohorts from the UK and Australia identified alleles at two strongly associated loci: 9p21 (MATP) and 22q13.1 (PLA2G6, a member of the phospholipase A2 superfamily of genes) [27]. The authors of this study were then able to associate the SNPs at these loci with melanoma susceptibility by analyzing two melanoma case-control samples. Interestingly, the signals

within 9p21 of this GWAS overlapped with those of the GenoMEL study. Furthermore, the authors of the GenoMEL study were able to replicate the association between the SNPs at 22q13 and melanoma risk as well. Given these associations of the SNPs identified in the nevus study with melanoma, as well as the well-known positive correlation between number of melanocytic nevi and risk for melanoma, it is reasonable to assume that the risk of developing melanoma at these loci with these SNPs is, at least in part, mediated through nevus count [28, 29]. The discovery of these and additional susceptibility genes may yield further insight into the biological pathways involved in melanomagenesis.

At present, there are no formal guidelines for genetic testing of melanoma patients, their kindred, or their tumor samples for the p16/CDKN2A mutation. Potential benefits of such testing include the possibility of increased compliance in preventive programs with a positive test result. A negative test result in a patient with relatives who have died from melanoma or pancreatic cancer with the germline mutation could be reassuring. Those opposed to such testing cite the lack of identifiable germline mutations in known high-risk genes in affected members of known melanoma families. Conversely, non-gene carriers in CDKN2A-positive families may still develop dysplastic nevus syndrome and develop melanoma. Thus, negative test results could confer a false sense of reassurance and be a disincentive to continued participating in screening/prevention activities. Finally, it is difficult to assign an absolute risk for development of disease in individuals who do have a germline mutation identified [30]. It is reasonable to conclude that genetic testing for germline CDKN2a mutations should be performed only in patients that have a significant risk of having a positive test result (i.e., those with three or more affected members) and only in conjunction with a thorough explanation of the limitations of the test, in conjunction with qualified genetic counseling and education [31].

16.3 Sporadic Melanoma

Research into the molecular basis of sporadic melanoma has implicated many genes, particularly those encoding proteins involved in the mitogen-activated protein kinase (MAPK) pathway [19, 20, 22, 23, 32, 33]. With up to 90 % of melanomas harboring constitutive activation of this pathway, great interest lies in detailing the precise mechanisms involved, with the hope of intervening at key junctions in an effort to thwart melanomagenesis and/or progression [33]. The MAPK pathway regulates cellular proliferation through signal transduction along the cascade of RAS, RAF, MAPK extracellular signal-regulated kinase (MEK), and extracellular signal-regulated kinase (ERK). Activated ERK induces several proliferative and survival processes, one of which is activation of the cyclin D-CDK4/6 complex (discussed above) upon translocation to the nucleus. Mutational analyses have yielded key aberrations in crucial players in the MAPK pathway, including BRAF, NRAS, c-Kit, and epidermal growth factor receptor (EGFR).

16.4 Molecular Diagnostic Tests

16.4.1 BRAF

There are three mammalian RAF proto-oncogenes—ARAF, BRAF, and CRAF. However, of these, only activating mutations of BRAF have thus far been shown to be present in a significant percentage of human malignancies, with the highest rate occurring in melanoma [19, 20, 22, 23]. BRAF-mutated melanomas tend to occur at anatomic sites exposed to intermittent, rather than chronic, sun damage. With approximately 70 % of cases harboring such a mutation, BRAF is the most commonly mutated proto-oncogene in cutaneous melanoma. Furthermore, a significant proportion of both benign and dysplastic melanocytic nevi have been shown to harbor mutations of BRAF as well, suggesting a relatively early event in melanomagenesis [19, 22, 23, 34].

Greater than 90 % of BRAF mutations in melanoma result from a single base missense mutation (T→A) at codon 1799 that leads to the substitution of valine in favor of glutamic acid at position 600 of the BRAF protein [35]. This alteration introduces a conformational change in BRAF's kinase domain, which can lead to a 480-fold increase in kinase activity when compared to wild-type BRAF [36]. This mutated BRAF induces uncontrolled proliferation in melanoma. Ongoing research of this molecule includes its role in inducing senescence of benign melanocytic nevi, the mechanisms involved in bypassing this senescent response in the development of melanoma from such nevi, and the mechanisms for acquired resistance to targeted inhibition of this molecule in patients undergoing anti-BRAF therapy [37–48].

A major breakthrough in the treatment of metastatic melanoma has been the successful treatment of BRAF-mutant melanoma with selective BRAF inhibitors such as vemurafenib and dabrafenib [49]. Since this agent shows tenfold greater activity for V600E-mutant melanoma compared to wild-type melanoma, most current clinical trials require pre-treatment assessment for the presence of this mutation [50]. The mutation is ascertained by means of a polymerase chain reaction (PCR) assay (TaqMan, Applied Biosystems), which involves hybridizing a probe specific to the 1799T->A substitution with DNA isolated from formalin-fixed, paraffin-embedded tumor tissue and determining the presence or absence of amplification after repeated chain-reaction cycles [51]. This assay is now routinely available at most major commercial reference laboratories. There is also a commercially available monoclonal antibody (VE1) that can be used to assess for the BRAF V600E mutation immunohistochemically. This antibody was 100 % sensitive and specific for the mutation compared to the gold standard of molecular analysis in a recent series of 44 cases [52]. There have also been attempts at predicting BRAF mutation status by analyzing various clinical and histologic features of primary tumors. In a recent study of 302 primary melanomas, groupings of various combinations of 17 such factors revealed that phenotypic features such as increased upward scatter, intraepidermal nesting, epidermal thickening, sharp lateral demaracation of the tumor, and the presence of rounded, highly pigmented tumor cells were distinct features of melanomas with BRAF mutation. In this study, classification tree analysis showed that BRAF mutation status could be correctly predicted in up to 82 % of

tumors using certain variables [53]. While there is certainly great interest in correlating easily analyzed clinicopathologic variables with mutation status, and active research aimed at this goal, at present, however, the gold standard for assessing tumors for BRAF status remains mutation analysis.

16.4.2 c-KIT

c-KIT, a proto-oncogene that encodes the type III receptor tyrosine kinase KIT, was first identified in 1987 as a result of sequence similarity to the Hardy-Zuckerman 4 feline sarcoma virus oncogene, v-KIT [54]. Upon binding its ligand, stem cell factor, KIT undergoes receptor dimerization, autophosphorylation, and activation of its intracellular tyrosine kinase domain [18, 55]. Once activated, KIT is capable of stimulating downstream signaling pathways, such as MAPK [18, 55, 56].

Activating mutations and/or gene amplification of KIT have been described in distinct subsets of melanoma [57–61]. In one study, aberrations were found in 39 % of mucosal melanomas, 36 % of acral melanomas, and 28 % of melanomas arising in chronically sun-damaged skin (as defined by the presence of solar elastosis on review of histopathology)—anatomic sites at which BRAF mutations occur far less frequently [60].

The most prevalent KIT mutations in melanoma occur in exons 11, 13, and 17 [62]. These mutations are thought to promote the constitutive activation of KIT either through precluding the protein from assuming its default autoinhibited conformation or by promoting its dimerization in the absence of stem cell factor [63, 64]. The precise manner in which constitutive KIT activation promotes melanomagenesis remains unclear. Nonetheless, recognizing activating mutations of KIT as well as copy number gains can be of great consequence, as this subtype of melanoma may respond the receptor tyrosine kinase imatinib [65–67]. Since the initial case report of a dramatic response to imatinib in c-KIT-mutated melanomas [65], interest has been raised in treating patients with acral and mucosal melanoma with this agent, leading to several ongoing phase II clinical trials (ClinicalTrials.gov) of imatinib and other c-KIT blockers such as sunitinib, dasatinib, and sorafenib. These studies require documentation of mutation or amplification of the c-kit gene. Currently, therefore, it seems reasonable that subgroups of patients with metastatic melanoma prone to c-kit mutations, such as primary mucosal or acral lentiginous tumors, be analyzed for KIT mutation status. If mutated, a therapeutic trial with a c-KIT blocking agent has been suggested. Testing, using PCR following by sequencing analysis of exons 11, 13, and 17 can be performed at several reference laboratories, including ARUP, as well as university laboratories such as those at University of Washington and University of Michigan.

16.4.3 N-RAS

The RAS proto-oncogene family is at the initiating point of the MAPK pathway, and theoretically activating mutations of these molecules should provide the

affected cell with continuous growth signals in the absence of extracellular stimuli. Indeed, RAS molecules have documented roles in melanomagenesis [18–20, 22, 23, 33]. Of the three members of this family (H-RAS, K-RAS, and N-RAS), only the latter has been shown to sustain activating mutations in melanocytes, as well as nearly one third of all melanomas [19, 23, 33]. The most commonly documented N-RAS aberration in melanoma is a missense mutation at codon 61 (Q61R), which results in the substitution of arginine in place of glutamine and impairs GTP hydrolysis, locking the protein in a state of constitutive activation [68]. However, therapeutic attempts at blocking the farnesylation, which is required for proper function of the molecule, using farnesyltransfer inhibitors, have yielded disappointing results [56, 69].

There has been a resurgent interest in the role of the N-RAS pathway in sustaining melanoma growth in the wake of the phenomenon of acquired resistance to BRAF inhibitors. Specifically, investigations into this phenomenon in cell lines have revealed that hyperactivation of the MEK-ERK1/2 pathway occurs rapidly after treatment with the BRAF inhibitor PLX4032, and this has been noted most prominently in cell lines containing the activating N-RAS mutation, which demonstrated resistance to apoptosis [70, 71]. Therefore, future trials of targeted therapies may require genotyping for N-RAS to stratify treatment.

16.4.4 Epidermal Growth Factor Receptor (EGFR)

EGFR is yet another receptor tyrosine kinase that is involved in activation of the MAPK and PI3K/Akt signal transduction pathways. The latter pathway is also activated by RAS and results in decreased apoptosis [19]. The selective expression of this receptor in human melanoma cells harboring an extra copy of chromosome 7 was first studied in 1985 [72]. Numerous studies have since investigated the importance of this receptor in melanomagenesis and/or progression with contradictory results. More recently, however, researchers have shown an association between polysomy 7 and EGFR gene amplification by fluorescent in situ hybridization (FISH), with significantly higher amplification in tumors that metastasized within 2 years of surgical excision of the primary lesion [73]. Implying a poorer prognosis in this latter subset of patients, further studies into the potential benefit of anti-EGFR therapy for those with EGFR alterations have been recommended. However, at this time there appears to be no clinical utility to testing tumors for EGFR overexpression.

16.4.5 Comparative Genomic Hybridization

The technique of comparative genomic hybridization (CGH), wherein lesional DNA as well as normal control DNA are hybridized to either normal metaphase chromosomes or an array of thousands of defined DNA probes (array CGH) in order to detect copy number changes, has been used to examine differences between

melanoma and benign melanocytic lesions. Specifically, gains of chromosomes 1q, 6p, 7p, 7q, 8q, 17q, and 20q and losses of chromosomes 6q, 9p, 9q, 10p, 10q, and 11q have been observed in greater than 95 % of melanomas [74]. Loss of chromosome 9, an early and common aberration in melanomagenesis, typically involves deletion of the distal region of the p arm that encompasses the 9p21 locus [75]. Of note, gain of 6p is a frequent aberration in both primary and malignant melanomas and may confer a poorer outcome [76, 77].

Melanocytic nevi rarely demonstrate chromosomal aberrations, with the exception of Spitz nevi, which may harbor gains on occasion [74, 78–80]. A gain of the entire short arm of chromosome 11 is the most commonly encountered aberration in Spitz nevus and is an abnormality not detected in primary cutaneous melanomas. Although most congenital nevi are readily distinguished from melanoma according to established histopathologic criteria, atypical nodular proliferations that arise in congenital nevi can be problematic [78]. These lesions simulate nodular melanomas microscopically, but tend to regress within a few months. CGH offers diagnostic aid in this area [78, 80]. While cases of atypical nodular proliferations show chromosomal aberrations by CGH, the pattern of involvement is distinct from that created by melanoma. Abnormalities in the former group involve only entire chromosomes. While gains or losses of entire chromosomes can frequently occur in melanoma as well, 95 % of such cases are paralleled by gains or losses of chromosomal fragments.

CGH has also underscored significant genetic differences between acral melanoma and superficial spreading melanoma [81], justifying the distinction between these subtypes by demonstrating disparity in pattern of chromosomal aberration between the two [80, 81]. While acral melanomas have been shown to invariably harbor gene amplifications (defined as greater than a threefold increase in copy number), superficial spreading melanomas, of comparable tumor thickness and patient age, contain an amplicon in less than 15 % of cases. The amplified regions frequently consist of areas containing known oncogenes. Study of the amplicons not harboring obvious candidate genes may provide further insight into the mechanisms of melanomagenesis.

Isolated melanocytes located beyond the microscopically-designated free margins of resection have been shown to harbor these genetic amplifications as well [80, 81]. Researchers believe that these field cells represent unrecognized radial growth of the acral melanoma and are likely responsible for local recurrences. If further studies support such a notion, then CGH may play an instrumental role in shaping future guidelines pertaining to the attainment of clear margins of resection.

16.4.6 Fluorescent In Situ Hybridization

A multiprobe fluorescent in situ hybridization (FISH) assay against several of the aforementioned chromosomal abnormalities detected in CGH studies has been developed and tested in several studies with promising results [3, 82]. The assay

identifies copy number alterations in chromosomes 6p25 (RREB1), 6q23 (MYB), and 11q13 (cyclin D1) as well as centromere 6. Results of these preliminary studies have demonstrated high sensitivity (~87 %) as well as specificity (~95 %) in distinguishing benign melanocytic nevi from melanomas using this particular panel of probes.

Therefore, this technique plays a potential role in classifying problematic melanocytic lesions. The area of most intense investigation of the applicability of this technique is in the distinction between spitzoid melanoma and benign Spitz nevus. Two recent studies have demonstrated sensitivity and specificity of 43–60 % and 50–80 % for determination of malignancy in ambiguous Spitzid lesions, using metastatic behavior as the gold standard [83, 84]. In another recent, albeit small, study evaluating the efficacy of FISH in distinguishing nodular nevoid melanomas from mitotically active melanocytic nevi with probes targeting these same for four loci, chromosomal aberrations were detected in each of the nevoid melanomas and in none of the melanocytic nevi [85]. Other scenarios for which FISH has shown potential benefit include distinguishing epithelioid blue nevus from blue nevus-like cutaneous melanoma metastasis [86], distinguishing intranodal nevus from metastatic melanoma [87], classifying superficial melanocytic neoplasms with pagetoid melanocytosis [88], and the microstaging of melanoma arising in association with a nevus [89]. Several commercial laboratories now offer this test for use in formalin-fixed, paraffin-embedded tissue using probes developed by Abbott Laboratories (Caris Life Sciences, Neogenomics Laboratories).

16.4.7 Immunohistochemical Staining for Prognostic Biomarkers

There has been abundant research aimed at identifying biomarkers for prognosis as well as treatment response. The widespread availability of immunohistochemical staining makes this technique one of the most widely investigated, because it can characterize patterns of protein expression while maintaining preservation of tissue and cellular architecture. There have been literally thousands of manuscripts devoted to potential molecular markers of melanoma that could identify patients at greatest risk for recurrence, or most likely to benefit from adjuvant therapy. Two recent systematic reviews of published data on immunohistochemistry-based prognostic molecular markers for cutaneous melanoma have been performed [90, 91]. The first of two reviews sought to report on associations between immunohistochemical expression of protein biomarkers and survival outcomes in cases of cutaneous melanoma. Molecular markers were then scored according to the protein's major biological function, as categorized by a modified Hanahan-Weinberg functional capabilities scheme. This review identified effectors of DNA replication, cyclin-dependent kinase inhibitors, growth-promoting transcription factors, and regulators of tissue invasion and metastasis as having a statistically significant impact on patient outcome. Ki-67, proliferating cell nuclear antigen, metallothionein, Ku70,

Ku80, and microtubule-associated protein-2 were consistently associated with disease-free as well as overall survival. Interestingly, cyclins and cyclin-dependent kinases were not associated with melanoma prognosis. More recently, a significant association between Ki-67 expression and melanoma-specific mortality—in addition to dermal mitoses, which remain a significant and independent prognostic factor—has been shown in a large and representative population of patients diagnosed with thin (≤ 1 mm in thickness) cutaneous melanomas [92]. With external validation in a similar population-based sample, it is possible that Ki-67 may be formally adopted into routine clinical practice, either as a prognostic factor for disease recurrence or a predictive factor of positive sentinel lymph node biopsy.

Expression of cyclin-dependent kinase inhibitors was significantly associated with mortality. For example, increased levels of p16 were associated with decreased all-cause and melanoma-specific mortalities, while increased levels of p27, which typically cause cells to arrest in the G_1 phase of the cell cycle, are paradoxically associated with increased mortality. The rationale behind this observation lies in the activation of p27 through cytoplasmic sequestration, rather than protein degradation. Cytoplasmic accumulation of p27 has been associated with metastatic potential [93]. Among proteins involved in growth signaling, statistically significant associations were most frequent among transcription factors, ATF-2 and AP-2α, and transcriptional coactivators, NCOA3/AIB-1. This observation suggests altered transcriptional regulation as a crucial step in regulation of melanoma-specific survival. Increased expression of three cellular adhesion molecules was shown to be associated with statistically significant worsening of disease-free survival: the melanocyte-specific melanoma cell adhesion molecule (MCAM/MUC18), neuron-specific L1 cell adhesion molecule (L1-CAM), and glandular tissue-associated carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1). These molecules are thought to promote tumor infiltration and distant spread through abnormal tumor-stroma interactions [94–97]. Also, elevated levels of osteopontin and tenascin-C—members of the matricellular protein family, which consists of secreted molecules that interface between cell surface receptors and the extracellular matrix [98]—were associated with statistically significant worsening of melanoma-specific mortality and disease-free survival, respectively. Among proteases, increased levels of tissue plasminogen activator and matrix metalloproteinase-2 were statistically significant for mortality outcomes.

16.5 Basal Cell Carcinoma

Basal cell carcinoma (BCC) is the most common invasive cancer in the Western world, and it accounts for 75 % of skin malignancies overall with increasing rates worldwide [99–101]. Ultraviolet (UV) radiation, more specifically of the UV B-wavelength, is the dominant etiologic agent thought to be responsible for most skin carcinomas [99, 102]. It does so by genetically altering genes involved in initiation, promotion, and progression of both melanoma and non-melanoma skin

cancers. Since the discovery of the hedgehog gene and its influence on the development of carcinoma in certain populations, great strides have been made in uncovering other genetic abnormalities and the possible use of specific inhibitors in treatment of the disease [103, 104]. These emerging genetic alterations, originally studied in inheritable disorders, such as xeroderma pigmentosum and nevoid basal cell carcinoma syndrome (also known as Gorlin syndrome), have offered new insight into its molecular basis.

16.5.1 Inherited Forms of Basal Cell Carcinoma

There are several major mechanisms involved in inherited forms of BCC: defects in detoxifying proteins, DNA repair, and embryonic signaling pathways. Defects in these pathways cause well-recognized skin cancer syndromes, including xeroderma pigmentosum, Gorlin syndrome, and familial cylindromatosis, and play a role in sporadic BCC as well. One of the most important components that protect the skin against oxidative stress after UV irradiation is the glutathione S-transferase (GST) enzyme system [105]. In humans, GST is expressed in melanomas, but only weakly in BCC [106]. Several GST polymorphisms have been identified in patients with high UV sensitivity and increased risk for BCC [105]. Impaired DNA repair, especially in patients with xeroderma pigmentosum, predisposes to non-melanoma skin cancer, preferentially of the squamous cell carcinoma (SCC) type (discussed later in this chapter). Of the embryonic signaling pathways, the most important is the hedgehog (Hh) signaling pathway.

PTCH1 gene is a member of the Hh signaling pathway, which encodes for a membrane receptor for three extracellular ligands: sonic hedgehog, Indian hedgehog, and desert hedgehog [103, 107]. Located on chromosome 9q22, this gene inhibits the Hh signaling pathway in its unbound configuration, thus functioning as a tumor suppressor gene. Over 90 % of sporadic BCCs harbor mutations on at least one allele. Malignant cells may also contain mutations in the trans-membrane protein Smoothened (Smo), normally inhibited by the unbound Hh receptor, allowing for decreased effectiveness of the PTCH1 gene [103, 108, 109]. In normal situations, Hh binding allows for the relocation of Smo to the cilium and subsequent activation of signal transduction. The end result of this unregulated pathway is the upregulation of the Gli family of transcription factors known as Gli [103, 108–110]. Since PTCH1 is overexpressed in BCC (up to 11-fold in one study) but not in other nonmelanoma skin cancers; it may be utilized as a tumor marker via in situ hybridization or reverse transcription-PCR to asses for elevated levels of either PTCH1 or Gli1 mRNA [107, 111]. In addition, Hatta et al. [112] found that both BCCs and trichoeptheliomas may be distinguished from other basaloid neoplasms using reverse transcription-PCR quantifications of Gli1 levels. There were consistently higher levels detected in these two tumors, with almost non-existent levels in others.

It has been shown that interruption of the Hh pathway in concert with chemotherapy enhances tumor response in animal models of pancreatic cancer [110, 113, 114]. Several small molecular inhibitors of Hh signaling pathway are under

investigation, and vismodegib was approved by the FDA in 2012 for patients with recurrent, locally advanced, or metastatic basal cell carcinoma. The targeted areas include Smo antagonists, Hh inhibitors, and Gli inhibitors, including cyclopamine, GDC-0449 (Phase I and II), IPI-926 (Phase I), LDE225 (Phase II), and BMS-833923 (Phase I) [110, 115, 116]. An in-depth discussion of Smo inhibitors on the horizon can be found in the recent review article by Peukert and Miller-Mosin [110].

Numerous Gli-mediated transcription inhibitors, as well as inhibitors targeting the Hh protein itself have been tested in animal models; however, these have not yet been brought to clinical trial. [110, 117, 118]. Because these mutations are so ubiquitous in sporadic BCC and always present in inherited BCC, there is no role for pre-treatment testing for their presence.

16.5.2 Sporadic Forms of Basal Cell Carcinoma

UV-induced mutations in both p53 and PTCH1 genes are thought to be the most common elements that induce the development of BCC [119]. The p53 gene is responsible for regulating the cell cycle and apoptosis induction in irreversible damaged cells, and mutations of the protein have been detected in 50–60 %% of BCCs [107, 119]. Although the usefulness of p53 is limited as a molecular diagnostic aid in BCC because it is overexpressed in numerous malignancies, levels are substantially increased in tumors with aggressive growth patterns [105]. This information may prove useful in determining prognosis or directing further medical management.

BCL-2 encodes for a protein that participates the regulation of cell death by blocking apoptosis [120]. In both BCC and SCC, overexpression has been noted, allowing for increased cell susceptibility to accumulation of harmful mutations. In contrast to the situation with SCC, where BCL-2 levels demonstrate a slow, graduated increase as lesions progress from an actinic keratosis to invasive carcinoma, in BCCs, BCL-2 expression is lost with increasing tumor progression or aggressiveness. Other studies have also attempted to characterize additional mutational events involved in basal carcinomas on a molecular level utilizing whole genome microarray, the details of which are beyond the scope of this chapter [111, 121]. The clinical implications of these new discoveries will be of great importance, impacting the diagnosis, treatment, and prevention of a variety of benign and malignant skin conditions.

16.6 Squamous Cell Carcinoma

Squamous cell carcinoma (SCC), the second most common skin malignancy, is postulated to demonstrate different pathogenetic mechanisms than BCC. While BCC generally develops de novo, SCC goes through a multistep process of development. Although p53 has been postulated to play a major role, the major signaling

transduction pathway(s) is presently unknown. SCCs display a great number of chromosomal aberrations; therefore, chromosomal instability likely plays a key role in its progression. Also, the differing frequencies of these tumors in organ-transplant patients compared to non-immunosuppressed (SCC being more common in the former) raise the probability that defects in immune surveillance play a much more important role in SCC development than in BCC [122].

SCC demonstrates alterations in several major areas, including p53 and RAS, as well as DNA repair mechanisms in xeroderma pigmentosum. p53 tumor suppressor gene mutations are present in approximately 40–50 % of cases, while RAS genes carry an incidence of 10–30 %. In addition, infection with human papillomavirus is also felt to play a large role in the development of SCC [123–125].

One study found that point mutations in the p53 gene were seen early in tumorigenesis and complete allelic loss correlated with progression to higher grade lesions [126]. More specifically, it has been shown that gain-of-function mutations may shift development into a more aggressive, spindle cell variant [127]. However, another study by Jensen et al. showed no difference in p53 activity [128]. Obviously, more investigations are needed before the role of p53 in tumor prognosis and progression is fully elucidated.

Although more than 100 HPV subtypes have been identified in cervical SCC, only a subgroup of high-risk types, including HPV-16, 18, 31, 33, 35 and 58, are believed to be a causative agent in the development of cervical cancer. The product of the viral gene *E6* is able to effect proteasomal degradation of p53, inhibiting cell cycle arrest and/or apoptosis. HPV DNA is frequently detected in skin carcinomas and is found in both immunocompetent (47 %) as well as immunosuppressed patients (75 %). However, no high-risk subtypes have yet been identified in cutaneous SCC [129]. Recently, attention has turned to HPV38, which has been detected in ∼50 % of skin carcinomas but only in 10 % of healthy skin. HPV38 was also demonstrated in 43 % of actinic keratoses, as well as 13 % and 16 % of SCCs and BCCs, respectively. At this point, however, the high frequency of HPV DNA in normal skin and hair follicles and the fact that more than one type of HPV may be found simultaneous in normal and tumor tissues make the role of HPV38 in skin carcinogenesis still elusive [129].

Recent insight into possible additional genetic markers with novel diagnostic or prognostic implications is included here for completeness. These include alterations in the c-Jun NH_2-terminal kinase (JNK) signaling cascade, MUC4, and collagen XVII protein. Emerging research by Ke et al. [130] has shown that the c-Jun NH_2-terminal kinase (JNK) signaling cascade is activated in at least 70 % of SCCs, and its interaction with RAS is necessary for glycolysis. In addition, they found that inhibition of JNK2 counteracts successful tumorigenesis of epithelial cells. MUC4, a high molecular weight glycoprotein, has been recognized as possible biomarker or prognostic indicator for several malignancies. A recent study has revealed its overexpression in cutaneous SCC, well to moderately differentiated, while it is only weakly expressed or negative in BCC and melanoma and normal cutaneous epithelium [131].

In late 2009, Chou et al. [132] published data concerning the effects of aloe-emodin (AE) on non-melanoma skin cancers. It was found that malignant cells were

selectively targeted and destroyed through induced apoptosis via elevation of p53 levels and ultimate reduction in BCl-2 levels. In addition, the utilization of both AE and 5-fluorouracil resulted in significantly higher cell death in mutated cell lines in human epidermoid carcinoma (A431) and human head and neck SCC (SCC25) cells than only the use of AE or 5-fluorouracil alone. In the future, interference in the aforementioned genetic aberrations may have therapeutic implications.

16.7 Merkel Cell Carcinoma

The discovery that viral gene sequences were integrally incorporated into the genome of a significant percentage of Merkel cell carcinomas was revolutionary for our understanding of the pathogenesis of this tumor. Although this rare neuroendocrine malignancy was known to have a strong association with immunosuppression and a predilection for areas of chronic sun exposure, the role of a virus was only discovered in 2008. Feng and colleagues were the first to demonstrate the presence of a fusion transcript between a previously unrecognized viral T antigen and a human receptor tyrosine phosphatase in 8 of 10 Merkel cell tumors tested. They performed sequence analysis and identified a 5387-base pair genome of a polyomavirus they named Merkel cell polyomavirus (McPyV) [133]. Since then, numerous publications have documented the presence of this virus not only in a significant portion (approximately 70–80 %) of tumor tissues but in other tumors, as well as normal tissue. The mechanism of viral pathogenesis is believed to be the result of integration of the virus into band 3p14 at the human protein tyrosine phosphatase, receptor type, G gene, a tumor suppressor gene. The viral T antigen is expressed as large T and small T antigens, and integrated MCPyV large T protein is truncated due to the deletion of the carboxyl terminal, which then suppresses autonomous viral replication [134]. However, much remains unknown concerning the modes of transmission and replication of the virus, as well as the associations between seropositivity and viral presence in tumor tissues. In particular, further research into the interplay between viral integration and further mutations, probably related to UV radiation, is needed.

Soon after the discovery of the MCPyV, a series of studies were undertaken to determine if the presence of viral genes was associated with outcome. The evidence from these studies has been conflicting, with some data suggesting that patients with viral gene-positive Merkel cell carcinoma experience better survival, some not showing a difference, and some indicating worse survival. Bhatia et al. demonstrated that patients with ≥ 0.06 viral copies per cell experienced higher median survival than those with ≤ 0.0035 viral copies per cell (86 months versus 20 months, $P = 0.015$) [135]. Sihto et al. also confirmed a positive association with viral load and outcome in a study of 114 Finnish Merkel cell carcinoma patients, reporting the 5-year survival to be 45 % for patients with virus-positive tumors versus 13 % for those with virus-negative tumors ($P < 0.001$) [136]. After adjusting for gender and the presence of nodal metastases, the presence of viral DNA was significantly

associated with improved survival (hazard ratio 0.42, 95 % confidence interval 0.25–0.71). In contrast, Garneski et al. evaluated the outcome of 30 Merkel cell carcinoma patients based on the presence or absence of viral sequences by real-time quantitative PCR. Using Kaplan–Meier analysis, no survival difference was noted between virus-positive and virus-negative tumors [137]. Becker et al. analyzed 75 samples from 53 MCC patients for the presence of MCPyV by real-time PCR. Their clinical follow-up data suggested that patients whose tumors contained the viral genome displayed a more aggressive behavior, but after stratifying for tumor stage there was no statistically significant difference between the groups [138]. Numerous studies continue to unravel the role this polyomavirus plays in the pathogenesis, prognosis, and outcome of Merkel cell carcinoma, but at present the assessment of viral presence is solely for research purposes.

16.8 Conclusion

The era of targeted therapy has ushered in a plethora of diagnostic tests aimed at optimal selection of cancer patients for treatment with novel agents. Beginning with the elucidation of the role of the p16 gene mutation in familial melanoma kindreds, continuing with the discovery of the BRAF V600E mutation, and more recently focusing on smaller subsets of patients such as those with nRAS and cKIT mutations, the explosion of molecular knowledge in the field of melanoma has led to new hope in patients with this aggressive tumor. While malignant melanoma has been the subject of the most active research, other forms of skin cancer are also the subject of ongoing, more recent studies. The discovery of the role of the Hh signaling pathway in BCC has opened the door to studies of targeted inhibitors of this molecule. Infectious agents have been shown to play a role in subsets of SCC and Merkel cell carcinoma, leading to new understanding of the underlying pathogenesis of these diseases. Molecular diagnostic testing for these four most prevalent forms of cutaneous malignancy will continue to play a major role in clinical decision making for prognostic and treatment purposes.

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Chapter 17
Molecular Diagnostics of Lymphoid Neoplasms

Deniz Peker, Jianguo Tao, and Ling Zhang

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Abstract According to 2008 World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissue, lymphoid neoplasms are divided into two, mature and immature (precursors), forms including mature B and T/NK cell leukemia/lymphomas and B and T-lymphoblastic leukemia/lymphomas (B-ALL/LBL, T-ALL/LBL). Nowadays a variety of molecular methods are introduced for the modern classification of lymphoid neoplasm system. Although morphological characteristics remain the cornerstone of the evaluation of lymphoid neoplasm, ancillary studies e.g. immunophenotyping and PCR study for T- and B-cell gene rearrangements are routinely implicated in daily service. Different from

D. Peker • J. Tao • L. Zhang, M.D. (✉)
Department of Pathology and Cell Biology, University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd, Tampa, FL 33612, USA

Department of Hematopathology and Laboratory Medicine, Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: ling.zhang@moffitt.org

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myeloid neoplasms, the application of molecular/genetic diagnosis and subclassification of lymphoid neoplasm are mainly limited in B-ALL. There are few known protooncogenes or cytogenetic abnormalities in the certain T/NK or B lymphoid malignancies. The chapter focuses on common molecular diagnostic approaches and molecules that implicated in therapeutic strategies, predicting prognosis and monitoring minimal residual disease.

Keywords Lymphoid malignancy • Cytogenetics • Molecular • Gene rearrangement • Diagnostic and prognostic implications • Novel molecular techniques

Abbreviations

ABC	Activated B cell-like type
ALK	Anaplastic large cell kinase
ALL	Acute lymphoblastic leukemias
ALTCL	Anaplastic large T cell lymphoma
ATM	Ataxia telangiectasia mutated gene
B-ALL	B-lymphoblastic leukemia/lymphoma
BL	Burkitt lymphoma
B-PLL	B-cell prolymphocytic leukemia
CDK6	Cyclin dependent kinase 6 gene
CLL/SLL	Chronic lymphocytic leukemia/small lymphocytic lymphoma
CNS	Central nervous system
CRA	Common region of amplification
DAPK1	Death-associated protein kinase 1
DLBCL	Diffuse large B-cell lymphoma
FFPE	Formalin-fixed and paraffin-embedded
FISH	Fluorescence in situ hybridization
FNA	Fine needle aspirate
GCB	Germinal center B-cell like
iAMP21	Intrachromosomal amplification of chromosome 21
LPL	Lymphoplasmacytic lymphoma
M-bcr	Major breakpoint cluster region
m-bcr	“minor” breakpoint cluster region
MF	Mycosis fungoides
MLPA	Multiplex ligation-dependent probe amplification
MRD	Minimal residual disease
NK	Natural killer
Non-GCB	Non-germinal center B-cell like
NPM	Nucleophosmin gene
PCR	Polymerase Chain Reaction
PTCL, NOS	Peripheral T cell lymphoma, not otherwise specified
RT-PCR	Reverse Polymerase Chain Reaction

SHM	Somatic hypermutation
SMZL	Splenic marginal zone lymphoma
SNP	Single Nucleotide Polymorphism Array
T-ALL	T-lymphoblastic leukemia/lymphoma
TCR	T cell gene rearrangement
TKI	Tyrosine kinase inhibitor
UDP	Uniparental disomy
WHO	World Health Organization

17.1 Introduction

Lymphoid neoplasms are clonal proliferation of mature or immature B cells, T cells and natural killer (NK) cells. According to 2008 World Health Organization (WHO) classification of hematopoietic and lymphoid neoplasms precursor T and B lymphoid neoplasms, mature B-cell neoplasms, mature T-cell and NK-cell neoplasms, Hodgkin lymphomas and immunodeficiency-associated lymphoproliferative disorders are defined in this group [1]. The classical diagnostic methods including histomorphology and immunophenotypic analysis have been widely used in diagnosis of lymphoid malignancies. In recent years, the advances in molecular techniques lead a better understanding of the role of genetic defects in the pathogenesis of neoplastic processes as a result of which molecular testing has become an important part of routine diagnostics including many leukemias and lymphomas. This chapter will address the key molecular tests used in diagnosis of lymphoid neoplasms as well as promising future diagnostic approaches.

17.2 Diagnostic Algorithms

A complete morphologic examination of involved tissues or body sites is the crucial first step of histopathologic evaluation of lymphoid neoplasms. A collection of substantial sample from fine needle aspirate (FNA), fine needle core biopsy or small resection biopsy is necessary in help of a diagnosis of lymphoid neoplasms. However, excisional biopsy of lymph node or extranodal tissue is recommended to more accurately assess the disease evolution or progression, to further subclassify lymphoid neoplasms as well as to identify composite neoplasms. A good quality of histology and staining technique prevents from misinterpretation, while a careful cytomorphologic examination of any single provided slide would avoid "tunnel-vision" or misdiagnosis.

Immunophenotyping refers to the technique of identifying the surface or cytoplasmic molecules or antigen that present in lymphoid cells. The three methods are commonly used for immunophenotyping including immunofluorescence, flow cytometry and immunohistochemistry. Using immunophenotyping strategy is able to

identify the majority of lymphoma/leukemias, so as to subclassify them. Nevertheless, it might generally not useful in distinguishing some low grade lymphomas from high grade ones, predicting clinical course or prognostic outcome. In addition to morphologic and immunophenotypic evaluation, molecular analysis of lymphoid neoplasms has become more important in classification of certain T/NK or B-cell lymphomas/leukemias with implication of therapeutic strategies, predicting disease prognosis and monitoring minimal residual disease (MRD). The most common use of molecular techniques in lymphoid malignancies can be divided into three categories: (1) detection of karyotypic abnormalities including gain or loss of chromosome(s), chromosomal translocations, deletions, and insertions some of which have shown to be recurrent in certain neoplasms; (2) detection of individual mutations associated with the malignancy; and (3) defining clonality in lymphomas and leukemias based on gene rearrangements on T and B cells. 2008 WHO classification and the most recent studies indicate that the molecular and genetic diagnostic tools seem more applicable as a diagnostic tool in precursor B-cell lymphoid neoplasms, particularly with regard to defined recurrent cytogenetic abnormalities [1–6].

B-lymphoblastic leukemia/lymphoma (B-ALL) commonly involves peripheral blood and bone marrow while T- lymphoblastic leukemia/lymphoma (T-ALL) is usually tissue-based, most common in the mediastinum. For the majority of cases, morphologic and phenotypic evaluation is often sufficient for diagnosis of ALL and making the distinction between B-ALL and T-ALL. However, further cytogenetic and/or molecular testing is required for further subclassification of B-ALL. Furthermore, some certain cytogenetic abnormalities are of importance in terms of prognosis and rational therapy. Mature lymphoid neoplasms often involve the lymphoid tissue as well as other body sites including visceral organs, skin and brain. Although for many lymphomas, morphologic and phenotypic assessment is usually sufficient to render a diagnosis and molecular testing might not be demanded, clonality studies has been widely used especially to differentiate a neoplasm from a reactive process. It is clear that molecular techniques facilitate the diagnosis of difficult cases [7–11]. Molecular studies also allow further categorization of some lymphomas regarding the prognosis and tracking subsequent minimal residual disease (MRD) [12].

Nowadays molecular and genetic diagnosis has been, without a doubt, integrated into daily pathologic practice and play an important role in diagnostic algorithm to differentiate lymphoma/leukemia from the other reactive processes (Fig. 17.1).

17.2.1 Molecular Methods Commonly Used in Lymphoid Malignancies

17.2.1.1 Standard Chromosomal Analysis

Conventional karyotyping is one of standard chromosomal analyses that have been widely used in diagnosis of myeloid leukemias as well as lymphoid neoplasms. Discovery of the recurrent cytogenetic abnormality in Burkitt lymphoma with t(8;14) and chronic myelogenous leukemia with t(9;22) in early 1960s. [13, 14], is

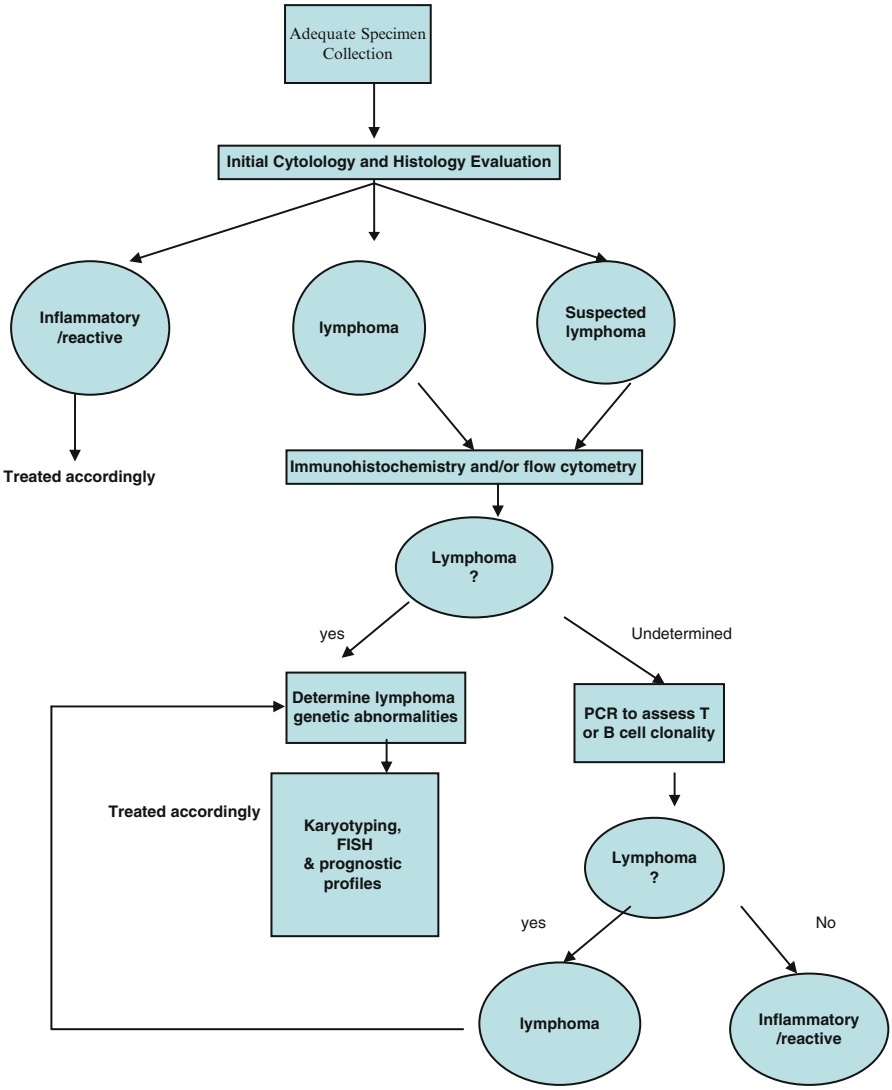

Fig. 17.1 The diagnostic algorithm for lymphoid malignancies commonly accepted in daily practice

followed by many chromosomal abnormalities described in lymphomas and leukemias. Up to date, certain precursor B lymphoid neoplasms, vast majority of the Burkitt lymphoma, mantle cell lymphoma, follicular lymphoma and a subset of marginal zone lymphomas are associated with recurrent translocations (Table 17.1) [1–3, 15, 16]. Diffuse large B cell lymphomas (DLBCL), can associate with single or multiple breakpoints and translocations, namely BCL6 at 3q and BCl-2 at 18q, "double or triple" hit lymphomas commonly involving *MYC* and *BCL2* and less

Table 17.1 The WHO classification of precursor lymphoid neoplasms

I. Precursor lymphoid neoplasms
B lymphoblastic leukemia/lymphoma, not otherwise specified (NOS)
B lymphoblastic leukemia/lymphoma, with recurrent genetic abnormalities
B lymphoblastic leukemia/lymphoma with t(9;22)(q34;q11.2); *BCR-ABL*
B lymphoblastic leukemia/lymphoma with t(v;11q23); *MLL* rearranged
B lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22); *TEL-AML-1 (ETV6-RUNX1)*
B lymphoblastic leukemia/lymphoma with hyperdiploidy
B lymphoblastic leukemia/lymphoma with hypodiploidy (Hypodiploid ALL)
B lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32); *IL3-IGH*
B lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); *E2A-PBX1(TCF3-PBX1)*
T lymphoblastic leukemia/lymphoma

frequently *BCL6* genes [17–20]. Nevertheless, only a minority of T cell lymphomas exhibit recurrent chromosomal translocations. Anaplastic large T cell lymphoma is a good example, often associated with t(2;5) involving *NPM1-ALK* gene fusion [21], though the other ALK partner genes also encountered.

Most cytogenetic abnormalities seen in lymphomas and leukemias are often detectable by conventional karyotyping by G-banding. Although it is considered to be the "gold standard" technique, some limitations prevent a wide use of this test, including the need to obtain a large volume of fresh tissue and rapid processing. Another problem encountered in cytogenetic testing is that the analysis requires high level of technical skills in culturing tissue, preparing the metaphase cells and analyzing complex karyograms which makes the method labor-intensive, expensive and time consuming [15].

17.2.1.2 Fluorescence In Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) is widely used as an ancillary method in diagnosis of lymphomas and leukemias. It is applicable to cytospin preparations from fresh harvested tissue as well as formalin-fixed and paraffin-embedded (FFPE) tissue without requiring tissue culturing. Thus, FISH is a less complex and rapid molecular technique with shorter turnaround time. The main limitation of FISH is the probe specific testing only for specific abnormalities with no genome-wide analysis.

17.2.1.3 Polymerase Chain Reaction (PCR), Reverse Transcription-PCR (RT-PCR)

PCR tests are widely used in lymphomas and lymphoblastic leukemias for clonality analysis of the immunoglobulin genes and T cell receptors (TCR). The testing can be performed on either fresh tissues or FFPE materials. DNA or RNA materials are extracted from submitted specimen according to the study design. Quantitative

RT-PCR is also very useful in detecting of the chromosomal translocations that lead to the production of fusion mRNA transcripts. A good example of this fusion transcript and protein generation is cyclin D1-IgH resulting in mantle cell lymphoma [22]. An advantage of PCR in lymphoma and leukemias is that it enables to amplify the fusion transcript; therefore it is superior in detection of minimal residual disease (MRD). PCR analysis for clonality is particularly advantageous in certain circumstances; (1) if the tissue is limited (e.g. small needle biopsies or fine needle aspirations) for immunohistochemical analysis and/or flow cytometry cannot be performed (e.g. FFPE tissue); (2) some B cell lymphoma cells may lack surface light chain antigens so that clonality may be equivalent by flow cytometry. (3) Phenotypic clonality evaluation has not been established for T cell neoplasms, therefore, gene rearrangement studies are helpful determining T cell clonality; (4) differentiation of reactive hematogone response vs. residual/recurrent precursor lymphoid neoplasms with increased immature lymphoblasts [23–26]. On the other hand, there are some limitations when using PCR for diagnostic purposes. False positive gene rearrangement results can be observed especially when testing a small biopsy with small number of lymphocytes as well as various reactive and inflammatory conditions [27, 28]. Furthermore, some T cell lymphomas may have false immunoglobulin (*IG)* gene re-arrangement [28]. False negative results can also be seen mostly due to technical or biologic factors [29].

17.2.1.4 Single Nucleotide Polymorphism (SNP) Array

SNP array is the most recently introduced molecular method that can analyze the whole genome with higher resolution than the other techniques. An important advantage of SNP array is the ability to detect not only copy number changes but also the regions with loss of heterozygosity without DNA loss or gain. This indicates that the both alleles are homozygous and derived from one parent, "uniparental disomy" (UDP), which is important in certain lymphomas [30–32]. Regions of UDP may harbor one or more genes with inactivating mutations (classical tumor suppressor genes) and since both alleles will have the mutation, the Knudson two-hit rule is fulfilled [15]. The main problem with SNP array is the difficulty in distinguishing acquired abnormalities from polymorphism which may be resolved by comparing to the germline DNA from the same patient [33]. SNP is so far not the replacement of cytogenetics and FISH laboratory, however, it is usually ordered for those patients with normal karyotyping or FISH results when seeking a possible disease related genetic findings.

17.3 Precursor Lymphoid Neoplasms

Precursor lymphoid neoplasms, also termed acute lymphoblastic leukemias (ALL), are one of the most common hematopoietic malignancies affecting pediatric population and a major cause of co-morbidity in adult. The current WHO classification

of ALL contains three main subgroups: (1) B-lymphoblastic leukemia/lymphoma (B-ALL), not otherwise specified, (2) B-ALL with recurrent cytogenetic abnormalities, and (3) T-lymphoblastic leukemia/lymphoma (T-ALL) [1] (Table 17.1). ALL is a greatly heterogeneous group of disorders with variety of associated genetic defects that have been defined recently or still unknown. The classical algorithm used in diagnosis of ALL includes a careful morphological examination of peripheral smear and/or bone marrow samples as well as involved tissue with appropriate phenotypic studies supported by relevant testing for karyotype analysis and molecular genetics. Cytogenetic abnormalities are seen in 60–80 % of ALL including recurrent genetic abnormalities.

17.3.1 B Lymphoblastic Leukemia/Lymphoma with Recurrent Genetic Abnormalities

This is a group of disease characterized by recurrent genetic abnormalities including chromosomal abnormalities and balanced translocations. The distinction of this B-ALL from other types is especially important as they have specific clinical and phenotypic features leading to important prognostic implications.

17.3.1.1 B Lymphoblastic Leukemia/Lymphoma with t(9;22)(q34;q11.2);BCR-ABL1

The Philadelphia (Ph) chromosome is the most common associated genetic anomaly in adult B-ALL, accounting for about 20–30 %. Only about 2–4 % of childhood leukemias harbor Ph chromosome. The lympho blasts have the translocation between the *BCR* gene on chromosome 22 and the *ABL* oncogene on chromosome 9 that creates the Ph chromosome. While the *ABL* breakpoint occurs in the same region (between exons a1 and a2) on chromosome 9, the *BCR* breakpoints on chromosome 22 can occur in two different regions. The m-bcr creates a fusion protein of 190 kDa (p190) and M-bcr results in a fusion protein of 210 kDa (p210) [34, 35]. The "minor" breakpoint cluster region (m-bcr) between exons 1 and 2 is present in vast majority of childhood Ph+ALL while a "major" breakpoint cluster region (M-bcr) spanning exons 12 to 16 is more common in adult Ph+ALL. The m-bcr creates a fusion protein of 190 kDa (p190) and M-bcr results in a fusion protein of 210 kDa (p210) [34, 35]. Phenotypically, Ph+B-ALL is typically CD10+, CD19+ and TdT+. The expression of myeloid antigens CD13 and CD33 is frequent. CD25 is highly associated with adult Ph+ALL [36].

Cytogenetics is still commonly used in detection of Ph chromosome. In cases of failed or normal cytogenetic results, FISH can be used as an alternative method. Quantitative RT-PCR is a rapid, accurate and sensitive method for the detection of *BCR/ABL* fusion transcripts. The advantage of test is to quantitatively track the copy number change of transcript before and after therapy. One major disadvantage is

that this approach is highly specific and cooperating abnormalities cannot be simultaneously identified [37]. The presence of t(9;22) is a poorer prognostic factor in both children and adults. Combination of tyrosine kinase inhibitor (TKI) with established antileukemic agents is promising in management of these patients.

17.3.1.2 B Lymphoblastic Leukemia/Lymphoma with t(v;11q23); MLL Rearranged

This is a group of acute leukemias harboring translocation between *MLL* gene at band 11q23 and any of the fusion partners. MLL translocations generate a new chimeric gene, in which the NH2-terminal portion of MLL is fused to the COOH-terminal sequence from multiple different partners [38]. The common result of many of these rearrangements is the expression of a DNA-binding protein that recruits additional histone methyltransferases such as DOT1L and leads to ectopic histone H3 lysine 79-dimethylation [39, 40].

These lymphomas exhibit unique clinical and biologic feature. *MLL* translocation occurs up to 70 % of infant B-ALL and less frequently in older patients. Typically patients present with very high white blood cell counts and often central nervous system (CNS) involvement at diagnosis. A characteristic feature of ALL with *MLL* translocations, mostly t(4;11) ALL, is the absence of CD10 in pro-B blasts often expressing myeloid antigen CD15. The expression of chondroitin sulfate proteo-glycan neural-glial antigen 2 (NG2) is also typical in these leukemias. Many fusion genes have been described in ALL with *MLL* translocation. t(4;11)(q21;q23)/*MLL-AFF1 (*previously known as *MLL-AF4*) comprise the largest group in ALL with *MLL*. Other common partner genes include *ENL* (19p13) and *AF9* (9p22). *FLT3* overexpression is also found in the presence of *MLL* translocations [41]. *MLL-AFF1* fusion is easily detectable by RT-PCR or cytogenetics. However, a more complex molecular analysis may be advantageous in detecting other common fusion partners. A dual color break-apart probe may provide detection of all chromosomal abnormalities involved in ALL with *MLL* translocations by FISH. Leukemias with *MLL* gene rearrangements usually have poor prognosis. The specific type B-ALL is also associated with adverse outcome, particularly when it occurred in infant <6 months.

17.3.1.3 B Lymphoblastic Leukemia/Lymphoma with t(12;21)(p13;q22); TEL-AML1 (ETV6-RUNX1)

This is the most common leukemia in childhood, accounting 25 % of all B-ALLs with a very favorable prognosis. It is not identified in infants and barely found in adults. It has similar clinical and phenotypic features to other types of ALL. CD13 myeloid antigen expression is common with no indication of mixed phenotype acute leukemia. t(12;21) results in the production of *TEL-AML1(ETV6-RUNX1)*, a fusion protein with important role in leukomogenesis [42]. The product protein

acts in a dominant negative fashion to interfere with normal function of the transcription factor *RUNX1*. It has been shown that the translocation occurs early in life, prenatally, as "preleukemic clone" but leukemia develops many years later [43]. There is evidence that secondary changes are needed for overt leukemia including frequent additional changes include losses of the *EBF1, ETV6* and *PAX5* genes and gains of chromosome 21 and chromosome arm Xq [44]. The fusion protein can be detected by RT-PCR. As t(12;21)(p13;q22) is invisible at cytogenetic level, FISH is usually used to detect the translocation applying an extra signal or dual color fusion probe. Furthermore, detecting deletion of second *ETV6* allele and additional fusion signals is also important in this type of B-ALL, possibly leading a longer survival [45]. FISH provides information on the status of second *ETV6* homologue.

17.3.1.4 B Lymphoblastic Leukemia/Lymphoma with Hyperdiploidy

Hyperdiploid B-ALL is characterized by blasts containing 51–65 chromosomes, in the absence of translocations or other structural alterations. This subtype accounts for 25 % of B-ALL. It is common in children and has a good prognosis with a survival rate up to 80 % at 5 years. Hyperdiploid B-ALL has no unique features to distinguish it from other subtypes. Hyperdiploid is characterized by gain of chromosomes, typically + X, +4, +6, +10, +14,+17, +18, and +21. The best method for the detection of chromosomal gain in hyperdiploid B-ALL is the standard karyotyping. Cytogenetics may fail in a portion of cases [46, 47]. FISH with centromeric probes specific for the trisomies of chromosomes 4, 10, 17, and 18 may be helpful to identify hidden hyperdiploidy [48, 49]. Identified trisomy 4, 10, and 17 link to a good prognosis. Flow cytometry to measure DNA index analysis can also be used.

17.3.1.5 B Lymphoblastic Leukemia/Lymphoma with Hypodiploidy (Hypodiploid ALL)

Hypodiploid B-ALL is characterized by blasts containing < 44–45 chromosomes including near-haploid ones (23–29 chromosomes). It is seen in both children and adults, although near-haploid ones are more common in children. This subgroup is associated with a worse prognosis. The overall clinical, morphologic and phenotypic features are not distinguishable from other B-ALL. By definition, there is loss of from single or more chromosomes to near haploid with or without structural abnormalities in the remaining chromosomes. The structural anomalies including translocations are more common in the cases with chromosome number between 30 and 40. Detection of hypodiploidy in this group could be problematic. One characteristic feature is the gain of some specific chromosomes onto the haploid chromosomes and the presence of a population of cells with an exact doubling of this chromosome number [50]. This may result in a near diploid or even hyperdiploid

karyotype. In such cases, standard karyotyping may dismiss the hypodiploid line. Flow cytometry can be used to detect hypodiploidy in these cases with the advantage of more rapid processing. Flow cytometry is also not affected by mitotic index of the cell population. However, flow cytometry does not identify the specific chromosomes with numeric changes, nor does show the additional structural anomalies which may have potential clinical and biologic importance [51]. Therefore, it is useful to use both techniques in tandem. FISH may also detect certain chromosomes with hypodiploidy; however, FISH detection may be difficult when near-haploid or doubled populations present.

In summary, hypodiploidy and near-haploidy in B-ALL carries a prognostic significance and molecular testing should be carefully applied in detecting chromosomal loss when this subgroup is clinically suspected.

17.3.1.6 B Lymphoblastic Leukemia/Lymphoma with t(5;14)(q31;q32);IL3-IGH

This subgroup is very rare and accounts for < 1 % of all ALL. It has been reported in all age groups. The eosinophilia is reactive and not part of the neoplastic process. The features and prognosis are otherwise similar to other B-ALL. The blast count has not shown to be a prognostic factor in this type of ALL. Patients typically have variable circulating eosinophilia due to overexpression of the *IL3* gene following the functional rearrangement between *IL3* gene and *IGH@* gene. The translocation may be detected using standard karyotyping, RT-PCR and/or less commonly FISH.

17.3.1.7 B Lymphoblastic Leukemia/Lymphoma with t(1;19)(q23;p13.3);E2A-PBX1 (TCF3-PBX1)

ALL with t(1;19) is fairly common in children, accounting for 6 % of all ALL. Clinical features are similar to those of other ALL patients. Although they used to be classified in the poor prognosis group, with therapeutic advances, they are regarded as standard risk [52, 53]. Although they do not differ morphologically from other types, blasts in this group are pre-B type and typically express CD19, CD10 and cytoplasmic μ (cμ) with less or no CD34. Strong CD9 expression is also characteristic. *TCF3* gene encodes two transcriptional proteins E12 and E47 which play a critical role in B-cell maturation while *PBX1* is a homeobox gene not expressed in B or T-cells [54]. Translocation between *TCF3* (previously *E2A*) gene, on chromosome 19, and *PBX1* gene, on chromosome 1, results in production of a fusion protein that has an oncogenic role. Recently, a variant subtype harboring translocation between *TCF3* and *HLF* genes related to a worse prognosis. Cytogenetics and FISH as well as RT-PCR are useful to detect the associated translocations in majority of the cases. The diagnosis of this subtype warrants the careful evaluation by both phenotypic features and translocation status, as an identical t(1;19) can be seen in a portion of B-ALL not necessarily associated with *TCF3-PBX1* translocation.

17.3.1.8 Other Cytogenetic Abnormalities Detected in B-ALL

Up to 50 % of all B-ALL show abnormalities involving genes associated with B cell development including *PAX5, TCF3,EBF1, LEF1, IKZF1* and *IKZF3* and cell cycle controlling genes such as *CDKN2A, CDKN1B* and *RB1* [55–59]. A cryptic translocation involving sex chromosomes, t(X;14)(p22;q32) or t(Y;14)(p11;q32), involving *IGH@* and *CRLF2* genes, and deletion within *PAR1,* resulting in *P2RY8-CRLF2* fusion has been reported [60–62]; possibly activating JAK-STAT signaling pathway and associated with a worse prognosis [63–65]. Intrachromosomal amplification of chromosome 21 (iAMP21) is a distinct chromosomal abnormality in B-ALL and associated with adverse prognosis and poor response to therapy [66]. Despite the variability between patients in the morphology of the abnormal chromosome 21, they consistently show multiple, extra copies of the *RUNX1 (AML1)* gene, tandemly repeated along the length of the abnormal chromosome [67]. Genomic and expression analysis has further characterized this abnormality, demonstrating a common region of amplification (CRA) between 33.192 and 39.796 Mb on chromosome 21 (which includes *RUNX1*) [68]. FISH can also be used to detect this abnormality.

17.3.1.9 B Lymphoblastic Leukemia/Lymphoma, NOS

Diagnosis of B-lymphoblastic leukemia/lymphoma (B-ALL), NOS is based on clinical, morphological and phenotypic findings when the aforementioned cytogenetic abnormalities are not indentified. Gene re-arrangement by PCR is a commonly used as a supportive diagnostic tool and also is also helpful in determining the minimal residual disease in some settings. Studies have showed that nearly all cases have clonal DJ rearrangements of the *IGH@* gene along with T cell gene rearrangement (TCR) in a majority of the cases (up to 70 %) [30, 69]. The B cell clonality in B-ALL can be detected by PCR amplification of the IG and/or TCR VDJ region. The secondary change of TCR gene in B-ALL is considered reactive with uncertainty in pathophysiology, being worthy of further investigation. SNP might be an alternative way to detect any underlying genetic aberration if it is applicable in clinical laboratory.

17.3.2 T Lymphoblastic Leukemia/Lymphoma

T lymphoblastic leukemia/lymphoma is a neoplasm of precursor T cells which are typically medium to large sized blasts with dispersed chromatin, inconspicuous nucleoli and scant cytoplasm. It involves bone marrow and blood (T-ALL) or lymphoid organs or extranodal sites (T-LBL). Both forms are more common in children than adults. The blasts usually express TdT with often T cell antigens CD2, cytoplasmic CD3, CD4, CD5, CD7, and CD8 and CD1a. Aberrant expression of myeloid

markers (CD13 and CD33) can be seen in up to 30 % of the cases [70]. Coexpression of CD117 occurs less frequently but often associated with *FLT3* mutation [71]. Multiple genetic aberrations encounter in T-ALL, but none of them is T-ALL specific. Translocations involving *TCR* loci are the most common cytogenetic anomalies observed in T-ALL/LBL. t(7;10)(q34;q24) and t(10;14)(q24;q11) are most common in adults involving *HOX11(TLX1)* gene whereas t(5;14)(q35;q32) involving *HOX11L2 (TLX3)* gene is commonly found in children [6]. As reported, dysregulation of HOX-type transcription factors is found in 30–40 % of cases of T-ALL [72]. Characteristically, *HOX*-type T-ALLs often accompany with activating *NOTCH*1 mutations and *CDKN2A* loss of function, *NUP214-ABL* amplifications, *C-MYB* tandem duplications, *PHF6* mutations, *PTPN2* deletions, and *WT1* mutations [6, 72–78], which are not present in non-HOX-type T-ALL. However, the role of these genetic changes in leukemogenesis is not well understood. The unique genetic features are not only helpful in view of diagnosis but also guide future clinical therapy. An in vitro study has demonstrated that a blockade of both TLX1/HOX and NOTCH could inhibit cell cycle progression in TLX1 T-ALL cell line 9,490 cells (Lesley A. 2011). Mutations that may occur in T-ALL/LBL are important. Mutation of *NOTCH* oncogene is the most common mutation and is seen about 60 % of cases. NOTCH proteins are transmembrane receptors and are important regulators of T cell differentiation and activation. Recent studies showed that *MYC* is upregulated by activated *NOTCH1* in T-ALL which stimulates the growth of T-ALL cells [46, 79]. *N-RAS* and *FLT3* are other mutation seen in T-ALL [80, 81]. Another common rearrangement is the formation of fusion genes, mostly in children. *SIL/TAL1* fusion gene results from a cryptic deletion of chromosome 1 and is seen in up to 30 % of childhood T-ALL. Important translocations resulting in fusion gene formation includes t(10;11) involving *CALM/AF10* genes in 10 % of all cases and less frequently t(11;19) involving *MLL* gene. Other *MLL* gene translocations {t(6;11), t(10;11), t(X;11) and t(4;11)} seen in some T-ALL cases [6]. Philadelphia chromosome has also been reported in rare cases [82]. Deletions may be detected in as many as 30 % cases. 9p21 involving *p16* gene and del(6q) are the important ones. Almost all cases show clonality in T cell receptors with up to 20 % accompanying *IGH@* gene rearrangement [83].

17.4 Mature Lymphoid Neoplasms

Lymphoid neoplasms are clonal tumors with malignant transformation of normal lymphoid cells at various stages of differentiation and comprise the sixth most common group of malignancies worldwide in men and women. Although certain infectious agents, autoimmune disorders and immunosuppression-related conditions have been linked to some lymphomas, the etiology in the majority of lymphomas remains unclear. Morphology and immunophenotype are generally sufficient to make the diagnosis of most lymphomas. Genetic features have begun to play an important role in classification and diagnosis of mature lymphomas. The recent advances in

molecular genetics have enlightened lymphoma biology and provide useful testing that can be integrated with the conventional diagnostic methods widely used in hematopathology. Although isolated use of molecular tests is not of any help in lymphoma diagnosis, the use of these tests has clear utility to facilitate the diagnosis, in particularly morphologically and phenotypically challenging cases. Also some molecular markers have prognostic implications in certain lymphomas. Mature B and T/NK cell lymphomas with recurrent cytogenetic abnormalities are discussed below.

17.4.1 Mature B Cell Neoplasms

Mature B cell lymphomas comprise over 90 % of lymphoid neoplasms worldwide. They are common in Western world and account for 4 % of new cancers each year [1]. The most common types are diffuse large B cell lymphoma and follicular lymphoma. The majority of the B cell lymphomas recapitulate the stages of normal B cell proliferation, except for some types, e.g. hairy cell leukemia, that do not correspond to any maturation stage (Table 17.2). Diagnosis of B cell lymphomas, like other mature lymphoid neoplasms, is usually based on histomorphologic and phenotypic features in conjunction with genetic and molecular profiles.

17.4.1.1 Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a neoplasm of monomorphic small B cells with a fairly unique phenotype. CLL is the most common leukemia in the Western world affecting mainly the elderly population. The neoplastic cells often coexpress CD5 and CD23 along with characteristically dim CD20, CD79b and dim surface immunoglobulin expression. CLL is the term used when long standing lymphocytosis ($\geq 5 \times 10^9$/L monoclonal lymphocytes for at least 3 months) is present in peripheral blood (PB) with or without bone marrow (BM) involvement [1]. The term SLL refers to the less frequent form with same phenotype to CLL but non-leukemic with the tissue morphology. The diagnosis of SLL requires lymphadenopathy, no cytopenias due to BM involvement by CLL/SLL and $<5 \times 10^9$/L peripheral blood B cells [1].

CLL is a heterogenous disease with variable clinical outcome in the light of variety of genetic abnormalities. Importantly, CLL has the highest familial genetic predisposition in all hematologic malignancies. A polymorphism in the death-associated protein kinase 1 (DAPK1) gene was associated with familial CLL in a single family, but this allele has not been found again in a larger number of cases [84]. The involved genes in genetically predisposed CLL are still yet to be defined. Approximately 80 % of cases have cytogenetic abnormalities. Although some of these abnormalities can be detected in about 40 % of cases by standard karyotyping, FISH is essential in evaluation [85]. The FISH CLL panel including probe CCND1 study is used to exclude mantle cell lymphoma and predict the prognosis of

Table 17.2 The WHO classification of mature B cell neoplasms

II. Mature B cell neoplasms
Chronic lymphocytic leukemia/small lymphocytic lymphoma
B-cell prolymphocytic leukemia
Splenic marginal zone lymphoma
Hairy cell leukemia
Splenic B-cell lymphoma/leukemia, unclassifiable
Splenic diffuse red pulp small B-cell lymphoma
Hairy cell leukemia-variant
Lymphoplasmacytic lymphoma
Heavy chain disease
Gamma heavy chain disease
Mμ heavy chain disease
Alpha heavy chain disease
Plasma cell neoplasms
Monoclonal gammopathy of undetermined significance (MGUS)
Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Monoclonal immunoglobulin deposition diseases
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone lymphoma
Follicular lymphoma
Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
Diffuse large B-cell lymphoma (DLBCL), NOS
T cell/histiocyte-rich large B-cell lymphoma
Primary DLBCL of the CNS
Primary cutaneous DLBCL, leg type
EBV positive DLBCL of the elderly
DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B-cell lymphoma
Intravascular large B-cell lymphoma
ALK positive large B-cell lymphoma
Plasmablastic lymphoma
Large B-cell lymphoma arising in HHV8-associated multicentric Castleman disease
Primary effusion lymphoma
Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma
B-cell lymphoma, unclassifiable, with features intermediate between DLBCL and Hodgkin lymphoma

CLL. The most common recurrent abnormality seen in CLL is deletion of 13q14, approximately 55 % of cases, which is also associated with the best prognosis [86]. Twenty percentage of cases have deletion of 11q22-23which corresponds to loss of *ataxia* telangiectasia *mutated (ATM) gene*. This abnormality is associated

with bulky lymphadenopathy and poor clinical outcome [87]. Deletion of 17p13 is seen in ~7 % of cases majority of which is related to loss or mutation of *TP53* tumor suppressor gene. Deletion 17p13 is often leads a poor prognosis with chemotherapy, particularly Fludarabine resistance in these patients [88–90]. *TP53* mutation with or without del(17p13) is associated with a worse prognosis [91, 92] CLL with trisomy 12 often has an atypical morphology and bright aberrant expression of CD11c on the neoplastic B-cells. FISH is sufficient in detecting the chromosomal anomalies but not gene mutations A novel molecular technique, Multiplex Ligation-dependent Probe Amplification (MLPA), can be used to complement FISH [16].

Status of immunoglobulin variable domain genes, Ig VH gene, in remains the "gold standard" prognostic feature in CLL patients. Presence of *IGH* variable domain gene somatic hypermutation (SHM) is seen 50–60 % of cases; whereas unmutated cases comprise 40–50 % with a worse prognosis [93]. Despite the prognostic importance, the test cannot be performed widely due to its complexity. In this case, the mutation status can be analyzed through a surrogate marker, such as ZAP-70 expression using flow cytometry or infrequently immunohistochemistry. The expression of ZAP-70 corresponds to the absence of *IGH* SHM in the vast majority of the cases [94, 95]. Recent studies also demonstrated *NOTCH1* activating mutations associated with worse prognosis in CLL [96].

17.4.1.2 B Cell Prolymphocytic Leukemia

B cell prolymphocytic leukemia (B-PLL) is the neoplasm of prolymphocytes involving peripheral blood, bone marrow and often spleen. Diagnosis requires prolymphocytes exceeding 55 % of the lymphocytes in the blood. It can be transformed from CLL. The typical morphology of prolymphocytes includes medium size with round nuclei and a prominent central nucleolus. Neoplastic cells strongly express surface immunoglobin, along with B cell antigens (CD19 and CD20). B-cells show often loss of CD23. B-cells with CD5 and CD23 co-expression are seen about 20 % of cases. Variable FMC-7 expression is noted. ZAP-70 and CD38 are expressed in half of the cases with no correspondence to *IGH* mutation status [97].

Immunoglobulin genes are clonally rearranged in half of the cases. Deletion of 17p and related *TP53* mutation has been demonstrated in 50 % of cases associated with poor prognosis and therapy resistant disease [98, 99]. Some case may also have 13q14 by FISH [97].

Initially, t(11;14)(q13;q32) had been demonstrated in "B-PLL"; however these cases are now considered leukemic forms of mantle cell lymphoma [100].

17.4.1.3 Splenic Marginal Zone Lymphoma

According to the WHO classification, splenic marginal zone lymphoma (SMZL) is neoplasm of small B lymphocytes which expansion of splenic white pulp, efface the

follicle mantle zone and emerge with marginal zone of larger cells; both small and large cells invade the red pulp [1]. Splenic hilar lymph nodes and bone marrow are usually involved as well as peripheral blood by circulating lymphoma cells with villous projections, usually with polar orientation. Lymphoma cells express surface IgM and often IgD along with B cell markers. Annexin A1 and cyclin D1 are negative with rare CD103 expression. Immunoglobulin heavy and light chains are often re-arranged with somatic hypermutation (SHM). Allelic loss of chromosome 7q31-32 has been reported in up to half of the cases [101]. Overexpression of cyclin dependent kinase 6 (*CDK6)* genes through chromosome 7q21 has been reported in few cases with possible role in lymphomagenesis [102]. *BCL2* and t(14;18) are absent. The presence of 7q deletion and unmutated IgVH in SMZL are possibly associated with adverse clinical outcome [103, 104].

17.4.1.4 Lymphoplasmacytic Lymphoma

Lymphoplasmacytic lymphoma (LPL) is characterized by clonal small B cells, plasmacytoid and plasma cells involving bone marrow and less frequently nodes and spleen. The neoplastic cells are typically IgM-secreting and clinically associated with hyperviscosity, autoimmune disorder, and cryoglobulinerimia. Phenotypically, LPL express both B-cell and plasma cell (subset) markers. *Immunoglobulin* genes are usually clonally arranged with hypermutation. No specific recurrent cytogenetic abnormality has been described in LPL. Deletion of chromosome 6q may be seen but not consistently and minority of patients showed trisomy 3 or 18 [105, 106]. Recently, Treson et al. reported that MYD88 L265P somatic mutation is detected in LPL/Waldenström's macroglobulinemia, which can be useful in distinguishing LPL from the other B-cell lymphoma with similar morphologic and immunophenotypic features [107]

17.4.1.5 Plasma Cell Myeloma

Plasma cell myeloma is the neoplasm of mature and clonal plasma cells involving bone marrow with widespread symptoms and findings from asymptomatic to prominent end-organ damage. Clinical, histomorphologic and phenotypic findings are often sufficient for a definitive diagnosis. Genetic evaluation is particularly important as for the prognosis and therapy effectiveness. Immunoglobin heavy and light chains are clonally arranged in myeloma patients. Plasma cell myeloma arises from post-germinal center B cells resulting in *IGH* SHM.

Standard karyotyping may reveal cytogenetic abnormalities in about one third of myelomas [108, 109]. The main difficulty in karyotype analysis of myeloma is the low-yield metaphases generated from myeloma cells due to low proliferation rate. Therefore; FISH is more commonly used in detecting myeloma related cytogenetics and reveals more than 90 % of the abnormalities [110–112]. When able to analyze sufficient sample, the karyotype in these patients is usually complex, mostly

hyperdiploidy. Hyperdiploidy has been defined in 40–65 % of myelomas [109, 113]. These gains in the chromosomes are random and often involve chromosomes with odd numbers including 3, 5, 7, 9, 11, 15, 19 and 21 [114]. Other less common type of karyotype abnormality includes structural re-arrangements in the absence of hyperdiploidy. Most of these cases include specific rearrangements involving the *IGH* gene located at 14q32 and comprise 55–70 % of all cases [115–118]. These rearrangements are typical reciprocal translocations with various chromosomal partners leading to upregulation of the target genes. The most frequent 14q32 partners are 11q13 (involving *CCND1 (CyclinD1)*; 20 % of patients) [119, 120], 4p16 (involving both *FGFR3* and *MMSET*; 15 % of patients) [121, 122], as well as 16q23 and 20q11 (targeting *MAF* genes; 2–5 % of patients each) [123–125]. Both *IGH@* translocations and hyperdiploidy appear to be early events in the oncogenesis in plasma cell neoplasms, unified by upregulation of various *cyclin D* genes (D1, D2, and/or D3) [126, 127].

Other chromosomal changes seen in myeloma include monosomy 13 which may be seen in about half of the cases considered an early event in the pathogenesis [110, 117, 128]. Additional two secondary events are also recurrent: gains of the long arm of chromosome 1 (1q21), in one third of patients, and del 17p, observed in approximately 10 % of patients which are related to disease progression from MGUS to myeloma as well as disease relapse [129, 130].

Activating mutations of *KRAS*- or *N-RAS* may be present in up to 40 % of cases and are likely associated with disease progression [117, 131]. Translocations involving *MYC* gene and activation of NF-kappa B pathway have also been shown in myeloma patients [132, 133]. Chapman et al. recently showed 4 % *BRAF* mutation in myeloma patients, a novel discovery in myeloma suggesting therapeutic implication [134]. Recent studies also emphasized the significance of Phosphatidylinositide 3-kinase (PI3K)/protein kinase B (PKB) pathway, also known as the Akt pathway, in myeloma [135]. The PKB/Akt kinase family includes 3 isoforms (PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3) that are encoded by separate genes and that are involved in the regulation of apoptosis, proliferation, motility, and energy metabolism. In many tumors including myeloma, activation of Akt is usually indicative of poor prognosis [136].

17.4.1.6 Extranodal Marginal Zone Lymphoma of Mucosa-Associated Lymphoid Tissue (MALT Lymphoma)

This is an often indolent extranodal lymphoma composed of heterogenous small B cells associated with various precursor lesions described, e.g. Helicobacter pylori gastritis and autoimmune diseases. Neoplastic lymphoid cells infiltrate the epithelium causing destruction of normal architecture and are characterized as "lymphoepithelial lesion". There is several recurrent cytogenetic changes described in MALT lymphoma. The t(11;18)(q21;q21) resulting in fusion of *API2* and *MALT1* genes is detected in approximately one third of cases by standard karyotyping. RT-PCR or FISH studies are helpful in detecting this defect and can be performed

on either fresh cells or PEFF tissue [46]. Importantly, the cases with t(11;18) are resistant to therapy targeting *H. pylori* [137]. Other described translocations associated with MALT lymphoma include t(1;14)(p22;q32), t(14;18)(q32;q21), (3;14)(p14.1;q32) and t(1;14) (p22;q32), showing fusion of the N-terminus of the API2 gene to the C-terminus of the MALT1 gene or trancriptional gene deregulation in BCL10, MALT1 or FOXP1 [138, 139, and 140]. t(1;14) (p22;q32), involving *BCL10* gene is usually seen in MALT lymphoma of lung and stomach [139, 140]. Immunohistochemical evaluation of BCL10 protein nuclear staining can be correlated with the presence of translocation [141]. t(14;18)(q32;q21), resulting fusion of IGH/MALT1, is associated with transcriptional dysregulation of *MALT1* and accumulation of cytoplasmic BCL10 [138]. This translocation is most common in parotid, liver and ocular MALT lymphoma [139, 140]. The translocation by karyotyping seems indistinguishable from that seen in follicular lymphoma and an attention should be paid when diagnosing MALT lymphoma. MALT lymphoma arising in thyroid, ocular adnexa and skin can be associated with t(3;14) (p14.1;q32) involving *FOXP1* and *IGH@* [140, 142]. Trisomy 3, 18, and less commonly other chromosomes may be seen less frequently in MALT lymphomas. *BCL6* translocation can also rarely be seen resulting in fusion to the *IGH@* gene [143].

17.4.1.7 Follicular Lymphoma

Follicular lymphoma (FL) is a clonal neoplasm of germinal center B cells including both small centrocytes and large centroblasts with a variable morphology and grading at the diagnosis. Approximately 90 % of the cases harbor the characteristic translocation, t(14;18), involving *BCL2* and *IGH@* genes. That can be detected by cytogenetic analysis or FISH as a more sensitive tool. *BCL2* overexpression results in decreased apoptosis in the lymphoma cells. Importantly, a subportion of cases, mostly high grade FL, may lack *BCL2* rearrangement [144]. A special attention needs to be paid in the situation in order to avoid a misinterpretation of "reactive germinal center cells". Morphologic features by devoiding of "tangible body macrophage", loss of normal polarity and significantly increased centroblasts are diagnostic clues when lack of the specific gene/protein expression. Furthermore, some FL, usually seen in elderly population, may lack *BCL2* but harbor *BCL6* re-arrangement. These cases have a different phenotype than typical FL; CD10-, BCL2- and BCL6+ as well as MUM1+ [145, 146]. The *BCL2* re-arrangement status may vary depending on the localization of primary FL. For instance, primary cutaneous FL is frequently BCL2 negative and often lack IgH-BCL2 fusion product/t(14;18) when analyzed by FISH [147]. Pediatric FL also lacks BCL2 and the translocation.

Other common cytogenetic abnormalities seen in 90 % of FL include 1p-, 6q-, 17p-, +7, +8, +12 and + X [148, 149]. Deletions of 6q25-q27 and 9p21 are associated with poor prognosis [150].

Transformation to a more aggressive lymphoma from FL has been described. Translocations resulting in inactivation of *TP53* and *p16* and activation of *MYC* are

associated with transformation to a higher grade disease [151–154]. Per literature, the majority of FL transforms into diffuse large B-cell lymphoma (DLBCL). Transformation to Burkitt-type lymphomas or precursor B-cell lymphoblastic lymphoma is also reported in a small subset of FL [155, 156].

17.4.1.8 Mantle Cell lymphoma

Mantle cell lymphoma (MCL) is a neoplasm of small to medium sized B cells with typical CD5 co-expression and *(CCND1)* translocation. Translocation between *CCND1* on chromosome 11 and *IGH@* on chromosome 14 leading to overexpression of *CCND1* is characteristic and seen in almost all cases. *CCND1* gene encodes a cell cycle protein, dysregulation of which by *IGH@* gene enhancer region results in possibly overcoming the cell cycle suppressors leading to MCL development [157, 158]. FISH analysis of *CCND1* is the preferred and commonly used method in diagnosis of MCL. Rare cases of MCL do not overexpress cyclin D, in which a higher expression of cyclin D2 and cyclin D3 is present and these cases have identical clinical and morphologic features and same genomic profile [159]. A subset of these cases harbor t(2;12)(p12;p13) or t(12;14)(p13;q32) fusing cyclin D2 to kappa light chain (*IGK@) or* IGH@, respectively. RT-PCR is superior in assessing cyclin D2 translocations. Several other recurrent genetic abnormalities have been demonstrated in MCL, including losses of 9p21.3, 11q22-q23, and 22q11.22, and gains of 10p11.23 and 13q31.3 [160, 161]. Specific mutations and deletions in *TP53 (*17p13), *p16 (CDKN2A), ATM,* and *CHEK2* have also been frequently noted in MCL [162, 163]. The cases with *TP53* are rather in blastoid morphology compared to conventional MCL and associated with poor prognosis. Deletion of 13q14 and trisomy 12 also can be seen in MCL [164]. MCL is often encountered as an aggressive lymphoma by nature. Recent studies demonstrated an indolent variant sharing a common genomic profile with conventional MCL. *SOX11* gene, which has a role in cell differentiation, is found to be associated with indolent variant MCL [165, 166]. Immunohistochemical testing is available to evaluate *SOX11* protein.

17.4.1.9 Diffuse Large B Cell Lymphoma

Diffuse large B cell lymphoma, not otherwise specified (DLBCL, NOS) is a very heterogenous neoplasm of large B cells with variable localization, morphology and clinical behavior. DLBCL, NOS accounts for 25–30 % of adult non-Hodgkin lymphomas and common in elderly population. It can arise as the primary or as transformation or progression from a lower grade lymphoma and clinically presents a rapidly enlarging mass. A subset of cases may be associated with EBV infection in the absence of immunodeficiency. DLBCL may arise in the nodal or extranodal sites, with gastrointestinal tract being the most common extranodal site. Common morphological variants include centroblastic, immunoblastic, and anaplastic as well as rare variants, e.g. with spindle morphology [1]. By immunohistochemical features,

DLBCL, NOS is divided into three groups; (1) CD5-positive DLBCL; (2) germinal center B-cell-like (GCB) and (3) non-germinal center B-cell-like (non-GCB). Molecular subgroups include germinal center B-cell-like (GCB) type and activated B cell-like (ABC) type [1].

Certainly, karyotyping and/or FISH study is useful in molecular subgrouping of DLBCL, differentiating between DLBCL and Burkitt lymphoma or hybrized variants. For example, detection of t(14;18) or BCL-2 gene rearrangement in DLBCL suggests follicular center origin (GCB type) while ABC type DLBCL often acquires gains of 3q, 18q21-q22 and losses of 6q21-q22 [167–169].

However, beyond aforementioned functions, cytogenetics study also helps to identify a subset of them with more aggressive clinical features. Abnormalities of *BCL6* gene on chromosome 3q27 is the most common cytogenetic change in DLBCL and is seen in approximately 40–45 % of cases [170]. *BCL6* is a transcription factor required for germinal center formation in B cells. Translocation of *BCL6* occurs in approximately 30 % while 10 % of cases have BCL6 targeted by somatic hypermutation [171–173]. *BCL6* related translocations involve multiple partner genes and prognosis is usually worse in non-*IG/BCL6* gene fusion [174, 175]. Translocations of *BCL2* and *BCL10* genes are the other common changes, seen in 30 % and 15 % of DLBCL, respectively [171, 176]. Translocation involving *MYC* gene also occurs up to 15 % of cases [171, 177, 178]. It has been shown that *MYC* translocation can be seen in *de novo* DLBCL, transformed DLBCL from an underlying low grade lymphoma or in the recently characterized B cell lymphoma unclassifiable, with features intermediate between diffuse large B cell lymphoma and Burkitt lymphoma (formerly atypical Burkitt lymphoma or Burkitt-like lymphoma) associated with poor prognosis [171, 179–181]. FISH testing is a valuable tool in detecting *MYC* amplification/translocation in large B cell lymphomas. Recent studies have been focusing on the implication of measuring Myc protein by immunohistochemical methods. Although some authors suggest that Myc protein may predict the *MYC* gene translocation status [182], it is still unclear if Myc protein is enough to evaluate the gene status requiring further studies. *MYC* translocation has also been found in HIV associated diffuse large B cell lymphomas with plasmablastic features [183]. Recently, "double hit" and "triple hit" lymphomas have been introduced to define DLBCL with often complex cytogenetics involving combinations of *MYC* gene rearrangement with *BCL2 and/or BCL6* gene detected by FISH [17–20]. These are considered high grade or aggressive lymphomas often associated with high proliferation fraction. They require intensive chemotherapy.

17.4.1.10 Other Large B Cell Lymphomas

Primary mediastinal (thymic) large B cell lymphoma (PMBL) is a subgroup of DLBCL that is usually seen in younger age females and associated with a favorable prognosis. Since the lymphoma is putative thymic B-cell origin, it differs from the other *de novo* DLBCL by its own pathohistologic and genetic features.

The genes involved in PMBL include *MAL, FIG 1, PDL1, PDL2, JAK2,* and *BCL11A* [184–187] but rarely BCL2, BCL-6, and c-MYC gene rearrangements are detected. Interestingly, PMBL overlaps with classical Hodgkin lymphoma (CHL) by morphology and sometimes it is difficult to distinguish between these two lymphomas. Recent studies investigated the gene profile of PMBL and CHL and found out PMBL showed the molecular gene-expression signature reminiscent of nodular sclerosis subtype of CHL; both lymphomas were shown to harbor 2p *(REL)* and 9p *(JAK-2)* amplification [188]. ALK-positive DLBCL is another rare subgroup of DLBCL. In contrast to its T cell counterpart ALK-positive anaplastic large T cell lymphoma which characteristically harbors t(2;5) with fusion between *ALK* and often *NMP1*, ALK-positive DLBCL shows fusion between *ALK* gene and *CLTC* (Clathrin) gene associated with t(2;17)(p23:q23) [189]. There are less than 1 % of ALK-positive anaplastic large T cell lymphoma having the same translocation [1]. As known, some cell markers (CD4, CD43 and MUM1) can also be aberrantly expressed in ALK-positive DLBCL, which could result in diagnostic challenging. Since karyotyping might not be requested on the submitted tissue sample, FISH positive for ALK gene rearrangement by using ALK break-apart probe could mislead to a diagnosis of anaplastic large T-cell lymphoma.

17.4.1.11 Burkitt Lymphoma

Burkitt lymphoma (BL) is a very high grade B cell lymphoma characteristically associated with t(8;14)(q24;q32) involving *MYC* gene. *MYC* translocation located at 8q24 region often involves immunoglobulin heavy chain at 14q32 region. Less frequently translocations between *MYC* and immunoglobulin light chains can be observed; at 22q11 region for kappa, t(8;22) or 2p12 region for lambda, t(2;8). FISH is the most valuable test to detect *MYC* gene translocations, however a small portion of cases, up to 10 %, may be negative by FISH [190, 191]. As a result of this finding, the recent studies have focused on alternative mechanisms resulting *MYC* deregulation. E2F1 is a transcription factor loss of which impairs *MYC*-mediated proliferation and lymphomagenesis [192]. Another mechanism that has been recently investigated is miRNA-related dysregulation of *MYC* without translocation [193]. Leucci et al. showed that down-regulation of hsa-miR-34b is associated with *MYC* negativity in BL [193]. Clinically, it is critical to distinguish between Burkitt's lymphoma and diffuse large-B-cell lymphoma in MYC negative cases by FISH since these two lymphomas require different treatments. In this setting, miRNA expression profiling appears to be promising diagnostic tool in highly suspected but *MYC* translocation negative BL cases.

A subset of Burkitt lymphoma demonstrates more than one gene aberration, also called complex abnormalities besides *MYC* [1]. Other genetic abnormalities involving Burkitt lymphoma include *p16*^{INK4a}*, TP53, p53, BAX, p130/RB2* and *BCL6* and chromosome 1q abnormalities [46], Despite the additional genetic changes do not alter the original diagnosis as Burkitt lymphoma when the other diagnostic criteria meet, the clinical significance of these additional cytogenetic abnormalities is still

Table 17.3 The WHO classification of mature T/NK cell neoplasms

Mature T- and NK-cell neoplasms
T-cell prolymphocytic leukemia
T-cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK cells
Aggressive NK cell leukemia
Epstein-Barr virus (EBV) positive T-cell lymphoproliferative diseases of childhood
Systemic EBV + T-cell lymphoproliferative disease of childhood
Hydroa vacciniforme-like lymphoma
Adult T-cell leukemia/lymphoma
Extranodal NK-T-cell lymphoma, nasal type
Enteropathy associated T-cell lymphoma
Hepatosplenic T-cell lymphoma
Subcutaneous panniculitis-like T-cell lymphoma
Mycosis fungoides
Sezary syndrome
Primary cutaneous CD30 positive T-cell lymphoproliferative disorders
Primary cutaneous peripheral T cell lymphomas, rare subtypes
Primary cutaneous gamma-delta T-cell lymphoma
Primary cutaneous CD8 positive aggressive epidermotropic cytotoxic T-cell lymphoma
Primary cutaneous CD4 positive small/medium T-cell lymphoma
Peripheral T-cell lymphoma, NOS
Angioimmunoblastic T-cell lymphoma
Anaplastic large T-cell lymphoma, ALK positive
Anaplastic large T-cell lymphoma, ALK negative

of further exploration. Under certain circumstances, a careful distinction between "double-hit" lymphoma and Burkitt lymphoma with complex cytogenetics is clinically warranted since these two different entities can greatly overlap by morphology, phenotype and genetically.

17.4.2 Mature T/NK Cell Neoplasms

T/Natural killer (NK) cell lymphomas are relatively uncommon with a significant variation in incidence in different geographical regions and racial populations [1]. The diagnosis of mature T/NK cell neoplasms, as in mature B cell neoplasms, is often based on histomorphologic and phenotypic features. In contrast to B cell counterpart, only anaplastic large T cell lymphoma has been associated with recurrent cytogenetic abnormalities with diagnostic implication up to date [12]. However, a variety of mostly random genetic abnormalities have been described in various mature T cell neoplasms. This section mainly focuses on the frequent genetic abnormalities in some T/NK cell lymphomas (Table 17.3).

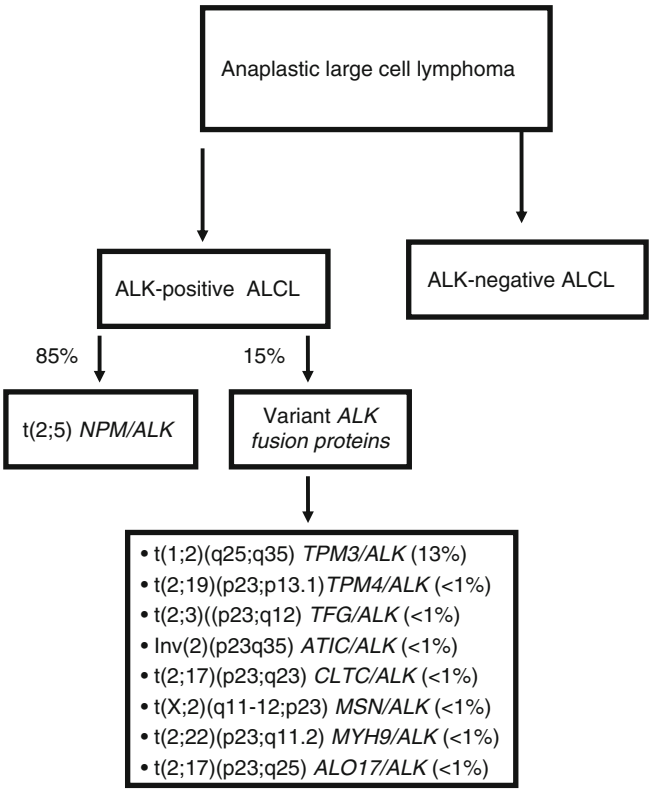

Fig. 17.2 The commonly detected partner genes to *ALK* gene in anaplastic large T-cell lymphoma

17.4.2.1 Anaplastic Large T Cell Lymphoma (ALTCL)

Anaplastic large T cell lymphoma (ALTCL) is characterized by CD30 expressing mostly large atypical T-cells, often displaying pleomorphic morphology and "C-shaped" or "horseshoe-shaped nuclei". Based on the genetic background, ALTCL can be divided into two subcategories by presence or absence of *ALK* (anaplastic large cell kinase) gene abnormality [21].

Anaplastic large T cell lymphoma (ALTCL), ALK positive, is a T cell lymphoma with defined recurrent cytogenetic abnormality. The most frequent translocation seen is t(2;5)(p23;q35) between *ALK* gene and often nucleophosmin (*NPM*) genes, found nearly 84 % of ALTCL [1]. This translocation can be detected by FISH using ALK break apart probe or karyotyping. There are also many other less frequent partners to *ALK* genes located on various chromosomes including *TPM3, TPM4, TFG, ATIC, CLTC, MOESIN, MYH9* and *ALO17* [1, 194–200] (Fig. 17.2). *ALK* gene rearrangement, with different translocations beyond t(2;5)(p23;q35), can also be found in the other hematopoietic or non-hematopoietic neoplasms

e.g. ALK + DLBCL involving t(2;17), non-small cell lymphoma, involving ALK fused to EML4 in an inv(2)(p21p23), and inflammatory myofibroblastic tumors involving t(1;2)(q25;p23).

A molecular diagnosis of ALK-negative ALTCL is challenging due to lack of recurrent primary cytogenetic aberration [1]. Careful examination of morphohistology and immunphenotyping are essentially needed to diagnose ALK-negative ALTCL. Notable efforts should be made in characterizing ALK-negative ALCL with gene profile.

17.4.2.2 T-Prolymphocytic Leukemia

T cell prolymphocytic leukemia (T-PLL) is an aggressive T cell neoplasm often associated with inversion of chromosome 14. The incidence of inv(14q) in T-PLL is high, approximately 80 % of cases, while 10 % of cases show a reciprocal translocation of chromosome 14 (t(14;14)(q11;q32) [179]. t(X;14) can rarely be seen [201]. As the results of inversion or translocation of 14q11, TCL1A and TCL1B oncogenes at the break region are activated [202]. Because of TCL1 is not present in mature T-cells and has a -high frequency found in T-PLL (70–80 % of patients) [202], identification of TCL1 activation by genetic or molecular tools helps a diagnosis of a T-PLL. In addition, a study showed that increased TCL1 is not only useful in diagnosis of T-PLL but also indicates a high proliferation in a subset of T-PLL in conjunction with intact T-cell receptor signaling [203]. Abnormalities of chromosome 8 and deletions of 11q23 are other rare genetic inactivation of *CDKN2A* as well as *PTEN* is described with possible association with disease progression [46, 204–206]. In addition, deletions or point mutations of *ATM* gene alleles have been demonstrated in some T-PLL cases suggesting that the dysfunction of this tumor suppressor gene play a role in the pathogenesis of this tumor [207].

17.4.2.3 The Other T Cell Lymphomas

There are sporadic cytogenetic changes noted in the other T-cell lymphomas, which have no known implications in diagnosis or prognosis of these T-cell lymphomas. As discussed in above, clonal T-cell gene rearrangement remains the sole molecular approach, though with low specificity.

Peripheral T cell lymphoma, not otherwise specified (PTCL, NOS) is usually associated with a complex karyotypes with recurrent chromosomal gains in chromosomes 7q, 8q, 17q, and 22q as well as recurrent losses in chromosomes 4q, 5q, 6q, 9p, 10q, 12q and 13q [1].

Mycosis fungoides (MF) is another T cell lymphoma of cutaneous origin. MF is often associated with complex karyotype, such as structural and numerical changes of chromosomes. Activation of *STAT3 and* inactivation of *CDKN2A* as well as *PTEN* are described with possible association with disease progression [46, 204–206].

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Chapter 18
Molecular Diagnostics of Myeloid Neoplasms

Xiaohui Zhang and Ling Zhang

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X. Zhang, M.D., Ph.D.
Department of Hematopathology and Laboratory Medicine, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA

L. Zhang, M.D. (✉)
Department of Pathology and Cell Biology, College of Medicine, University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612, USA

Department of Hematopathology and Laboratory Medicine, H. Lee Moffitt Cancer Center & Research Institute, 12902 Magnolia Drive, Tampa, FL 33612, USA
e-mail: ling.zhang@moffitt.org

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Abstract According to 2008 World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissue, myeloid neoplasms include (1) myeloproliferative neoplasms (MPN), (2) myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PFGFRB, or FGFR1, (3) myelodysplastic/myeloproliferative neoplasms (MDS/MPN), (4) myelodysplastic syndromes (MDS), and (5) acute myeloid leukemia (AML) and related precursor neoplasms. The diagnosis and subclassification of myeloid neoplasms is critical and approaches are multifaceted. In an era of advanced molecular biology, the diagnosis of myeloid neoplasms requires an integration of morphology, clinical presentation, laboratory results and immunophenotying with cytogenetics and molecular studies. Emerging novel molecular genetic technologies e.g. DNA microarray, single nucleotide polymorphism (SNP) array, whole genomic sequencing will aid further subclassification and characterization of disease entities of myeloid neoplasm. The common or fundamental molecular markers and associated cytogenetic aberrations, together with the diagnostic approaches are emphasized and reviewed in this chapter.

Keywords Myeloid neoplasm • Myeloproliferative neoplasm • Myelodysplastic syndrome • Acute myeloid leukemia • Molecular diagnosis

Abbreviations

aCML	Atypical chronic myeloid leukemia
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APL	Acute promyelocytic leukemia
B-ALL	B lymphoblastic leukemia
CARs	Commonly affected regions
CBBF	Core binding factor translocations
CBF	Core-binding factor
CDR	Common deletion region
CEL	Chronic eosinophilic leukemia
CML	Chronic myelogenous leukemia
CMML	Chronic myelomonocytic leukemia
CyR	Cytogenetic response
DIC	Disseminated intravascular coagulation
EMH	Extramedullary hematopoiesis
ET	Essential thrombocythemia
FAB	French-American-British
FGFR1	Febroblast growth factor receptor 1

FISH	Fluorescence in-situ hybridization
GEP	Gene expression profiling
GIST	Gastrointestinal stromal tumors
HR	Hematological response
JMML	Juvenile myelomonocytic leukemia
MDS	Myelodysplastic syndromes
MPN	Myeloproliferative neoplasms
MR	Molecular response
MPN-U	Myeloproliferative neoplasms, unclassifiable
MR	Molecular response
MS	Mastocytosis
NOS	Not otherwise specified
PDGFR	Platelet derived growth factor receptor
PMF	Primary myelofibrosis
PML	Promyelocytic leukemia
PV	Polycythemia vera
RAEB	Refractory anemia with excess blasts
RARA	Retinoic acid receptor gene, alpha
RARS	Refractory anemia with ring sideroblasts
RCMD	Refractory cytopenia with multilineage dysplasia
RCUD	Refractory cytopenia with unilineage dysplasia
RFLP	Restricted fragment length polymorphism
SM-AHNMD	Systemic mastocytosis with associated clonal hematopoetic non-mast cell lineage disease
SPOC	Spen paralog and ortholog C-terminal
T-ALL	T-lymphoblastic leukemia
WHO	World Health Organization

18.1 Introduction

According to 2008 World Health Organization (WHO) classification of hematopoietic and lymphoid neoplasms [1], myeloid neoplasms include myeloproliferative neoplasms (MPN), myeloid and lymphoid neoplasms with eosinophilia and abnormalities of PDGFRA, PFGFRB, or FGFR1, myelodysplastic/myeloproliferative neoplasms (MDS/MPN), myelodysplastic syndromes (MDS), and acute myeloid leukemia (AML) and related precursor neoplasms (Table 18.1). In recent years, there have been significant advances in hematopathology, making the diagnosis rely not only on morphology, but also heavily on immunophenotying, cytogenetics, and molecular studies. This is significantly different from the previous classification known as the French-American-British (FAB) classification, which

Table 18.1 The 2008 WHO classification of myeloid neoplasms

Myeloproliferative neoplasms
Chronic myelogenous leukemia, *BCR-ABL1*-positive
Chronic neutrophilic leukemia
Polycythemia vera
Primary myelofibrosis
Essential thrombocythemia
Chronic eosinophilic leukemia, NOS
Mastocytosis
Myeloproliferative neoplasm, unclassifiable
Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* or *FGFR1*
Myelodysplastic/myeloproliferative neoplasms
Chronic myelomonocytic leukemia
Atypical chronic myeloid leukemia, *BCR-ABL1* negative
Juvenile myelomonocytic leukemia
Myelodysplastic/myeloproliferative neoplasm, unclassifiable
Myelodysplastic syndromes
Refractory cytopenia with unilineage dysplasia
Refractory anemia with ring sideroblasts
Refractory cytopenia with multilineage dysplasia
Refractory anemia with excess blasts
Myelodysplastic syndrome associated with isolated del(5q)
Myelodysplastic syndrome, unclassifiable
Childhood myelodysplastic syndrome
Acute myeloid leukemia (AML) and related precursor neoplasms
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFβ-MYH11*
Acute promyelocytic leukemia with t(15;17)(q22;q21); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
AML with mutated *NPM1*
AML with mutated *CEBPA*
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to down syndrome
Blastic plasmacytoid dendritic cell neoplasm

was based on morphology and cytochemical stains. Besides, the approaches to prognostication and therapeutic guidance have been transformed and have been increasingly useful molecular markers for clinical practice. Since the molecules involved in myeloid neoplasms are extensive, we will address only those molecular markers that have been widely used or will be potentially used in practical hematopathology.

18.2 Diagnostic Algorithms

The morphologic examinations on peripheral blood smears, bone marrow smears and bone marrow biopsies are always the first step on diagnostic algorithms, which are followed by immunophenotyping, cytogenetics, molecular cytogenetics and molecular genetics studies. The initial morphologic evaluation of the available specimen including peripheral blood and/or bone marrow aspirate is critical for the subsequent ordering of ancillary studies.

Generally, the presence of 20 % or more blasts in bone marrow or peripheral blood warrants the diagnosis of acute leukemia. However, when certain cytogenetic changes e.g. t(8;12)(q22;q22), inversion 16/t(16;16)(p13.1;q22), t(15:17)/(q22;q12) are confirmed, the diagnosis of leukemia can be made even when the blast count is less than 20 %. Careful assessment of morphology of blast with monocytic differentiation is needed since monoblasts and promonocytes are both counted as blasts, while atypical monocytes are not. Blasts with bilobed, or cup-shape or concaved nuclei with or without fine azurophic granules and blasts with Auer rods in cytoplasm are also important to recognize, which could suggest acute promyelocytic leukemia (APL). In this situation, a laboratory work up for disseminated intravascular coagulopathy (DIC) is warranted and molecular genetic study of t(15;17)(q22;q12)/*PML-RARA* should be performed to confirm the diagnosis. Blasts with perinuclear hofs and chunky orange, or salmon-pink cytoplasmic granules are also important to recognize, not to be misinterpreted as myelocytes, which are unique features of acute leukemia with t(8;21)(q22;q22)/*RUNX1-RUNX1T1*. An overt increase of abnormal eosinophils with large, basophilic granules, along with increased myeloblasts, are features of acute leukemia with inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB-MYH11*. Immunophenotyping by flow cytometry is required to define the lineage differentiation of the blast cell population. Cytogenetic studies by conventional karyotyping or FISH analysis should be performed to detect the recurrent cytogenetic abnormalities. According to studies, approximately 55 % of acute myeloid leukemia cases have various kinds of cytogenetic changes [2]. Some of the cytogenetic aberrations also predict disease prognosis. Mutation analysis of certain genes, such as NPM1, CEBPA, FLT3 internal tandem duplications (FLT3-ITDs) and FLT3 kinase domain mutation (FLT3-TKD), have been included in the 2008 WHO classification, and these mutations are not only related to the nature of AML but also tend to be considered as important prognostic markers. Moreover, clinical findings should be integrated into the diagnosis consideration, as in patients with myeloid proliferations related to Down syndrome and therapy-related myeloid neoplasms. Schematic algorithms of clinical and genetic diagnostic approaches for acute myeloid leukemia is

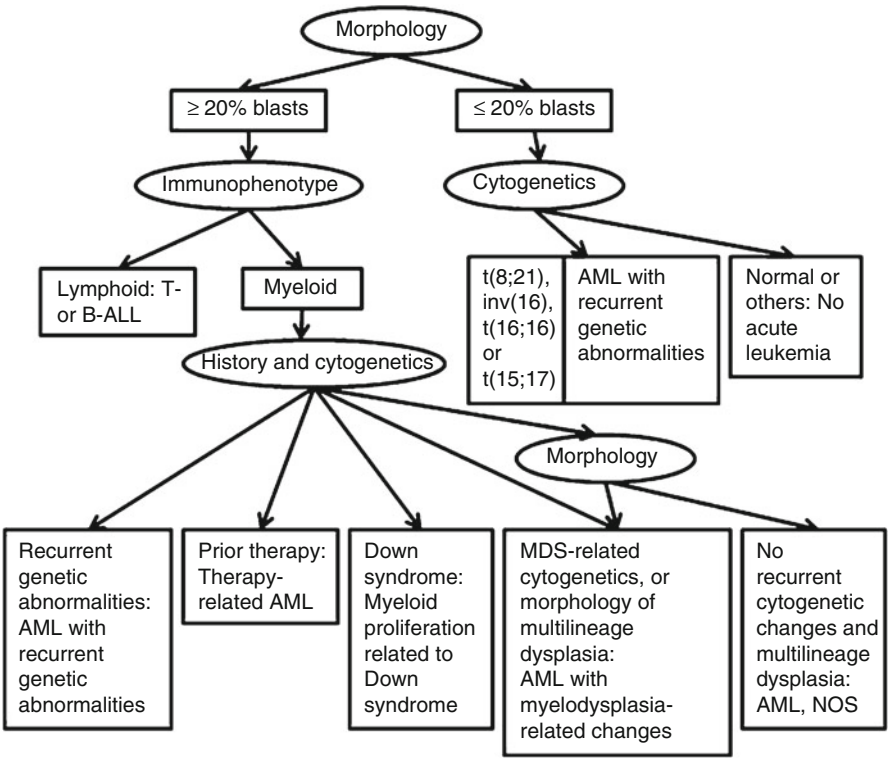

Fig. 18.1 Algorithmic approach to the classification of acute leukemia (Modified from http://www.uscap.org/site~/98th/pdf/companion21h02.pdf)

summarized below (Fig. 18.1) (modified from http://www.uscap.org/site~/98th/pdf/companion21h02.pdf; accessed Jan 10, 2011) [3].

For myeloproliferative neoplasm (MPN), myelodysplastic syndromes/myeloproliferative neoplasm (MDS/MPN), and myelodysplastic syndrome (MDS), the diagnostic algorithms integrate clinical and laboratory data, histologic findings, and molecular markers. The 2008 WHO classification criteria combine all three factors [1]. Clinical and laboratory data are important. For example, in patients with hemoglobin level greater than 18.5 g/dL in men or 16.5 g/dL in women, polycythemia vera (PV) would be suspected if the patients do not have any other etiology that could cause a secondary erythrocytosis or elevated hemoglobin, and testing JAK2 mutation (V671F, or exon 12) is needed to confirm the diagnosis. A patient with essential thrombocythemia would have a platelet count of more than 450×10^9/L, persistent, often accompanying thrombotic episodes. Bone marrow morphology is an important diagnostic tool, which is non-replaceable to distinguish among the categories of MPN, MDS/MPN, and MDS, but plays only a minor role in distinguishing between reactive and clonal myeloproliferation [1]. True MPN could be

distinguished from MDS and MDS/MPN by the absence of dyserythropoiesis, dysgranulopoiesis and monocytosis, and the morphology of hyperplastic megakaryocytes provides important diagnostic clues [4]. MDS will be considered when morphologic dysplasia is present in one or more major myeloid cell lineages with associated cytopenia. The availability of molecular markers, namely, BCR-ABL1, JAK2 V617F, JAK2 exon 12, MPL 515L/K, c-Kit D816V are widely applied in the diagnosis of myeloproliferative neoplasm. Among them, detection of t(9;22)(q34;q11.2)/*BCR-ABL1* translocation is necessary to diagnose chronic myelogenous leukemia (CML), which makes the diagnostic criteria dramatically different from other categories of myeloid disorder. Different from the prior versions of WHO classification, novel 2008 WHO classification has conjunction with the cytogenetic aberration and certain molecular markers into major diagnostic criteria of many categories of myeloid disorders [5]. We will discuss the important diagnostic features and correlated clinicopathologic findings, and prognostic outcomes of myeloid neoplasm as below in this chapter.

18.3 Myeloproliferative Neoplasms (MPN)

The MPN are clonal hematopoietic stem cell disorders characterized by proliferation of one or more of the myeloid lineage [1]. The bone marrow is hypercellular with effective hematopoietic maturation and the peripheral blood has increased numbers of granulocytes, red blood cells, and/or platelets. This category includes eight entities: Chronic myelogenous leukemia (CML), BCR-ABL1-positive, chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia (CEL), NOS, mastocytosis (MS), and myeloproliferative neoplasm, unclassifiable (MPN, U).

18.3.1 BCR-ABL and Chronic Myelogenous Leukemia (CML)

CML is a clonal proliferation of myeloid cells at all stages of differentiation with the chromosome translocation t(9;22)(q34;q11.2) leading to the formation of the *BCR-ABL* fusion gene. Patients usually present with marked leukocytosis and basophilia in the peripheral blood and bone marrow with splenomegaly. An initial chronic phase usually lasts for 3–5 years and the disease progresses to accelerated phase and blast phase, which have increased blast counts. The blast phase has more than 20 % of blasts and it can be of myeloid or lymphoid lineage or mixed lineages.

As early as the 1960s, Philadelphia (Ph) chromosome, a minute chromosome 22 resulting from a translocation of chromosome 22q11.2 to chromosome 9q34 [t(9;22)(q34;q11.2)], was identified and has been associated with CML since 1973 [6, 7]. Reportedly 90–95 % of CML cases have this translocation at diagnosis [1],

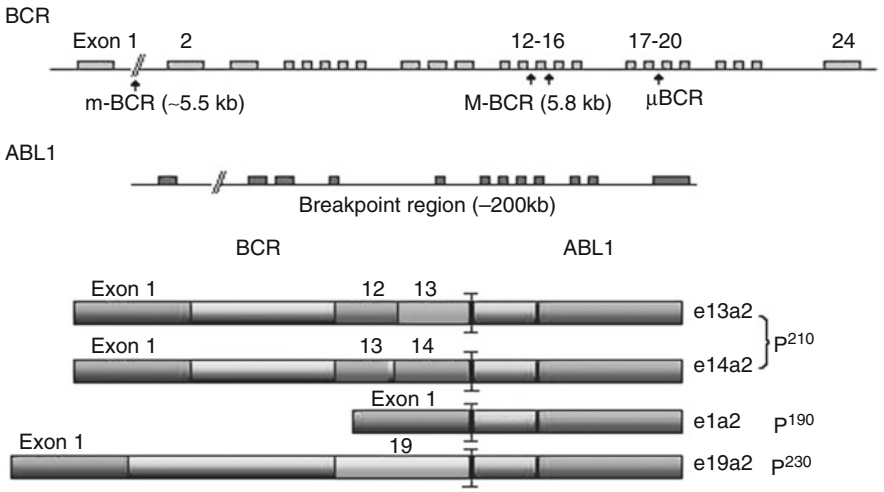

Fig. 18.2 Schematic BCR-ABL1 fusion proteins. *Arrows* indicate breakpoint sites for M-BCR (p210), m-BCR (p190), and **μ**-BCR (p230). The breakpoints in BCR are variable and can occur in the major (M-BCR), minor (m-BCR), or micro (**μ**-BCR) breakpoint cluster region. The breakpoint in ABL1 occurs upstream of 2a. The resulting fusion transcripts are e13a2, e14a2, e1a2, e19a2 encoding p210, p190, and p230, respectively

and the remaining cases have either variant translocations involving chromosome other than chromosomes 22 and 9, or cryptic translocations which cannot be identified by conventional cytogenetic analysis. All of these cases have molecular level detectable BCR-ABL1 transcript, a fusion product of *BCR* gene on chromosome 22 with *ABL1* gene on chromosome 9 [8]. The *BCR-ABL1* fusion gene is present in CML by definition, and has been designated in 2008 WHO classification as CML, *BCR-ABL1* positive. However, *BCR-ABL1* gene rearrangement is not specific for CML, as it is also found in acute lymphoblastic leukemia (ALL), AML, and rarely, MDS [9–12].

As known, the wild-type *BCR* gene encodes a serine/threonine kinase and might be involved in signal transduction pathways [13]. The wild-type *ABL1* gene product is a tyrosine kinase involved in cell cycle regulation [14]. The *BCR-ABL1* fusion gene produces a chimeric oncoprotein, BCR-ABL1, which is an aberrantly activated tyrosine kinase and activates several different growth factor mediated signaling pathways including RAS, STAT, NFκB, PI3K/AKT, and MAPK pathways [15].

The breakpoint in the *BCR* gene may occur at three different sites: major (M-BCR), minor (m-BCR), and micro (μ-BCR). M-BCR spans exons 12–16, and encodes an abnormal protein, p210. M-BCR is the most common site in CML, occurring in 95 % of CML patients. m-BCR, spanning exons 1–2, encodes a shorter protein, p190. μ-BCR occurs rarely; it spans exons 17–20 and encodes a larger fusion protein, p230 (Fig. 18.2). μ-BCR (p230) is associated with prominent neutrophilic maturation and/or conspicuous thrombocytosis [1]. m-BCR (p190) is most frequently associated with Ph chromosome positive ALL. However, p190 can be seen in rare cases of CML with monocytosis which resembles chronic

myelomonocytic leukemia and a small amount of p190 can be seen in the majority of p210 CML as a result of alternative splicing [1]. A more recent study reported p190 BCR-ABL CML, although rare, is associated with a poor outcome and irresponsive to tyrosine kinase inhibitor therapy [17].

To diagnose CML, it needs a correlation of clinical features, laboratory findings, bone marrow morphology, and cytogenetic and molecular studies. Detection of Ph chromosome or *BCR-ABL1* fusion gene is required. Conventional cytogenetics has been the gold standard, but fluorescence in-situ hybridization (FISH) and reverse transcriptase PCR (RT-PCR) methods are more sensitive for diagnosing the disease and monitoring for minimal residual disease. FISH can detect a cryptic translocation as well as deletions of derivative chromosome [18]. RT-PCR is most sensitive and real-time PCR methods can be used for quantification [19, 20].

18.3.2 CML Transformation, Therapy Monitoring and Imatinib Resistance

The accelerated and blast phases of CML frequently demonstrate secondary cytogenetic changes, including extra Ph chromosome, +8, +19, or i(17q) [1]. Translocations characteristic of *de novo* AML are occasionally detected in blast phase of CML, such as t(8;21)(q22;22), t(3;21)(q26;q22), t(7;11)(p15;p15), inv16(p13q22), and t(15;17)(q22;q12-21), which are, though with the same chromosome breakpoints, not associated with a better prognosis as they are noted in *de novo* AML. Associated gene changes include *TP53, RB1, MYC, p16INK4a, RAS, AML1 and EVI-1,* but their significance in diagnostics is unknown [1].

Targeted on the BCR-ABL1 tyrosine kinase activity, imatinib mesylate is very effective to control CML, particularly when used early in the chronic phase. Imatinib mesylate induces hematologic remission in nearly all CML patients [21]. The evaluation of response to treatment is based on the hematologic, cytogenetic, and molecular criteria. A hematological response (HR) is indicated by normal peripheral blood cell counts and bone marrow morphology. Complete, major, minor, and minimal cytogenetic response (CyR) can be achieved by detection of 0 %, < 35 %, 36–65 % and 66–95 % Ph chromosome positive (Ph+) bone marrow cells [21]. Molecular response (MR) is defined by reduction in the *BCR-ABL1* transcript, which can be measured by quantitative RT-PCR. The real-time technology provides sensitive and reproducible results. A ratio of the *BCR-ABL1* to control genes, usually ABL1, is reported, and the treatment plan is changed accordingly [22].

Resistance to imatinib mesylate occurs when subclones of CML cells acquire point mutations on ABL1 kinase domain that prevent the binding of imatinib mesylate to the kinase. Up to 90 % of the resistant patients have detectable mutations, and more than 35 mutations have been described [23]. Detection of emerging mutations can be analyzed by PCR followed by direct sequencing of the *BCR-ABL1* kinase domain. However, it should be pointed out that kinase mutation independent imatinib-resistance also exists in CML, e.g. LYN kinase overexpression, and increased

BCR-ABL1 expression [24, 25]. A study demonstrates a role of constitutive activation of the PI3K/AKT1 pathway in *BCR-ABL1*-independent imatinib resistance [26]. Additionally, some patients are resistant to imatinib primarily, which is defined by inability to achieve complete hematologic response by 3 months or molecular complete response by 6 months of imatinib treatment, and the molecular mechanism remains unclear.

18.3.3 JAK2 and MPN

In 2005, several groups independently identified *JAK2* V617F as the major molecular mutation event in *BCR-ABL1* negative MPNs. This somatic mutation is found in more than 95 % of PV patients, and 40–50 % of ET and PMF patients [27–30]. With this regard, PV, ET and PMF may be considered as a spectrum of diseases sharing a common molecular etiology. There is also a rare *JAK2* exon 12 mutation which has similar functional effects as *JAK2* V617F [1, 31].

JAK2 (Janus Kinase 2) is a tyrosine kinase that mediates activation of receptors for erythropoietin, thrombopoietin, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor. It is also involved in signaling by receptors with tyrosine kinase activity, such as *KIT* [32]. *JAK2* mutations can activate the downstream signaling pathways (STAT, ERK1/2, MAPK, PI3K/AKT) and cause uninhibited growth, independent of cytokines [33, 34].

Virtually all patients with PV have either *JAK2* V617F or *JAK2* exon 12 mutation, thus, it is a sensitive diagnostic marker for PV. Homozygous mutation of *JAK2* V617F has been more frequently detected in PV and associated with the clinical picture of classic PV and with a higher tendency to secondary myelofibrosis but without increased leukemia [35]. For ET or PMF, only about half of patients have the *JAK2* mutation and a small portion of patients (5 %) have *MPL* mutation (*MPL* W515K/L) [36], so the diagnostic use of *JAK2* mutation is limited. *JAK2* V167F is not specific to any of the MPNs and its absence does not exclude MPN; at the same time, its presence does support the diagnosis of an MPN if other clinical and laboratory data are supportive. Importantly, its presence excludes reactive myeloproliferative causes [4]. In addition, concurrent *MPL* and *JAK2* V617F mutations have also been reported, which indicate that these two mutations may have functional complementation in myeloproliferative disease [36]. Of note as well, *JAK2* mutation could occasionally be seen in MDS [37], MDS/MPN [38] and rarely coexist with CML [39].

For the diagnosis of PV, ET and PMF, an initial *JAK2* V617F test is routinely performed. If the result comes back negative, a *JAK2* exon12 mutation test can be done if PV is still suspected, while an *MPL* W515K/L mutation should be performed if ET or PMF is suspected. Either DNA or RNA from the peripheral blood or bone marrow aspirate is applicable for the mutation analysis. Qualitative as well as quantitative methods, including restricted fragment length polymorphism (RFLP), direct Sanger sequencing, and real time PCR, can be selected. Real time PCR is a preferred method with better sensitivity.

18.3.4 Polycythemia Vera (PV)

PV is characterized by increased red blood cell production independent of the mechanisms that normally regulate erythropoiesis. The proliferation involves not only the erythroid lineage but also the granulocytes and megakaryocytes. The diagnosis of PV is based on clinical and laboratory parameters. According to 2008 WHO classification, PV is suspected in patients with hemoglobin levels greater than 18.5 g/dL in men or 16.5 g/dL in women, or hemoglobin greater than 17 g/dL in men or 15 g/dL in women if associated with a documented and sustained increase of at least 2 g/dL from a previous base line [1]. Serum erythropoietin level is usually lower than that in the other pseudo PVs and also below the normal range in healthy patients (3.3 IU/L) [40]. Bone marrow biopsy shows marked hypercellularity with pan-hyperplasia. Lack of stainable iron particles is a common phenomenon noted in marrow aspirate from PV patient. *JAK2* V617F or other functionally similar mutations such as *JAK2* exon 12 mutation is a required molecular test. Identification of an abnormal karyotype would be helpful but not necessary for a diagnosis since only about 20 % of patients with PV have cytogenetic abnormalities. Presence of Ph chromosome and/or *BCR-ABL* fusion excludes PV. The most common recurring cytogenetic abnormalities include +8, +9, del (20q), del (13q) and del (9p); sometimes +8 and +9 are found together [41].

18.3.5 Essential Thrombocythemia (ET)

ET is characterized by sustained thrombocytosis $> 450 \times 10^9$/L in the peripheral blood and increased numbers of large, mature megakaryocytes in the bone marrow. Bone marrow cellularity usually retains in normal range. There is no significant erythrocytosis or leukocytosis with left shifted mutation. Clinically the patients usually have episodes of thrombosis and/or hemorrhage. The diagnosis is after exclusion of other causes of reactive or neoplastic thrombocytosis, including other MPN, inflammatory, iron deficiency anemia, infectious or non-hematopoietic neoplastic disorders. The presence of a *BCR-ABL1* fusion gene excludes the diagnosis of ET. As mentioned above, approximately 40–50 % of ET patients have the *JAK2* V617F or a functionally similar mutation. It has been recently found that carrying homozygous *JAK2* V617F mutation in patients with ET increased a risk of recurrent thrombosis [42]. *MPL* mutation, namely *MPL* W515K/L, has been found in 1 % of cases of *JAK2* V617K negative ET [1]. *MPL* is a thrombopoietin receptor, and it is spontaneously activated by the mutation in amino acid position 515 (*MPL* W515K/L). The mutation leads to thrombocytosis and myelofibrosis [36]. Worthy of mention is that W515L/K has not been detected in patients with PV according to a large series of studies including 1,182 patients with myeloproliferative and other myeloid disorders [6]. Thus, presence of W515L/K narrows the diagnosis down to ET or PMF. Presence of *JAK2* or *MPL*

mutation helps to make the diagnosis, nevertheless, neither *JAK2* nor *MPL* mutations is specific for ET.

There is no consistent karyotypic abnormalities noted in ET, but +8, abnormalities of 9q, and del (20q) have been reported [43].

18.3.6 Primary Myelofibrosis (PMF)

PMF is characterized by a proliferation of predominantly megakaryocytes and granulocytes in the bone marrow associated with excess reticulin and collagen deposition and extramedullary hematopoiesis (EMH). In cellular phase, PMF is often associated with myeloid preponderance, with less intensive myelofibrosis besides atypical megakaryocytic proliferation. Historically, PMH should have no other myeloproliferative neoplasm diagnosed before and no *BCR-ABL1* fusion gene documented. As aforementioned, approximately 50 % of patients with PMF have the *JAK2* V617F mutation [27–30]. Up to 5 % of PMF cases have *MPL* W515K/L mutation [27–30, 36], although none of the mutations are specific to the disease entity. Similar to ET, the presence of *JAK2* V617F or MPL mutation is helpful to rule out reactive marrow fibrosis, e.g. chronic inflammatory condition, autoimmune disorder, and drug-related situations. Exclusion of other primary and secondary malignancies with secondary myelofibrosis, namely lymphoma, metastatic carcinoma, is also needed.

Approximately 30 % of patients have cytogenetic changes, including partial trisomy 1q, 13q deletion, 20q deletion, trisomy 8, and chromosomes 1, 7, and 9 abnormalities [44–46]. The presence of either del(13)(q12–22) or der(6)t(1;6)(q21–23;p21.3) is strongly suggestive but not diagnostic of PMF [44, 47].

Since some cytogenetic abnormalities, e.g. 20q deletion and trisomy 8, can also be associated with MDS, differential diagnosis should include fibrotic MDS. A careful morphologic assessment and correlation with clinical presentation would be helpful. In general, splenomegaly is less commonly associated with MDS. Presence of characteristic syncytial clustering of megakaryocytes, extrasinasoidal hematopoiesis and osterosclerosis favors PMF over fibrotic MDS.

18.3.7 Mastocytosis

Mastocytosis is a new addition to the MPN category in 2008 WHO classification. It is a clonal proliferation of mast cells that accumulate in one or more organ systems, and it is characterized by the presence of multifocal compact clusters or cohesive aggregates/infiltrates of abnormal mast cells [1]. Cutaneous and systemic mastocytosis are the two most common clinicopathologic settings. Systemic mastocytosis with associated clonal hematopoetic non-mast cell lineage disease (SM-AHNMD) has been observed and considered a variant of systemic

mastocytosis [1]. Mastocytosis is separated from mast cell hyperplasia by distinct morphological and/or molecular characteristics.

KIT mutations at codon 816 in exon 17, most commonly D816V, have been reported in 95 % or more of adult patients with systemic mastocytosis or one third of cutaneous mastocytosis in pediatric patients [48]. The *KIT* mutation results in a constitutively activated kinase, signaling from which appears to be essential for the survival and proliferation of mast cell precursors in the marrow. The D816V mutation also confers resistance to imatinib therapy [49]. Other mutations on *KIT* including D816Y, D816H and D816F are rarely seen. The latter (D816F) is reported and relatively more common in the pediatric group [50]. Pyrosequencing or mutation-specific quantitative PCR on DNA extracted from microdissected bone marrow or skin biopsy specimens, or from sorted mast cells from bone marrow aspirates are recommended methods [51, 52].

In the setting of SM-AHNMD, seeking additional disease specific cytogenetic or molecular abnormalities is warranted. For example, *JAK2* mutation can be detected in MPN. In addition, since D816V is also observed in cases of AML, particularly in tumors with core binding factor (CBF) translocations [53, 54], correlation with clinical information and morphologic findings is suggested before a final diagnosis is rendered.

18.3.8 Chronic Neutrophilic Leukemia (CNL)

CNL is very rare, characterized by persistent neutrophilia (>80 % of white blood cells, white blood cells at least $>25\times10^9$/L) in peripheral blood, bone marrow hypercellularity and hepatosplenomegaly. Diagnosis is made by exclusion of other causes of neutrophilic leukocytosis. There is no Philadelphia chromosome or *BCR-ABL1* fusion gene. No *PDGFRA, PDGFRB, FGFR1* gene rearrangements are detected. *JAK2* mutation has been found in 20 % of patients [55]. In the presence of *JAK2* mutation, further investigation is requested to completely exclude any *JAK2* positive MPN as well as MDS/MPN.

18.4 Myeloid and Lymphoid Neoplasms with Eosinophilia and Abnormalities of *PDGFEA, PDGFB,* or *FGFR1*

Myeloproliferative and lymphoid neoplasms associated with rearrangement of *PDGFRA, PDGFRB* and *FGFR1* includes three rare diseases. They have some shared clinical and pathological features and all of them have chromosomal rearrangement of one of three tyrosine kinases: platelet-derived growth factor receptor *(PDGFR) alpha (A), PDGFR beta (B)*, or fibroblast growth factor receptor 1 (*FGFR1*) [1]. The fusion genes encode aberrant tyrosine kinases. Eosinophilia is characteristic but not invariable. All three diseases could represent as AML, MDS/

MPN or MPN, depending upon their partnership. The most common partner genes involved are *FIP1L1, ETV6/TEL*, and *ZNF198* for PDGFRA, PDGFRB, and *FGFR1*, respectively [56]. Cytogenetic and molecular genetic analysis should be performed. *FIP1L1-PDGFRA* fusion gene usually results from a cryptic del(4)(q12). Clinically, the specific gene fusion is representative of a picture of chronic eosinophilic leukemia (CEL) with eosinophilia with dysplasia. *ETV6-PDGFRB* results from t(5;12)(q31~33;p12), which has hematological features resembling CMML, CEL, aCML with eosinophilia and MPN with eosinophilia [57–59]. Translocations involving an 8p11 breakpoint lead to a fusion gene with *FGFR1* [1]. There are multiple partner genes associated with *FGFR1*. Among them, one of the partners located at 6q27 creates a fusion gene, *FGFR10P1/FGFR1*, is observed in some patients with PV [60]

To diagnose *PDGFR* or *FGFR* related disorder, FISH or PCR should be performed. FISH analysis with any *PDGFA, PDGFRB* or *FGFR1* probe is widely accepted to identify the gene rearrangement. PCR is an alternative approach. Recently, generic quantitative RT-PCR has also been developed to detect diverse *PDGFRA* or *PDGFRB* [61].

18.5 Myelodysplastic/Myeloproliferative Neoplasms (MDS/MPN)

MDS/MPN neoplasms have both MDS and MPN features, comprising chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia, *BCR-ABL1* negative (aCML), juvenile myelomonocytic leukemia (JMML), and MDS/MPN, unclassifiable.

The manifestation of CMML is persistent peripheral monocytosis greater than 1×10^9/L and fewer than 20 % blasts (including promonocytes). Philadelphia Chromosome/BCR-ABL1 gene or rearrangement of *PDGFRA* or *PDGFRB* should be absent. Dysplasia involves one or more myeloid lineages. Dysplasia is not a necessary condition for diagnosis of CMML if the other criteria are met. The other diagnostic criteria include a persistent monocytosis lasting over 3 months without any other explainable reactive process, or a clonal cytogenetic or molecular genetic aberration is acquired. The most common numerical and structural chromosome abnormalities associated with CMML are trisomy 8, deletion of 7 or 7q and 12p. In view of molecular changes, as many as 40 % of patients with CMML have *RAS* gene point mutations [62, 63].

However, the diagnosis of CMML is based on clinical, laboratory and morphological observations. Nonspecific cytogenetic changes are found in 20–40 % of patients [1]. It is important to know that cases with abnormalities of 11q23 suggest the diagnosis of AML instead of CMML. Cases with p190 *BCR-ABL1* is classified as CML, regardless of persistent high monocyte count over 10 % of WBCs or 1×10^9/L [64].

Atypical chronic myelogneous leukemia (aCML) is characterized by leukocytosis with dysplastic neutrophils and their precursors. *BCR-ABL1* fusion gene is

absent. *PDGFA* or *PDGFB* genes are also excluded. Similar to *BCR-ABL1* positive CML, patients with aCML commonly present with elevated WBC count with granulocytic predominance and splenomegaly. It is distinguished from CML by dysgranulopoiesis with nuclear aberrations and cytoplasmic hypogranulation [64, 65] and from CMML by less than 10 % monocytes [64]. About 30 % of cases have acquired mutations of *NRAS* or *KRAS* [66]. Cytogenetic abnormalities have been reported in up to 80 % of patients with aCML, most commonly +8 and del(20q).

JMML affects children of 0–14 years of age. There is no *BCR-ABL1* fusion gene. PTPN11 mutations occur in 35 % of patients [67, 68], and oncogenic mutation of the *RAS, NRAS, KRAS*, and *NF1* are each seen in approximately 20 % of patients. Most of the patients with JMML (65 %) have normal karyotype. Monosomy 7 is found in about 25 % of patients. Other cytogenetic abnormalities are also noted in 10 % of patients [1].

18.6 Myelodysplastic Syndrome (MDS)

MDS is a group of clonal hematopoietic stem cell diseases characterized by cytopenia, dysplasia in one or more of the major myeloid cell lines, ineffective hematopoiesis, and increased risk to transform to AML. According to 2008 WHO classification, the diseases include refractory cytopenias with unilineage dysplasia (RCUD), refractory anemia with ring sideroblasts (RARS), refractory cytopenia with multilineage dysplasia (RCMD), refractory anemia with excess blasts (RAEB), myelodysplastic syndrome – unclassified (MDS-U), and MDS associated with isolated del(5q). This classification integrates prognostically different subgroups, and certain chromosome aberrations, which have prognostic significance and may predict response to therapeutic drugs. For example, MDS associated with isolated del(5q), which usually has a favorable prognosis and may benefit from lenalidomide therapy [69, 70].

Cytogenetic abnormalities have been found in approximately 50 % of patients with MDS. Table 18.2 shows the common recurring chromosomal changes. The presence of these chromosomal abnormalities and refractory cytopenia of undetermined origin could suggest a diagnosis of presumptive MDS if no convincing morphologic evidence of dysplasia. Exceptions are sole +8, −Y or del(20q), which are not sufficient for a diagnosis of MDS if morphological criteria are not met. These recurring changes can also be used in monitoring of the disease during follow up. It is known that certain cytogenetic changes are associated with some characterized clinical or morphologic features. For example, del(5q) occurs primarily in elderly women with refractory macrocytic anemia, and the associated MDS is characterized by megakaryocytes with non-lobated or hypolobated nuclei, and has a favorable clinical course. Loss of 17p is associated with pseudo Pelger-Huët anomaly, small vacuolated neutrophils, TP53 mutation and unfavorable clinical course [1, 71]. Inversion of 3q21q26 or t(3;3;)(q21;q26.2) is often associated with atypical megakaryocytic hyperplasia [72]. Complex karyotype which is defined by at least three independent chromosomal

Table 18.2 Common recurring cytogenetic abnormalities and their frequency [1]

Cytogenetic abnormalities	Frequency in MDS	Frequency in t-MDS
Unbalanced		
+8	10 %	
−7 or del(7q)	10 %	50 %
−5 or del(5q)	10 %	40 %
del(20q)	5–8 %	
−Y	5 %	
i(17q) or t(17p)	3–5 %	
−13 or del(13q)	3 %	
del(11q)	3 %	
del(12p) or t(12p)	3 %	
del(9q)	1–2 %	
idic(X)(q13)	1–2 %	
Balanced		
t(11;16)(q23;p13.3)		3 %
t(3;21)(q26.2;q22.1)		2 %
t(1;3)(p36.3;q21.2)	1 %	
t(2;11)(p21;q23)	1 %	
inv(3)(q21q26.2)	1 %	
t(6;9)(p23;q34)	1 %	

t-MDS therapy-related MDS

abnormalities, typically includes chromosomes 5 and/or 7 [−5/del(5q), −7/del(7q)], and is associated with an unfavorable clinical course [1, 73].

Molecular defects associated with the cytogenetic changes are largely unknown. Often times, the breakpoints are not clustered in a small area, but are distributed in a relatively large commonly affected regions (CARs) [74]. Only a few molecular markers have been identified.

Genes located at 5q- have been investigated. In common deletion region (CDR), there is many tumor suppressor genes identified. For example, *RPS14* has been identified as a del(5q) related gene [75], and *SPARC* may also be involved in the pathogenesis of 5q- syndrome and response to lenalidomide [76].

MDS1/EVI1 gene in 3q26 is found to be involved in translocations like inv(3)(q21q26), t(3;3)(q21;q26), and t(3;21)(q26;q22). It can form a fusion gene with *AML1* gene in 21q22, or overexpression may be the consequence of the translocations [77–79].

Unrelated to the cytogenetics, many gene mutations have been reported in MDS, including *TET, KIT, G-CSFR, PDGFRB, FLT3, FMS, N-RAS, K-RAS, JAK2, RUNX1, CEBPA, PU.1, GATA-1, p53* and *MLL* [74]. It is noteworthy that *JAK2* V617F can be found in MDS, most often associated with marrow fibrosis and thrombocytosis [80, 81], however, it is less frequent than in MPN or MDS/MPN [82]. A small subset of patients with isolated del(5q) may show a concomitant *JAK2* V617F mutation [83]. *JAK2* has been found frequently in refractory anemia with ring sideroblasts associated with marked thrombocytosis (RARS-T), which belongs

to MDS/MPN, and should be distinguished from pure RARS. Despite the progress to date, the molecular markers are mostly under research and they have not been widely accepted in diagnosis and therapy of MDS.

18.7 Acute Myeloid Leukemia (AML)

AML represents a group of hematopoietic stem cell disorders characterized by clonal proliferation of immature hematopoietic cells, or blasts that accumulate in bone marrow, and interfere with normal hematopoietic function. It can develop *de novo*, or secondary to myelodysplastic syndrome, myeloproliferative neoplasm, chemotherapy or radiation. In 2008 WHO classification, AML and related precursor neoplasms consists of seven subcategories: (1) AML with recurrent genetic abnormalities; (2) AML with myelodysplasia-related changes; (3) therapy-related myeloid neoplasms; (4) acute myeloid leukemia, not otherwise specified (NOS), which is essentially a modified FAB classification predominantly based on traditional morphologic and cytochemical criteria; (5) myeloid sarcoma; (6) myeloid proliferations related to Down syndrome and (7) blastic plasmacytoid dendritic cell neoplasm [1].

Besides AML with recurrent genetic abnormalities, cytogenetics and molecular studies are also an integral part of 2008 WHO classification for those AML under the remaining six subcategories and are recommended for risk stratification in disease progression. In addition, cytogenetics and gene mutation status have been used to stratify the patients into favorable, intermediate, and poor prognostic groups, and are also used to predict the patient's response to therapies [84].

18.7.1 AML with Recurrent Genetic Abnormalities

This category includes seven neoplasms associated with specific genetic abnormalities. These genetic abnormalities have prognostic significance and are correlated with morphology and immunophenotypes in most types. The most common abnormalities are balanced chromosomal translocations including t(8;21)(q22;q22), inv(16)(p13.1q22)/t(16;16)(p13.1;q22), t(15;17)(q22;q12) and t(9;11)(p22;q23) [1]. As mentioned earlier, the presence of t(8;21), inv(16), or t(15;17) is diagnostic of acute leukemia even when the blasts are less than 20 %. The fusion genes resulted from the translocations can be detected by FISH, and RT-PCR. RT-PCR is a more sensitive method.

18.7.1.1 t(8;21)(q22;q22); *RUNX1-RUNX1T1*

The t(8;21) (q22;q22) is observed in 8 % of AML cases. The translocation results in a fusion gene involving the *RUNX1* (also known as core-binding factor α, and *AML1*) gene and *RUNX1T1* (also known as *ETO*) gene. The *RUNX1-RUNX1T1*

fusion gene product represses normal transcriptional activity of *RUNX1* which contributes to the leukemic transformation. Cases with t(8;21) have distinct morphological and immunophenotypic features, with long, thin Auer rods and salmon-colored cytoplasmic granules (AML-M2 in FAB classification), and expression of B cell lineage markers (CD19) [1]. AML with t(8;21) is one of the core-binding factor (CBF) leukemias, along with inv(16) and t(3;21) in AML and t(12;21) in pediatric acute lymphoblastic leukemia (ALL). These CBF leukemias have a favorable prognosis [85].

18.7.1.2 inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFβ-MYH11*

The inv(16)(p13.1q22) or t(16;16)(p13.1;q22) is observed in 10 % of adult and approximately 6 % of childhood AML cases. Both of the inv(16)(p13.1q22) and t(16;16)(p13.1;q22) result in a fusion protein composed of β subunit of the core-binding factor (*CBFβ*) and smooth muscle myosin heavy chain (*MYH11*). *CBFβ-MYH11* acts as a transcriptional repressor [86]. The characteristic morphology is acute myelomonocytic leukemia with abnormal eosinophils (AML-M4Eo in FAB classification). The inv(16)(p13.1q22)/t(16;16)(p13.1;q22) is a subtle rearrangement by conventional cytogenetic analysis, therefore FISH and RT-PCR may be necessary to detect the changes. This type of AML has favorable prognosis.

18.7.1.3 t(15;17)(q22;q21); *PML-RARA*

This translocation is present exclusively in acute promyelocytic leukemia (APL) (AML-M3 in FAB classification). APL patients commonly present with coagulopathy, due to disseminated intravascular coagulation (DIC), thus a prompt diagnosis is essential. Therefore, morphologic recognition or molecular detection of genetic abnormalities is important. Detection of the genetic changes alone is diagnostic, regardless of the blast count

There are two types of APL identified, hypergranular and hypogranular variants. Hypergranular APL represents 60–70 % of cases. The abnormal promyelocytes have numerous large cytoplasmic red to purple granules and Auer rods. Hypogranular or microgranular APL (AML-M3v in FAB classification) promyelocytes have fine granules and often bilobed (“apple-core” or “butterfly” shaped) nuclei.

The translocation results in fusion of the promyelocytic (*PML*) gene with the retinoic acid receptor, alpha (*RARA*) gene. The break point in *RARA* occurs consistently within intron 2. The breakpoints in the *PML* gene occur in three different sites, introns 6, 3 and exon 6. Accordingly, three forms of *PML-RARA* may be formed: long form (bcr-1), short form (bcr-3) and variable form (bcr-2) [87] (Fig. 18.3).

Wild-type *PML* functions as a tumor suppressor gene, and *RARA* has been shown to promote differentiation and to suppress growth. The fused *PML-RARA* acts as a dominant negative regulator preventing transcription of genes, thus interfering with myeloid maturation [88].

Cytogenetic analysis, FISH, or RT-PCR is necessary for detection of the *PML-RARA* fusion. In FISH, APL leukemic cells have a “microspeckled” pattern in

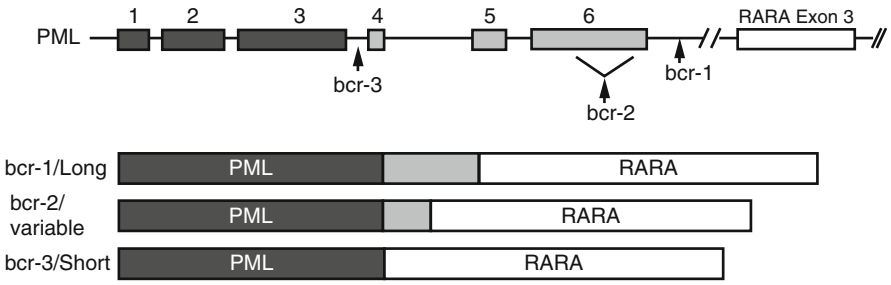

Fig. 18.3 Schematic representation of *PML* breakpoints and *PML-RARA* fusion transcripts. The 3 PML breakpoint cluster regions (bcr−1, −2, and −3) are indicated by *arrows*, and the resultant long, variable, and short *PML-RARA* isoforms are diagrammed

contrast to the normal speckled or macrospeckled pattern of staining for PML protein. RT-PCR is also a sensitive method for minimal residual disease monitoring.

18.7.1.4 t(9;11)(p22;q23); *MLLT3-MLL*

Translocations involving the MLL gene on chromosome 11q23 are associated with more than 70 different partner genes [89]. They are observed in approximately 6 % of AML cases [84]. The MLL protein is a histone methyltransferase that assembles in protein complexes that regulate gene transcription [1]. The t(9;11)(p22;q23) is associated with AML with monocytic features. It typically occurs in children with an intermediate prognosis. Using Southern blot or FISH, MLL probe can detect most translocations involving 11q23. PCR is not used routinely because of the large number of partner genes, but can be used for minimal residual disease, and multiplex RT-PCR is developed using multiple primers [90].

18.7.1.5 t(6;9)(p23;q34); *DEK-NUP214*

The t(6;9)(p23;q34) is observed in approximately 1 % of AML cases. The translocation results in a fusion of *DEK* with *NUP214*, and the fusion protein acts as an aberrant transcription factor and also affects transport factors. AML with t(6;9)(p23;q34); *DEK-NUP214* may have blasts with Auer rods and may exhibit monocytic features. It is often associated with basophilia and multilineage dyspoiesis [91].

18.7.1.6 inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*

The inv(3)(q21q26.2) or t(3;3)(q21;q26.2) occurs in 1–2 % of AML cases. The translocation involves the oncogene *EVI1*, or its longer form *MDS1-EVI1*, and *RPN1*. *RPN1* acts as an enhancer of *EVI1* expression, resulting in increased cell proliferation, and impaired cell differentiation [1]. High expression of *EVI* also suggests a poor prognosis. The morphologies are variable, including AML-M1, M4,

and M7 subtypes in FAB classification. Cytogenetics may not be able to detect the cryptic changes, which can be identified by FISH [92].

18.7.1.7 t(1;22)(p13;q13); *RBM15-MKL1*

The t(1;22) occurs rarely in infantile AML, taking approximately 1 % of childhood AML. The translocation results in a fusion of RNA binding motif protein-15 (*RBM15*), which encodes RNA recognition motifs and a spen paralog and ortholog C-terminal (SPOC) domain, and megakaryocyte leukaemia-1 (*MKL1*), which encodes a DNA-binding motif involved in chromatin organization [1]. The fusion protein may modulate chromatin organization, HOX-induced differentiation and extracellular signaling pathways [1]. The blasts show typical features of megakaryoblasts with a small amount of agranular cytoplasm with budding or blebs [84].

18.7.2 Other AML Subtypes

Among the other AML subtypes are AML with myelodysplasia-related changes, therapy-related myeloid neoplasms, and AML, not otherwise specified (NOS). AML with myelodysplasia-related changes has morphological features of myelodysplasia or a prior history of myelodysplastic syndrome (MDS) or myelodysplastic/myeloproliferative neoplasm (MDS/MPN), or MDS-related cytogenetic abnormalities. There are no specific genetic abnormalities. Cytogenetic changes are similar to MDS. Therapy-related myeloid neoplasms include therapy-related acute myeloid leukemia (t-AML), myelodysplastic syndrome (t-MDS) and myelodysplastic/myeloproliferative neoplasms (t-MDS/MPN) occurring as late complications of cytotoxic chemotherapy and/or radiation therapy. Following a long latent period after alkylating agent and/or radiation therapy, unbalanced chromosomal aberrations develop, mostly involving whole or partial loss of chromosomes 5 and/or 7, and are often associated with additional chromosomal abnormalities. These changes compose of 70 % of the patients. In contrast, following a short period after therapy, balanced chromosomal translocations may develop, including rearrangements of 11q23 and other abnormalities such as t(15;17) and inv(16). These happen in the remaining 20–30 % of the patients [1].

AML, NOS, includes AML categories based on morphologic and cytochemical criteria modified from FAB classification. The cases should not have recurrent genetic abnormalities, a history of prior therapy, or Down syndrome [1].

18.7.3 Molecular Markers in AML

Specific gene mutations in AML include frequent mutations of *FLT3* (fms-like tyrosine kinase 3), *NPM1* (nucleophosmin), *CEBPA* (CCAAT/enhancer-binding

protein alpha), *KIT, MLL* (mixed lineage leukemia), *WT1* (Wilm's tumor 1), *N-RAS* and *K-RAS*. By WHO classification, mutations can be divided into two classes: class I and class II mutations. Class I mutations constitute *FLT-3, KIT* and *RAS*, while class II mutations include mutation of *CEBPA* and probably *NPM1* [1]. Class I mutations occur late in AML or relapsed AML, integrating to more than one subtype of AML and play roles in predicting prognosis. Class II mutations, also including *PML-RARA*, *RUNX-1-RUNX1T1*, *CBFB-MYH11*, appear to be in earlier AML and affect hematopoietic differentiation and subsequent apoptosis [1]. Among aforementioned, mutations of *FLT3, NPM1* and *CEBPA* have been known to have a great impact on prognosis.

18.7.3.1 FLT3

FLT3 is located on chromosome 13q12, and it encodes a receptor tyrosine kinase. It is primarily expressed on normal myeloid and lymphoid progenitors as well as on the blast cells in AML [93]. An activation of FLT3 regulates a number of different cellular processes, including transcription, proliferation and apoptosis, and it plays critical roles in normal hematopoiesis and stem cell proliferation [94].

There are two distinct FLT3 mutations in juxtamembrane and kinase domains. Internal tandem duplications (FLT3-ITD) has been identified in a high proportion of patients with AML (75–80 %) [95]. The second most common (20–35 %) mutation involves codons 835 or 836 of the tyrosine kinase domain at exon 20 (FLT3-TKD, D835 or D836) [96].

FLT3-ITD mutations are duplication of a fragment within the juxtamembrane domain coding region, exons 14 and 15. The length of duplicated DNA can be between 3 and more than 400 base pairs [97]. The mutations lead to the receptor activation, and further stimulate cell proliferation [98, 99]. FLT3-ITD is the most common mutation in hematologic malignancies, occurring in AML (20–40 %), as well as in CML (5–10 %) and MDS (5–10 %) [1, 100]. In AML, it is most commonly in AML with t(6;9)(p23;q34), acute promyelocytic leukemia and AML with a normal karyotype [101, 102].

Importantly, FLT3-ITD is a significant prognostic factor, which is associated with an adverse clinical outcome. FLT3-TKD mutation remains controversial [1]. Detection of FLT3-ITD is critical to predict the prognosis of most cytogenetically normal AML.

18.7.3.2 *NPM1*

NPM1 is located on chromosome 5q35, and is a nucleocytoplasmic shuttling protein. It has multiple functions, including regulating *p53* and *ALK* tumor suppressors [103]. It can be involved in several translocations, such as *NPM-ALK*, *NPM-RARA*, and *NPM-MLF1* [104]. The mutations most commonly occur in exon 12, and rarely in exon 9 and exon 11. In exon 12, the mutations are usually duplications of TCTG

tetranucleotide at position 956–959. There are more than 40 *NPM1* mutation variants detected [105]. *NPM1* mutations occur in about one third of adult AML and in approximately 50–60 % of adult AML with normal karyotype [106, 107].

The presence of *NPM1* mutation, as an independent factor without combining *FLT3-ITD*, suggests a good prognosis and improved response to chemotherapy, while *NPM1* mutation combined with *FLT3-ITD* suggests an intermediate prognosis [108]. A provisional entity of AML with mutated *NPM1* is proposed in 2008 WHO classification [1]. In this AML type, blasts show monocytic differentiation and lack of CD34 expression, and are largely (85 %) with normal karyotype. It has a good response to induction therapy [105, 109]. Genetic features of AML with mutated *NPM1* include: It is specific for AML, mostly *de novo* AML; Usually all leukemic cells carry the mutation; It is mutually exclusive with other AML with recurrent genetic abnormalities; It is consistently retained at relapse; It usually precedes other associated mutations (e.g., *FLT3-ITD*); It has unique gene expression profiling (GEP) signature and distinct microRNA profile.

18.7.3.3 *CEBPA*

CEBPA (CCAAT/enhancer-binding protein-α) is located on chromosome 19q13.1, and it is a basic leucine zipper (bZIP) transcription factor. The normal protein functions in control of proliferation and differentiation of myeloid progenitors [110]. There are two types of *CEBPA* mutation: N-terminal nonsense mutations resulting in a truncated isoform with dominant negative activity, and C-terminal mutations in the bZIP domain interfering with DNA binding and homodimerization of the transcription factor [110]. *CEBPA* mutations are observed in 6–15 % of all AML [1]. Like *NPM1* mutations, *CEBPA* mutations are also frequently observed in AML with a normal karyotype.

CEBPA mutations are associated with a favorable prognosis in AML with normal karyotype. The clinical significance of combined *FLT3-ITD* and *CEBPA* mutations is still uncertain. A provisional entity of AML with mutated *CEBPA* is proposed in 2008 WHO classification. The AML subtype is equivalent to AML with maturation or AML without maturation and characterized with high blast count in peripheral blood.

18.7.3.4 Other Markers

C-KIT is a tyrosine kinase receptor of the platelet-derived growth factor family, located at 4q11-12. Gain-of-function mutations of *C-KIT* occur in multiple diseases, including gastrointestinal stromal tumors (GIST), germ cell tumors, mastocytosis and AML. The most frequent *KIT* mutations reported are point mutations at codon 816 of exon 17 and insertions/deletions in exon 8. Exon 17 mutations of *KIT* are associated with poor prognosis in AML with t(8;21)(q22;q22) and inv(16)(p13.1q22)/t(16;16)(p13.1;q22) [111]. The relevance of exon 8 mutations is still not clear.

Wilm's Tumor 1 (*WT1*) gene mutations have been observed in approximately 10 % of AML with normal karyotype, and the patients are usually younger than 60 years old [112]. *WT1* mutations are associated with poor prognosis in AML patients with a normal karyotype.

Mixed Lineage Leukemia (*MLL*) gene, located on chromosome 11q23, is involved in multiple chromosomal translocations with 71 partner genes [113]. *MLL* related hematologic malignancies are found in patients with AML related to topoisomerase II inhibitor therapy [114], as well as in ALL, MDS/tMDS, *de novo* AML, and acute leukemias of ambiguous lineage [115]. *MLL*-rearranged leukemias represent about 10 % of all leukemia cases [113]. The mechanism of *MLL*-related AML is largely elusive [113]. Speaking of mutation, a partial tandem duplication of *MLL* has been reported in 5–10 % of AML with normal karyotype and in patients with isolated trisomy 11 [1]. Presence of *MLL* gene has an adverse prognostic significance in AML with normal karyotype.

RAS mutations occur in 12–27 % of AML patients [116]. *NRAS* is mutated and constitutively activated in 10–20 % of AMLs, *KRAS* in 5–15 % of patients, and *HRAS* is rarely mutated [117]. *RAS* mutations do not have consistent prognostic significance.

Furthermore, the expression levels of *BAALC* gene (Brain And Acute Leukemia, Cytoplasmic), *ERG* (ETS-related gene), MN1 (Meningioma 1), and *CXCR4* genes, as well as gene expression profiles and microRNA expression patterns may also provide useful markers for AML [97].

18.8 Future Perspectives

The last decade saw the rapid progress in myeloid neoplasm diagnosis, response prediction, monitoring of minimal residual disease, and prognosis. Molecular studies are playing more and more important roles in the diagnosis and management of the patients with myeloid neoplasms. It is very likely that in the coming years, more genetic abnormalities, molecular markers or prognostic factors will be identified and the stratification of the diseases will change accordingly.

In a view of technological aspect, gene expression profiling (GEP) and microRNA profiling are the emerging diagnostic tools which may lead to more precise classification, with their enormous capacity to simultaneously quantify the expression of tens of thousands of genes [118, 119]. Moreover, these approaches may also contribute to prognostic stratification, targeted therapy and the detection of new molecular markers.

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