Ulrich Pfeffer *Editor*

Cancer Genomics

Molecular Classification, Prognosis and Response Prediction

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Chapter 1
Genomic Pathology of Lung Cancer

Kenneth J. Craddock, Shirley Tam, Chang-Qi Zhu, and Ming-Sound Tsao

Abstract Genome-wide studies of lung cancer began with RNA expression microarrays, followed by DNA copy number microarrays. More recently, microRNA profiling and high-throughput sequencing studies have entered the arena. Cancer genomics is a quickly moving field. Here, we summarize the pertinent findings of lung cancer genomics studies to date, with an emphasis on diagnostic, prognostic, and predictive findings that have been validated or confirmed by multiple studies.

Keywords Lung cancer • Genomics • NSCLC • SCLC • Adenocarcinoma • Squamous cell carcinoma • Mutations • Amplifications • Deletions • CNAs • RNA expression • miRNA expression • Prognosis • Predictive

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1 Introduction

Lung cancer is the leading cause of cancer-related death worldwide for men, and second leading cause for women [1]. In 2008, lung cancer was globally the cause of 1.38 million deaths, representing 23% of all cancer deaths [1]. Despite recent data showing decreasing incidence in developed countries and significant advances achieved in its treatment and early detection, lung cancer is projected to be the sixth leading cause of death by 2030, advancing from the ninth position in 2002 [2]. In North America, the mortality rate of lung cancer is greater than the three next most common cancers combined, colorectal, breast and prostate cancer [3]. Smoking is well known as the most important etiology for lung cancer, but many other environmental carcinogens have also been implicated, including arsenic, nickel, and radon [4]. It is estimated that the health benefits of smoking cessation would take approximately 20 years to realize, since as many as half of current new lung cancer patients are former smokers who have quit for more than 10 years [5]. Importantly, lung cancers of never-smokers demonstrate distinct clinical features compared to those of smokers, accounting for 15–20% of lung cancer cases worldwide [6]. Thus, lung cancer will remain a major global health burden for decades to come.

Currently, the overall 5-year survival rate for all lung cancer patients is about 15% [3]. This poor rate is largely due to diagnosis at a late stage of disease; about two-thirds of patients are initially diagnosed at a stage too advanced to be cured by surgery. The management of lung cancer patients is largely based on the histologic type and stage of the disease. Despite the complexity of lung cancer classification according to histology [4], two major subtypes with different biology and therapeutic responses are recognized: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is a highly aggressive cancer with almost all patients presenting with systemic metastases at diagnosis. Therefore, SCLC patients are treated primarily by chemotherapy. Yet, despite high initial response rate, very few SCLC patients are cured [7]. However, the incidence of SCLC has been decreasing during the last 30 years. The survival of NSCLC is correlated with the tumor stage at diagnosis [8].

Currently only one third of NSCLC patients are diagnosed with the tumor being localized still to the lung, and potentially curable by surgical resection [9]. Therefore, the greatest potential in reducing lung cancer mortality would come from an effective screening program, to discover the cancers in their earliest stage. A recent randomized lung cancer screening trial comparing chest X-ray and low dose spiral computed tomography (CT) has confirmed the effectiveness of the latter detection technique, which is capable of detecting sub-centimetre cancers [10]. Nevertheless, the risk of these "early stage" NSCLC patients to develop metastatic recurrences after surgery is still 30–60% [9]. While adjuvant chemotherapy can improve significantly the survival of these surgically treated patients, there remains a need for biomarkers to identify poorer prognosis patients, for whom adjuvant chemotherapy might improve survival [11].

For advanced or recurrent NSCLC patients, the standard first line treatment has been chemotherapy, with overall response rates of 30–40% [12, 13]. More recently,

however, it has been demonstrated that more effective therapies can be achieved by drugs that are designed to target specific molecule or pathway to which a tumor is "addicted." Two such targets have so far been identified, epidermal growth factor receptor (EGFR) [14–16] and anaplastic lymphoma kinase (ALK) [17, 18]. The protein product of these genes become bona fide targets when they are activated constitutively, by activating mutations in the kinase domain, high level amplification of the gene copy number, or a translocation which may bring the gene under a new transcriptional control, or form a novel chimeric fusion gene. Clinical trials have demonstrated that when patients with tumours "driven" biologically by these aberrant genes are treated by drugs targeting their kinase activities, response rates of >60% have been achieved. Targeted therapies can improve cancer treatment outcome only when they are used selectively on patients that harbour the targeted aberrant proteins. Thus, in order to tailor therapy, cancer diagnostics need to include profiling of these specific genetic abnormalities including gene copy number aberrations (CNAs) and mutations.

2 Somatic Mutations and Molecular Classification

The most frequently mutated gene in lung cancers overall is TP53, which is present in approximately 70% of SCLC [19], and 50% of NSCLC [19], more frequently in squamous cell carcinoma (SqCC) than adenocarcinoma (AdC) [20]. TP53 has broad tumour suppressive actions, mediating multitude effects including anti-proliferative, pro-apoptotic, transcriptional, DNA repair and metabolic pathways in response to a variety of stresses, including in particular DNA damage [20]. Most TP53 mutations occur within the DNA-binding domain [20], but may occur throughout the gene.

2.1 Mutations in Lung Adenocarcinoma

Aside from TP53, the most frequent mutations in lung cancer discovered so far have largely been restricted to adenocarcinoma histology [21]. A recent broad sequencing study of 623 cancer genes in 188 AdC of the lung found 17 significantly mutated genes [22]. These included in decreasing order of frequency TP53, CDKN2A, STK11, NF1, ATM, RB1, APC, LRP1B, PTPRD, and PTEN, and oncogenes KRAS, EGFR, NRAS, ERBB2, ERBB4, EPHA3 and nine other ephrin receptor genes, NTRK genes, KDR and other VEGFR family members, FGFR4, and other FGFR family members. Mutations in oncogenes BRAF and PIK3CA have also been reported previously [22, 23].

The most common mutated oncogenes involve *KRAS* and *EGFR*. *KRAS* mutations are found in 30–35% of lung AdC [19], most commonly on codon 12, and less frequently in codons 13 and 61 (193) [24]. The *RAS* genes encode a family of membrane-bound GTP-binding proteins that regulate cellular proliferation, differentiation, motility and survival by activating the MAPK, STAT, and PI3K

signaling cascades [25]. Point mutations in the GTP-binding pocket impair the intrinsic GTPase activity of RAS, causing it to accumulate in its constitutionally active GTP-bound state [20, 24].

Mutations on the tyrosine kinase domain of *EGFR* gene occurs in 10–50% of NSCLC [24], with significant geographic and ethnic variations. It occurs most commonly in AdC and uncommonly in SqCC. Mutations occur mainly on exons 18–21 [20, 24]. Approximately 90% are either short deletions of 4–6 amino acids on exon 19 or missense point mutation that result in a leucine-to-arginine substitution at codon 858 (L858R) [24]. However, many other mutations have also been reported. While exon 19 deletions and L858R mutation sensitize tumour cells to the apoptotic and/or anti-proliferative activity of EGFR small molecule tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, some mutations do not render the cells dependent on the EGFR pathway or are insensitive to the TKIs; the latter are called primary resistant mutations. In contrast, the T790M mutation has been identified in patients who develop clinical progression after initial response to TKIs, thus is called secondary resistant mutation.

The first recurrent gene fusion in lung cancer was reported to occur between two genes on the short arm of chromosome 2, anaplastic lymphoma kinase (ALK) and echinoderm microtubule associated protein like 4 (EML4), which are joined by a paracentric chromosomal inversion [26]. Subsequent studies have found this mutation to be present in up to 5% of lung AdC. Similar to EGFR, ALK is also a tyrosine kinase receptor, which has previously been identified as rearranged in anaplastic large cell lymphoma and inflammatory myofibroblastic tumour, among others. Fusion of exons 1–13 of EML4 with exons 20–29 of ALK creates a fusion gene that is under the promoter control of EML4, generating a fusion cytoplasmic protein that is constitutively dimerized and activated, thus triggering its downstream signaling pathways including MAPK, PI3K, and STAT3 that affect cell growth, proliferation, survival, motility and invasion.

2.2 Mutations Found in Squamous Cell Carcinoma of the Lung

Aside from TP53 mutations, which are found in a majority of SqCC of the lung, additional tumour suppressor gene mutations or oncogene "driver" mutations, have been elusive [27]. PIK3CA is one of the most commonly mutated oncogenes in human cancers [28], and recent studies have found mutations in 2–4% of NSCLC, more commonly in SqCC (4–6.5% compared to 1.5% in AdC) [21, 29, 30]. PI3Ks are lipid kinases that can be activated downstream of multiple receptor tyrosine kinases including EGFR [28]. Mutations in the helical and catalytic domains of its catalytic subunit (encoded by PIK3CA) on exons 9 and 20 result in elevated lipid kinase activity, leading to activation of downstream Akt signaling pathway [29]. The most common mutations that have been detected to date are E542K,

E545K and H1047L/R. Notably, the PIK3CA gene lies in a region of the long arm of chromosome 3 (band q26) that is within an amplicon that is significantly more common in SqCC than in AdC [29].

APC gene mutations have been described in up to 5% of SqCC and SCLC [31]. More recently, mutations in NOTCH1 and NOTCH2 were described in 5 of 40 SqCC of the lung [27]. Notch receptors participate in a highly conserved signal transduction pathway that regulates cell growth and survival [27]. In addition, the oncogenic kinase DDR2 is activated in ~5% of lung SqCC [27], and a DDR2 mutated cell line was shown to be sensitive to dasatinib, a multi-targeting tyrosine kinase inhibitor. The Cancer Genome ATLAS program is currently conducting a comprehensive molecular profiling of 500 resected lung SqCC using the next generation sequencing (NGS) technology. The results are expected to be published in 2012 and are anticipated to identify large number new mutations.

2.3 *Mutation Patterns in Lung Carcinomas*

Some mutations in lung carcinomas are mutually exclusive or tend to occur together. This is becoming easier to study with newer technologies capable of detecting multiple mutations in the same analysis, such as NGS. Ding et al. [22] sequenced 623 cancer genes 188 AdC of the lung and found the following mutations that tend to occur together in AdC: PIK3C3 and PTPRD; NTRK2 and PDGFRA; FGFR4 and NTRK2; FGFR4 and PDGFRA; EGFR and PIK3A; and RB1 and TP53. Mutually exclusive mutations included EGFR and KRAS; EGFR and other tyrosine kinase receptor genes; and ATM and TP53.

Mutually exclusive mutations are likely due to redundant effects on pathway dysregulation, while mutations that co-occur likely have cooperative effects on the same or parallel signaling pathways. Multiple studies have demonstrated that the two most common oncogene mutations in lung AdC, mutations on the *KRAS* and *EGFR* genes, are mutually exclusive [19, 24, 32]. The recently discovered *ALK-EML4* gene fusion is also reported to be mutually exclusive to *EGFR* and *KRAS* mutations [33, 34]. *PIK3CA* mutations on the other hand are not mutually exclusive to *EGFR* or *KRAS* mutations [28].

Ding et al. also found that mutations of *TP53*, *PRKDC*, and *SMG1* were each associated with higher rates of mutations involving other genes [22], which likely reflects that genomic instability is a consequence of mutations involving these three genes.

2.4 *Associations of Mutations with Patient Demographics*

Some notable clinical associations have been described with the more common mutations. *EGFR* mutations are significantly associated with female, never-smoking

and East Asian ethnicity, while *KRAS* mutations are more common among Caucasians and African Americans, and are significantly associated with smoking [19, 22, 24, 25]. *EGFR* mutations have been reported to occur in up to 75% of East Asian lung AdC never-smoked patients [35], while *KRAS* mutations occur in only 2% [36]. Also associated with smoking are mutations of TP53 [24] and STK11 [22], and an increase in the total number of detectable mutations in a given lung cancer [22], while ALK-EML4 mutations are more common in younger, never-smokers, with a comparable prevalence in Asian and Western patients and without gender preference [33, 34, 37].

2.5 Associations of Mutations with Histology

The low frequency of many newly identified mutations in lung carcinomas makes association studies with histology, prognosis, and therapies very difficult. However, the more common mutations, including *TP53, EGFR, and KRAS* have been well studied in this regard. KRAS mutations are most common in mucinous bronchioloalveolar carcinoma and/or goblet cell morphology, with expression of CK20 and CDX2, although a significant proportion of non-mucinous AdC also harbour mutations, while mutations are uncommon in SqCC [20, 24]. A recent study of 95 biomarker-verified SqCCs found that EGFR and KRAS mutations are completely absent in SqCC, and asserted that any such mutations described in SqCC were due to adenosquamous (mixed histology) carcinomas, or poorly-differentiated AdC that were misclassified as SqCC [21]. This study also reported absence of mutations in BRAF, NRAS, ERBB2, and MAP2K1 in SqCC, although the sample size is small.

EGFR tyrosine kinase domain mutations are found most commonly in AdC, while less common in other types [24]. Among AdC, these mutations appear to be significantly associated with bronchioloalveolar (recently changed to lepidic) and papillary subtypes, and are least likely to be found in the solid subtype [24, 38].

ALK-EML4 is much less common than the previously mentioned abnormalities, but has been associated with mucin producing AdC that demonstrate solid and signet ring morphology [33], or with an acinar or papillary predominant pattern [37]. An association has been reported with TTF-1 expression by IHC [37].

TP53 mutations have a somewhat higher frequency in SqCC (60–75%) than AdC (40–50%) [20, 32]. In Ding's study of 623 genes in 188 lung AdC, TP53 mutations were associated with higher tumour grade and a solid histology, and increased numbers of detectable mutations were associated with higher tumour grade and stage [22]. Some less frequent mutations were associated with grade as well, including LRP1B, INHBA, and PRKDC, while mutations of NTRK2, BRAF, EPHA7, PRKCG, and FLT4 were associated with higher stage [22]. However, the findings in these uncommon/rare mutations remain to be confirmed in larger number of tumor samples. BRAF mutations, although uncommon, have been reported to have a high incidence of papillary and lepidic histology patterns in AdC [23].

2.6 Prognostic Significance of Gene Mutations in Lung Cancer

Reports of prognostic significance of mutations in lung cancer have been variable. Several studies have reported an association of EGFR mutations with a favourable prognosis, irrespective of treatment received [20, 39–41]. This association may not be independent of other established prognostic factors such as smoking status and stage [42]. Mascaux et al. performed a meta-analysis of 53 published studies that assessed the prognostic value of KRAS mutations, and found an association with poor prognosis (hazard ratio for death of 1.50) in lung AdC [43]. However, in a phase 3 randomized adjuvant chemotherapy trial, *KRAS* mutation status was not prognostic for survival in early-stage NSCLC by univariate or multivariate survival analysis [44]. In a separate study, KRAS mutations were associated with poor prognosis and EGFR mutations with a good prognosis, by univariate analysis; however, multivariate analysis revealed only smoking status and stage to be significant predictors of survival [42].

TP53 mutation status has not been consistently associated with prognosis in multiple studies [20, 42, 44]. Cancers with both TP53 and KRAS mutations were found in one study to show a borderline poor prognostic significance, but the number of patients was low [20].

2.7 Mutations as Predictive Markers in Lung Cancer Treatment

The most exciting advancements in lung cancer treatment have come recently with the discovery of two targetable mutations in AdC; EGFR and ALK. In advanced NSCLC patients being considered for first line treatment with EGFR TKI or chemotherapy, sensitizing (exon 19 deletions and exon 21 L858R) EGFR TKD mutations are predictive of significantly higher response rate and greater progression free survival to TKI therapy [19]. The effect is pronounced, with 70–80% response rate for patients with EGFR mutation as compared to 10% response in those who have EGFR wild type tumours [45]. However, these pivotal trials have not shown differential improvement in overall survival, perhaps due to treatment cross-over confounding the results [46].

Despite pronounced initial responses to TKI therapy, almost all EGFR-mutant AdCs develop resistance at a median of 10 months [47]. The mechanisms for development of TKI resistance have been studied extensively, and the two most common mechanisms of resistance being a secondary mutation in the kinase domain of EGFR, and activation of other molecules downstream or in similar parallel pathways [40, 48].

The single most common resistance alteration, found in 50–60% of resistant cases, is a secondary mutation T790M, which occurs on the same allele (in cis) as the original activating EGFR mutation [40, 48]. Various EGFR TKIs including

some that irreversibly inhibit the EGFR TK domain are currently being investigated [49]. Strategies to overcome this may require the use of multiple pathway inhibitors that have yet to be devised and trialed.

Examples of alternate pathway activation as a mechanism for resistance to EGFR TKIs include MET amplification and PTEN loss [47, 49]. MET encodes for the receptor tyrosine-kinase that is stimulated by hepatocyte growth factor; amplification of this gene has been reported to account for TKI resistance in ~20% of cases [49]. PTEN encodes for the tumour suppressor phosphatase and tensin homologue, which negatively regulates the activation of phosphatidylinositol-3-kinase (PI3K) and the serine/threonine kinase Akt. These molecules are activated by EGFR, but loss of PTEN expression results in increased signaling of the PI3K-Akt pathway, independent of EGFR activation, and it has been reported to be associated with primary resistance to erlotinib and gefitinib [50].

In addition, some EGFR mutations may be associated with primary resistance to TKI inhibitors. While point mutations in exon 18 (G719A/C) and 21 (L858R and L861Q) and in-frame deletions in exon 19 are associated with TKI sensitivity [40], in-frame insertion mutations in EGFR exon 20, accounting for 3% of EGFR mutations [24], has been reported to decrease the sensitivity to erlotinib and gefitinib by 100-fold [24, 51].

In contrast to *EGFR, KRAS* mutant cancers tend to show poor or lack of response to EGFR TKI treatment [52, 53]. The pivotal JBR10 adjuvant chemotherapy trial reported that patients with wild-type *KRAS* status showed a significant benefit from adjuvant chemotherapy (cisplatin and vinorelbine) in stage IB-II NSCLC, while patients with *KRAS* mutations did not demonstrate benefit from adjuvant chemotherapy [44]. However, the interaction effect between chemotherapy, *KRAS* status, and response was not statistically significant. In contrast, another study found that response rate for patients treated with carboplatin and paclitaxel did not differ significantly by *KRAS* mutation status [25].

The most recent breakthrough in lung cancer therapy has been in patients with their tumours bearing the ALK-EML4 re-arranged genes. A pivotal Phase I trial demonstrated an objective response rate of 56% to the small molecule ALK inhibitor crizotinib in ALK rearranged patients and a median progression-free survival of 9.2 months in 105 patients with EML4-ALK-positive NSCLC [54]. However, these cancers eventually develop resistance to crizotinib, usually within 1 year [55]. Recent studies on tumors biopsied after patients experienced progression during crizotinib treatment have already identified secondary mutations of ALK, most frequently L1196M, C1156Y, and F1174L as a mechanism of resistance [55–58]. An in vitro study demonstrated that a more potent ALK inhibitor, TAE684, maintained substantial activity against mutations that conferred resistance to crizotinib [56]. However, secondary ALK mutations may be the mechanism for resistance in only a subset of cases; activation of bypass signaling pathways including KIT and EGFR have also been observed as a potential mechanism [55].

2.8 Molecular Classification of Lung Cancer

The emergence of subsets of AdC that are highly responsive to molecularly-targeted therapies (EGFR mutated, ALK rearranged), has spurred the concept of a molecular classification of lung cancer. Currently, the major categories in this classification are EGFR-mutated, KRAS mutated, and ALK-rearranged. This classification system is currently not formalized as the full mutation spectra is as yet to be determined and validated and the their clinical impact in term of prognosis and treatment remains to be fully elucidated.

3 Gene Copy Number Alterations (CNAs) in Lung Cancer

3.1 Analytical Platforms

There are many platforms to identify gene copy number changes in cancer cells. For single gene copy number changes, fluorescent in situ hybridization (FISH) is the most commonly used standard method. More extensive gene copy number profiling is usually performed by array comparative genomic hybridization (aCGH) [59, 60]. DNA from a tumor and a reference sample are differentially labelled and co-hybridized to a microarray containing DNA probes representing loci of the genomic sequences. Alternately, a single sample can be hybridized to individual arrays, and hybridization signals can be imaged and compared to other samples computationally. While early aCGH studies on lung cancer have commonly used array platforms focusing on specific regions of chromosomes [61–63], more recent studies have used arrays with genome wide coverage [64–67], including single nucleotide polymorphism (SNP) based oligonucleotide probe elements to deduce allelic information in addition to DNA dosage. However, the resolution of array platforms is significantly influenced by the density and the distribution of probes throughout the genome [68]. Most platforms have been applied to fresh or frozen tumour or cell line material, but few studies have successfully been carried out on formalin-fixed, paraffin-embedded (FFPE) tissue. Many array CGH platforms have been used for investigating CNAs in lung cancer genomes (Table 1.1). Comparison of tumor DNA against non-cancerous tissue from the same patient yields somatic alterations in the tumor genome. However, matched normal DNA is not always available for comparison, especially from archived biopsy materials. In such cases, the genome profiles of the HapMap panel are often used as a common reference [69–71]. Differences between a tumor and the reference profile may be attributed to copy number variation between individuals rather than somatic alteration. Much effort has been applied to catalogue the remarkable variations in segmental DNA copy in the human population [72, 73].

Table 1.1 Platforms used in array-CGH publications of lung cancer

Type of array	Number of references	# of loci	FFPE samples	Source
BAC	21	800–26,363	Yes	Research Institute (British Columbia Cancer Agency and Peter Mac), Nimblegen, Macrogen
cDNA	7	8,000–39,632	No	Agilent, Invitrogen
Oligonucleotide	13	25,000–1,000,000	No	Agilent
SNP	17	114,000–784,544	No	Affymetrix, Illumina

3.2 CNAs in NSCLC

The karyotypes of NSCLC invariably show polyploidy (in the range of 58–102 chromosomes per cell) and multiple complex chromosomal aberrations, resulting in net gain or loss of genetic material, indicative of genomic instability [74]. Lung cancer is a very heterogeneous disease, yet the copy number aberrations (CNA) profiles of the major histologic subtypes of NSCLC are remarkably similar, with frequent gains involving 5p, 8q, 3q, and 1q, frequent losses at 3p, 8p, 9p, 13q, and 17p [75]. Amplifications are commonly observed in the form of double minutes. Knowledge of the order or progression of these aberrations remains very preliminary; some investigators have speculated that early events include trisomy 7, loss at 3p and trisomy 12.

High resolution aCGH can identify CNAs in great detail, including genomic amplification and deletion involving only a few genes. Figure 1.1 depicts the frequency of copy number gains and losses across the genome in NSCLC. Consequently, the list of genes implicated in NSCLC is growing rapidly. The most commonly amplified regions in lung cancer include *MYC*, *TERT*, *CCND1*, and *EGFR*, but many other amplification targets have been identified in smaller percentages of cases. Tables 1.2 and 1.3 list the most frequent amplifications and deletions found in lung cancer by microarray studies, respectively, and their associated oncogenes and tumour suppressor genes [66, 77–79].

3.3 NSCLC Histology Subtype Specific CNAs

Many aCGH studies have focused their analysis on detecting differences in genomic copy number profile between different histological subtypes of lung cancer. A majority have examined specifically squamous cell carcinoma (SqCC) and adenocarcinoma (AdC), the two most common histologic types of NSCLC. Overall the patterns of aberrations were similar between AdC and SqCC; supervised or unsupervised clustering methods have for the most part been unable to differentiate them. However, focal distinct associations have been identified. By far the most

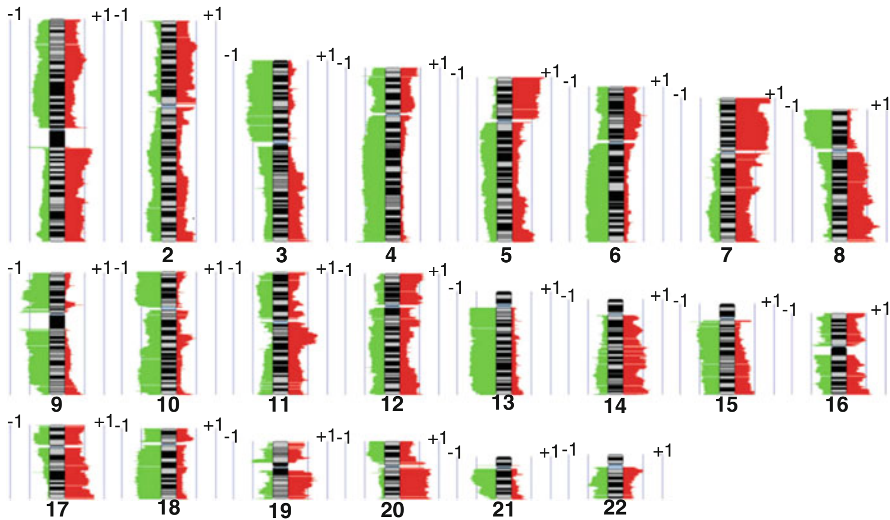

Fig. 1.1 Common DNA copy number changes in NSCLC. *Red* horizontal lines to the *right* of the chromosomes mark the frequency of gain at that location, and *green* horizontal lines to the *left* of the chromosomes mark the frequency of loss at that location. Vertical lines to the *left* and *right* of the chromosomes mark the 50 and 100% frequency levels (Reproduced with permission from Garnis et al. [76])

striking difference has been gains on long arm of chromosome 3 spanning a large 30 Mb region including bands q25 to q29, which is commonly found in SqCC [32, 77, 80]. The gains on 3q appear to occur in early stages of the cancer [80, 81]. Candidate oncogenes at this region include PIK3CA, TP63, SOX2, DCUN1D1, EVI1, MECOM, TP73L, FGF12, SST, and GLUT2. One of the obvious candidates in this group is SOX2, as identified by two recent high-resolution analyses [67, 82]. SOX2 plays critical roles in foregut development and is postulated to be a lineage-defined oncogene for squamous cell differentiation, proliferation and survival. SOX2 is considered one of the "magical four" crucial transcription factors capable of cooperating to reprogram differentiated cells into an induced pluriopotent stem cell-like phenotype. In the same region, TP63 is another notable gene [83], as immunohistochemistry for p63 protein is used commonly in clinical laboratories as a sensitive and specific marker for SqCC of multiple organs [84]. However, evidence is building that multiple genes are likely contributing to oncogenesis at the 3q26-29 region in SqCC [85].

A region of 8p12 has been reported as amplified in around 20% of SqCC, while AdC often show losses involving 8p [81]. Candidate oncogenes at this location include FGFR1, BRF2, and WHSC1L1. FGFR1 is discussed later in the text as a potential target for molecularly-directed therapy [78]. Other copy number alterations reported by more than one study to be significantly associated with SqCC include 2q gain, 3p loss, and 12p gain [32, 76, 80, 85].

Table 1.2 Major amplifications in lung cancer

Cytoband	Histology	Candidate genes
1p34.3-p34.2	NSCLC	BMP8B, COL9A2, MYCL1, PPT1, RLF, ZMPSTE24, PPIE, CAP1, HEYL, HPCAL4, TRIT1, OXCT2, NT5C1A, MFSD2, TMCO2, FLJ31434, YRDC, FLJ45459
1q32.2-q21.2	All	ARNT, LAMB3
2q14.2	SqCC	GLI2, TFCP2L1
3q11.2-q29	SqCC	PIK3CA, TP63, SOX2, DCUN1D1, EVI1, MDS1, TP73L, FGF12, SST, TGFA, and GLUT2
5p15.33-p14.3	All	BASP1, DAP, CTNND2, TERT, BRD9, IRX2, IRX1, ADAMTS16, MED10, SRD5A1, POLS, FASTKD3
6p21.1-p12.1	NSCLC	TINAG, C6orf142
7p11.2	NSCLC	**EGFR**, SEC61G, VSTM2A
7q11.21-q11.22	SCLC	PMS2L3, HIP1, ELN, FZD9, LIMK1, BAZ1B, GTF2IRD1, GTF2I, GTF2IRD2, GTF2IRD2B
8p12	SqCC	FGFR1, FLJ43582, WHSC1L1, LETM2, BRF2
8q24.21	NSCLC	**MYC**
11q13.2-q13.3	NSCLC	CCND1
12p12.1	NSCLC	**KRAS**, BCAT1, LRMP, SOX5, FLJ32894, LOC196415
12q13.2-q24.23	All	ERBB3, CDK4, MDM2, DYRK2
14q13.3	AdC	**NKX2-1**, MBIP
14q32.13-14q32.31	All	KIAA1622, RTL1
17q24.3-q25.3	SCLC	SOX9, CBX2
18q11.2-q12.3	NSCLC	hsa-mir-1-2, SNRPD1, MIB1, ESCO1, ABHD3, DSC1, DSC2, DSG2, PIK3C3, RIT2, SYT4
19q12-q13.33	NSCLC	CCNE1, C19orf12, LTBP4, NUMBL, SPTBN4, ADCK4, ITPKC, SHKBP1
20q11.21-q13.32	NSCLC	BCL2L1, TPX2
22q11.21-q12.2	NSCLC	MTMR3, ASCC2, HORMAD2

Adapted from references [66, 77–79]

Table 1.3 Major deletions in lung cancer

Cytoband	Histology	Candidate genes
4q21.3-q32.2	SqCC	MAPK10, GPR103, PCDH18, RAPGEF2, FSTL5
7q11.22-q34	NSCLC	TAC1, LOC154761
9p21.3	NSCLC	**CDKN2A**, **CDKN2B**, DMRTA1
10q23.2-q23.31	NSCLC	**PTEN**, ATAD1
11q11-11q21	All	OR4C11, OR4C6, OR4V1P, MAML2
13q12.11-13q32.3	All	**RB1**, HSMPP8, PSPC1, FOXO1, ELF1, ITM2B, FARP1

Adapted from references [66, 77–79]

For AdC, the main histology-specific CNA has recently been recognized in two large and comprehensive studies. Weir et al. [66] identified 14q13.3 amplifications containing NKX2-1 (TITF1) in 6–12% of 528 AdC samples by aCGH, and 12% of a separate set of 330 AdC by FISH. Kwei et al. [86] noted a similar proportion,

11% of their 36 AdC, while only 1 of 40 SqCC showing this amplification using aCGH. Notably, NKX2-1/TTF1 amplification has not been identified in a total of 385 cancers of the breast, prostate, colon, and pancreas, providing further evidence for its specificity for lung AdC.

NKX2-1 is a tissue-specific transcription factor required during normal lung development and differentiation of pulmonary epithelial cells. It is expressed in approximately 80% of AdC, but rarely in SqCC [87]. Its lineage-specific expression is routinely employed by pathologists to support the pulmonary origin of an adenocarcinoma, despite its role as a lineage marker of thyroid follicular cells and carcinoma.

3.4 SCLC Specific CNAs

A major barrier in studying the genetic lesions of SCLC has been the lack of adequate tissue for studies, since these tumours are rarely resected by surgery. Consequently, aCGH studies on primary SCLC samples are relatively few; instead many researchers have focused on examining cell lines [88–90]. Tables 1.2 and 1.3 also include common amplifications and deletions that have been associated with primary SCLC.

Few studies have compared the CNAs of SCLC versus NSCLC by aCGH. Coe et al. [91] found some striking similarities including gains at 5p, 7p, and 11q and losses at 3p, 4q, but also identified differences at multiple loci on 1p, 2p, 3q, 5q, 6q, 7p, 8q, 9p, 10q, 12q, 13q, 17q, 18p, 18q, 19p, and 21q (Fig. 1.2). Most striking among these were common gains at 21q11.2-22.3 in SCLC, while the same region was frequently deleted in NSCLC lines. The lack of 8q21.2-22.3 gains in SCLC lines was also apparent compared to the very frequent gains in NSCLC at this region. It was postulated that cell cycle upregulation in SCLC and NSCLC occurs through distinctly different mechanisms, suggesting a need for differential therapeutic target selection in these two malignancies. Peng et al. [92] also found that gains at 18q11-22 and losses at 1p and 16q were infrequently in NSCLC compared to SCLC, suggesting these regions might play a pathogenetic role specific to high-grade neuroendocrine tumours. These findings are similar to those reported with conventional CGH analyses [74, 93].

3.5 CNAs in Large Cell Neuroendocrine Carcinoma (LCNEC)

Peng et al. [92] also examined 31 primary LCNEC and observed similar frequencies of most frequent CNAs in both SCLC and LCNEC, consistent with results of previous allelotyping, chromosomal CGH, and karyotyping analyses [74]. However, losses at 3p and 4q were significantly associated with SCLC, while 2q gains and 6p

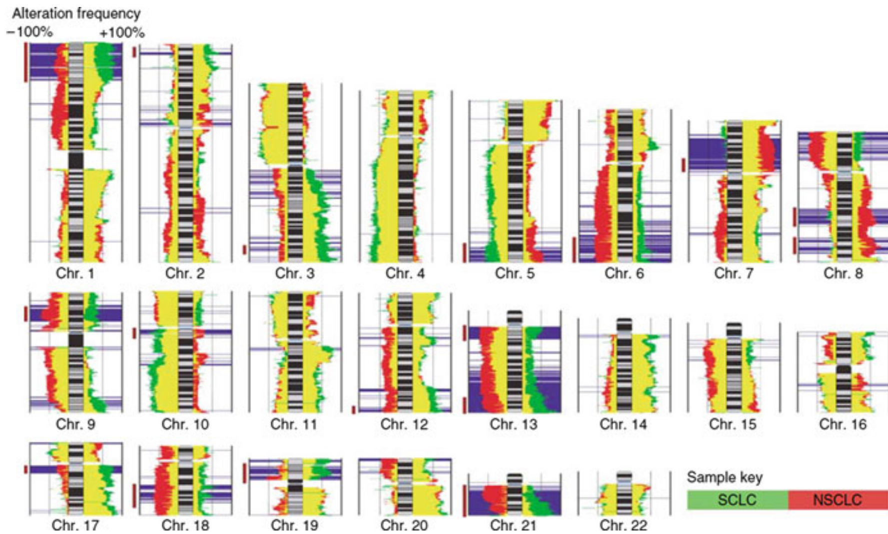

Fig. 1.2 Comparison of copy number changes in SCLC and NSCLC. Horizontal lines to the *left* and *right* of the chromosomes represent frequency of loss and gain, respectively, from 0 to 100%. SCLC frequencies are marked in *green*, and NSCLC frequencies are marked in *red*. Where overlapping, the lines are *yellow*. Vertical bars to the *left* and *right* of the chromosomes represent 50 and 100% frequency marks (Reproduced with permission from Coe et al. [91])

losses were associated with LCNEC. These CNAs were present even in early stage I tumours, suggesting that they may be early events in tumour development, thus potentially play a role in the histological divergence between these two types of high grade neuroendocrine carcinomas.

3.6 CNAs in Bronchial Carcinoid

Aside from SCLC, Voortman et al. [79] also studied 19 carcinoid tumors of bronchial and nine gastrointestinal origins. When comparing to SCLC, the carcinoids revealed significantly less complex alterations often localized to narrower gene regions. CNAs that were very infrequently observed in carcinoids included 17p loss involving TP53, and amplifications involving MYC gene family members. High copy number gains found mainly in four distinct cytobands in SCLC were not observed in carcinoids. On the other hand, deletion of 11q13 involving the MEN1 gene was relatively specific for bronchial carcinoids, and much less frequent in carcinoids of the GI tract. A number of other alterations, involving 203 genes and 59 microRNAs, were common to both tumour types, including most notably loss of RB1 and gain of DLK1-DIO3 domain. The latter has been postulated to be specific for neuroendocrine tumours.

3.7 CNAs Associated with Early Stages of Neoplastic Progression in NSCLC

Evaluations of heavy smokers by serial bronchoscopies and biopsies have established the histologically defined epithelial lesions that epitomize neoplastic progression leading to SqCC. These lesions include squamous metaplasia (SM), increasing grades of dysplasia and carcinoma-in-situ [61, 62, 81, 94, 95]. van Boerdonk et al. [96] reported that copy number alterations at 3p, 3q, 9p, and 17p in SM were predictive of subsequent development of invasive SqCC. Along the same vein, Schneider et al. [97] have proposed SOX2 amplification on 3q as a sensitive marker for screening or early detection of SqCC in bronchial washings or possibly sputum samples.

Atypical adenomatous hyperplasia (AAH) and bronchioloalveolar carcinoma are regarded as putative precursor lesions of peripheral invasive lung adenocarcinoma [98, 99]. Bronchioloalveolar carcinoma is defined as AdC with tumor cells growing in a lepidic pattern along pre-existing lung alveolar septae, without histological evidence of invasion [4]. In a more recent proposal for new classification of lung AdC, bronchioloalveolar carcinoma has been replaced by the term lung adenocarcinoma in situ (AIS), recognizing the pre-invasive nature of these tumors and the 100% survival of patients following complete surgical resection [100]. To identify markers of invasion, Aviel-Ronen et al. [65] used a tiling bacterial artificial chromosome (BAC) arrays to compare the CNAs of bronchioloalveolar carcinoma to invasive AdC with bronchioloalveolar carcinoma-like features (AWBF). They were able to demonstrate a progression with respect to numbers of CNAs between bronchioloalveolar carcinoma and AWBF, but not between pure bronchiolocarcinoma and bronchioloalveolar carcinoma with focal areas of invasion. They generated a list of 113 differentially gained genes in AWBF compared to bronchioloalveolar carcinoma, and furthermore found that a fair proportion of these genes were prognostic markers for early-stage AdC, using publically available expression database.

3.8 DNA Copy Number Abnormalities as Prognostic Markers

Relatively little conclusive information is available regarding the application of CNAs detected by aCGH for prognosis in lung cancer. Many studies do not include patient follow-up in their analysis, and among those that do, the patient numbers are often too small to make strong conclusions. Of the associations that have been made, most have not been validated on separate patient cohorts, thus could represent chance associations within their particular dataset. However, a few survival associations have been made in multiple independent studies. Gains of 8q and loss of 13q material have both been associated with poorer disease-free survival (DFS) in AdC, and with distant metastases in SqCC [101, 102]. In addition, 8q gains

have been associated with prognosis in LCNEC patients [92]. Candidate genes at 8q include MYC, PVT1, GLI4, RECQL4, CDH17, SPAG1, RAD21, MTBP, HAS2, and ANGPT1, and at 13q LCP1 has been suggested.

Notably, three of the four studies reporting the association of 8q gains with poor patient outcome were in the Japanese patients. Given the known geographic and ethnic differences in the occurrence of other genetic aberrations in lung cancer, most notably EGFR mutation, it is possible that these prognostic associations may be ethnicity or region specific.

Another potential prognostic marker is a deletion at 4q which were found to be associated with lymph node metastases in SqCC in one study, as well as presence of disseminated tumour cells in the bone marrow and metastasis to the brain in another study [102, 103]. Candidate genes in this region include MAPK10, GPR103, PCDH18, RAPGEF2, FSTL5 [78].

3.9 DNA Copy Number Abnormalities as Predictive Markers

Applications of aCGH as predictive markers for lung cancer are currently very limited; however this will likely change in the near future, as more and more molecularly targeted therapeutics are developed for their high efficacy against specific genetic lesions. The latter include activating mutations and/or amplification involving oncogenes, which render tumor cell dependence on these aberrations. The following examples illustrate how focal amplifications can expose specific oncogenes that are driving the growth of a given lung cancer, and how removing this driver using an appropriately-directed therapy can result in dramatic treatment response.

EGFR mutations and amplifications in lung AdC have been established as predictive of higher response rate to treatment by EGFR tyrosine kinase inhibitors (TKIs) [14–16, 104, 105]. While TK domain mutations are superior predictive marker of response, amplification may also be predictive of survival outcome when TKIs were compared to no treatment in advanced NSCLC patients who have failed multiple previous chemotherapies. Nevertheless, it is clear that a majority of EGFR mutant tumors demonstrate amplification of the mutant allele [106, 107]. Most copy number studies have employed FISH to detect the presence of amplification. In practice most centres have chosen to test for common mutations in order to select patients for EGFR TKI therapy, but this approach may be missing a subset of patients with amplification in the absence of mutation, who may derive significant benefit from TKI therapy [105].

In more recent studies, FGFR1 amplifications have been demonstrated in up to 20% of SqCC, associated with current smoking status [78], and cell lines carrying FGFR1 amplifications were shown to be sensitive to PD173074, an FGFR inhibitor [78]. Similarly, a Bcl-2 antagonist, ABT-737, has shown effectiveness in cell lines with increased BCL2 copy number, which could be potentially useful in the subset of SCLC that carry amplifications at this site [89]. These experiments are likely just

the tip of the iceberg. Directed inhibitors are currently available or in development for every major molecule in every known oncogenic pathway, and lung cancers frequently show amplifications involving one or more of these oncogenes.

4 RNA Expression in Lung Cancer

4.1 Prognostic RNA Expression Signature

The emerging use of biomarkers may enable physicians to make decisions based on the specific characteristics of individual patients and their tumors, instead of population statistics [108]. The search for the terms "gene expression signatures AND non-small cell lung cancer OR NSCLC OR adenocarcinoma OR squamous cell carcinoma AND prognostic OR prognosis" in PUBMED as of December 18, 2011 identified 1,205 papers. However, when focusing specifically on reports in which signature identification was attempted, 37 pertinent studies were found (Table 1.4). Overall, minimal overlap has been observed among these signatures (depicted in Fig. 1.3 for studies after 2005 and Lau et al. [128] for earlier studies, genes in small gene sets classifiers are listed in Table 1.5). However, protein-protein-interaction analysis has shown that they interconnect to form pathways [138, 139], suggesting that these pathways may be critical in lung cancer oncogenesis and progression. To date, there is no fully validated gene signature that is used clinically to predict risk of death or metastatic recurrence in lung cancer [142].

4.2 Analytical Platforms for mRNA Expression Profiling

Multiple technologies have been used for systematic and high-throughput interrogation of mRNA expression of large number of genes, such as microarrays, reverse transcriptase quantitative PCR (RT-qPCR). The DNA microarray platform has the scalability that enables global studies to measure mRNA expression of nearly all annotated genes from the human genome. While the overall technical performance and characteristics of the platform have steadily improved, microarrays have certain limitations in analytical specificity and sensitivity. In contrast, the qPCR method lacks in scale, but provides greater dynamic range, higher sensitivity, specificity, greater flexibility in experimental setup, thus has been regarded as a validation "gold-standard".

Currently several high-density oligonucleotide arrays are commercially available [e.g., Affymetrix (Santa Clara, CA), and Agilent (Santa Clara, CA), and Illumina (San Diego, CA)] and they differ in the length of probes (25 or 60 nucleotides) that are arrayed, the method for arraying the probes (e.g., photolithography, inkjet-type printing, bead-based, direct spotting), and the preferred methods for processing

Table 1.4 Gene expression signatures in NSCLC

Study	Histology	Stage	Survival outcome	Number of patients		Platform (method)	Classifier development	Genes in classifier
				Training	Testing			
Garber et al. [109]	AdC	I–III	OS	31	NA	Stanford 24K	Clustering	835
Beer et al. [110]	AdC	I, III	OS	43	127 (M)	HUgeneFL	Risk score	50
Bhattacharjee et al. [111]	AdC	I–III	OS	125	NA	U95Av2	Clustering	169
Miura et al. [112]	AdC	I–II	5-y OS	18	NA	Custom	Clustering	27
Wigle et al. [113]	NSCLC	I–III	DFS	39	NA	OCI 19.2K	Clustering	22
Gordon et al. [114]	AdC	I	4-y OS	75	157 (M)	HUgeneFL	Fold change ratio	4
Ramaswamy et al. [115]	AdC	I–II	OS	62	279 (M)	U95Av2	Supervised clustering	17
Blackhall et al. [116]	NSCLC	I–III	DFS	38	92	qPCR	Clustering	11
Endoh et al. [117]	AdC	I–IV	OS	85	21	qPCR	Risk Index	8
Liu et al. [118]	AdC	NA	OS	Cell line	18	OCI 19.2K	Clustering	117
Parmigiani et al. [119]	NSCLC	I–III	OS	242	NA	U95Av2	None	14
Tomida et al. [120]	NSCLC	I–III	OS	50	6	Invitrogen	Clustering	25
ibid	SqCC	I–III	OS	16	3	Invitrogen	Clustering	19
ibid	non-SqCC	I–III	OS	34	3	Invitrogen	Clustering	12
Chen [121]	NSCLC	I–III	OS	63	208	Custom + qPCR	Risk score	5
Guo et al. [122]	AdC	I, III	5-y OS	86	84 (M)	HUgeneFL	Feature-selection	37, 8
Lu et al. [123]	NSCLC	I	OS	197	127 (M)	Multiple	Risk score	64
Raponi et al. [124]	SqCC	I–III	OS	126	36	U133A	Risk score	50
ibid	NSCLC	I–III	OS	212	72	Multiple	Risk score	50, 50
Bianchi et al. [125]	AdC	I	OS	195	79 (M)	Multiple	Risk score	10
Larsen et al. [126]	AdC	I–II	Rec	48	95 (M)	Operon 22k	Risk score	54
Larsen et al. [127]	SqCC	I–III	Res + DSD	51	129 (M)	Operon 22k	Risk score	111

Lau et al. [128]	NSCLC	I–III	OS	147	214 (M)	qPCR	Risk score	3
Sun et al. [129]	SqCC	I–III	OS	129	89	U133A	Risk score	50
ibid	AdC	I, III	OS	86	302	HUgeneFL	Risk score	50
Raz et al. [130]	AdC	I–III	OS	107	NA	qPCR	Risk score	4
Skrzypski et al. [131]	SqCC	I–III	OS	66	26	qPCR	Risk score	3
Hsu et al. [132]	NSCLC	I–III	OS	111	442 (M)	Multiple	Risk score	4
Xie et al. [133]	NSCLC	I		442 + 55FFPE	228 (M)	Multiple	PCA	59
Chen et al. [134]	NSCLC	I–III	5-y OS	442	672 (M)	Multiple	PCA	120
Chen et al. [135]	NSCLC	I–III	5-y OS	680	101	Multiple	Random survial forest	91
Guo et al. [136]	AdC	I–III	OS	256	186	U133A	Relief selection	13
Shedden et al. [137] method A	AdC	I–III	5-y OS	256	186 (M)	U133A	Risk score	100
ibid method B	AdC	I–III	5-y OS	256	186 (M)	U133A	Risk score	52
ibid method D	AdC	I	5-y OS	117	119 (M)	U133A	Clustering	42
ibid method F	AdC	I–III	5-y OS	79	186 (M)	U133A	PCA	42
ibid method G	AdC	I–III	5-y OS	256	186 (M)	U133A	PCA	38
ibid method H	AdC	I–III	5-y OS	80	186 (M)	U133A	Risk score	313
ibid method C	AdC	I–III	5-y OS	177	186 (M)	U133A	Clustering	26
Zhu et al. [138]	NSCLC	I–III	DFS	62	375 (M)	U133A + qPCR	MARSA	15
Zhu et al. [139]	SqCC	I–III	5-y OS	129	182 (M)	U133A + qPCR	MARSA	12
Navab et al. [140]	AdC	I–III	5-y OS	218	218	U133A	MARSA	11
Wan et al. [141]	AdC	I–III	OS	256	186 (M)	U133A	Risk score	21 sets

OS Overall survival, *5-y OS* 5-year overall survival, *AdC* Adenocarcinoma of the lung, *SqCC* Squamous cell carcinoma, *NSCLC* Non-small cell lung cancer, *PCA* Principal Component Analysis, *MARSA* MAximizing R Square Analysis, *M* Validated in multiple data sets, *NA* Not applicable

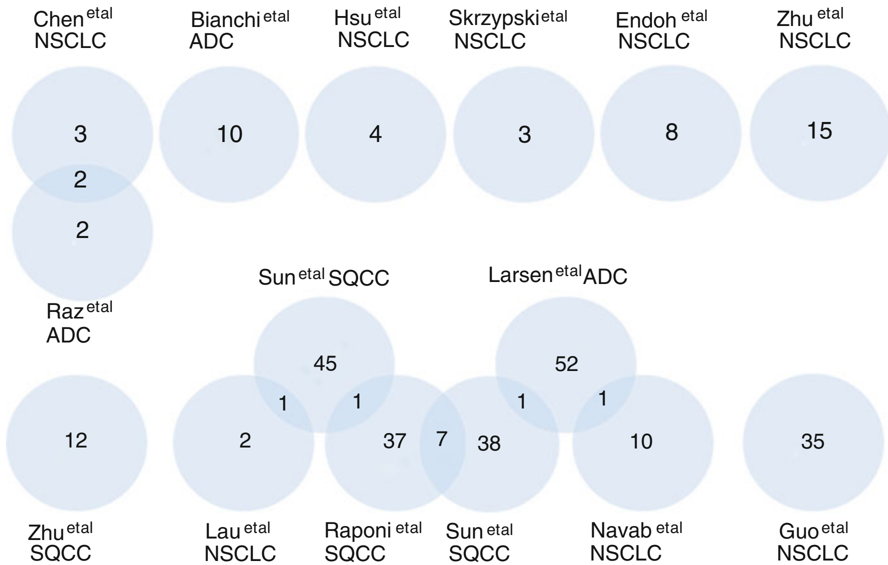

Fig. 1.3 Lack of overlaps in RNA gene expression signatures: prognostic gene sets from Microarray or qPCR studies (2005–2012)

RNA samples to be hybridized (e.g., sample amplification and labeling). A majority of previous studies on gene expression signatures in lung cancer have employed the Affymetrix "GeneChip" arrays of three generation versions, the Hu95, U133A and U133plus 2 arrays. The MicroArray Quality Control (MAQC) consortium was formed to conduct a systematic and detailed evaluation of the technical reproducibility and performance of multiple microarray formats as compared to each other and to real-time qPCR. The group has determined that current commercially produced microarrays have good reproducibility among and between different formats [143], and this can be attributed to proper laboratory and data analysis practices [144]. In addition, microarray-based gene measurements showed good concordance with other quantitative gene expression platforms, particularly qPCR [145, 146].

Several detection methods have been developed for RT-qPCR that either involve the presence of a non-specific DNA-intercalating dye (e.g., SYBR Green) or some form of a gene-specific probe that contains a fluorescent dye (e.g., Taqman, Molecular Beacon) [147]. The qPCR has emerged as the preferred method to validate microarray results for smaller gene sets (reviewed in [116, 117, 125]). Several recent NSCLC prognostic gene expression studies have relied on this platform, possibly also for potential rapid transfer of the assay to a clinical laboratory setting [121, 125, 128, 131]. Due to its limited scale for screening, qPCR is used mainly in studies on gene sets that have been previously identified as putative prognostic genes by microarray studies or due to their assumed role in malignant processes [117, 121, 125, 128, 131].

Table 1.5 RNA expression signatures with small number of genes

8-gene [117]	5-gene [121]	3-gene [128]	10-gene [125]	3-gene [131]	4-gene [140]	4-gene [110]	15-gene [139]	12-gene [138]	11-gene [120]	13-gene [136]
PTK7	DUSP6	STX1A	NUDCD1	CSF-1	WNT3a	ANKRD49	ATP1B1	RPL22	ICAM-1	DNAJA2
CIT	MMD	HIF1A	E2F1	EGFR	ERBB3	LPHN1	TRIM14	VEGFA	THBS2	EEF1B2
SCNNIA	STAT1	CCR7	HOXB7	CA IX	LCK	RABAC1	FAM64A	G0S2	MME	FBXO31
PGES	ERBB3		MCM6		RND3	EGLN2	FOSL2	NES	OXTR	GRHL2
EST-LB4D	LCK		SERPINB5				HEXIM1	TNFRSF25	PDE3B	GRK6
ERO1L			E2F4				MB	DKFZP586P0123	CLU	GTPBP1
ESTAA434256			HSPG2				L1CAM	COL8A2	B3GALT2	IRF3
ZWINT			SF3B1				UMPS	ZNF3	EVI2B	KIAA0040
			RRM2				EDN3	RIP5	COL14A1	MAGEB4
			SCGB3A1				STMN2	RNFT2	GAL	MRPL39
							MYT1L	ARHGEF12	MCTP2	RPS6KA1
							IKBKAP	PTPN20A/B		TNNI1
							MLANA			UGT2B4
							MDM2			
							ZNF236			
Secondary validations										
qPCR	qPCR MicroAR	MicroAR	qPCR MicroAR	qPCR	None	MicroAR	qPCR MicroAR	qPCR MicroAR	MicroAR	MicroAR

qPCR Validated in independent cohort profiled by qPCR, *MicroAR* Validated in independent cohorts profiled by microarray

The next generation sequencing (NGS) platforms, or massively-parallel sequencing, provide genome-wide information with applications ranging from RNA expression, chromatin immunoprecipitation, mutation mapping and polymorphism discovery to non-coding RNA discovery [148]. NGS has the added advantage of detecting alternatively spliced variant or fusion (re-arranged gene) transcripts. The nCounter® Gene Expression Assay (NanoString Technologies, Seattle, WA) is a new platform bridging the gap between genome-wide (microarrays) and targeted (qPCR) expression profiling for detecting the expression of up to 800 genes in a single reaction with high sensitivity and linearity across a broad range of expression levels [149]. As these are as yet no reports of gene expression signatures using these two new techniques, these will not be discussed further.

4.3 RNA Expression Data Preprocessing and Filtration

Microarray is characterized as a powerful but relatively "noisy" platform for genome-wide gene expression profiling. While some of the early studies employed customized or home-made cDNA arrays, Affymetrix, Illumina, and Agilent chips dominate the field nowadays. These arrays are characteristically designed having multiple probes for each gene. An algorithm is then required to assimilate the data from the set of probes into a single expression value. There are tangible advantages provided by the use of multiple probes but the high complexity provides both a challenge and an opportunity to develop alternate algorithms in attempt to gain greater accuracy. A data preprocessing is needed to gain quantitative measurements of mRNA expression and also attempt to correct for technical variation and noise. Microarray Analysis Suite 5 (MAS5) [150] has been used extensively for analysis of data generated by the Affymetrix chips, however, independent groups have developed alternate preprocessing algorithms for array normalization that include Robust Multichip Average (RMA) [151] or its variant Gene-Chip RMA (GCRMA) [152] and dCHIP [153] since 2004. These have been the most commonly used methods in NSCLC studies. Although these methods differ in the specific strategies that are employed, they all perform several common functions that include subtraction of background noise, correction for potential biases arising from differences in probe hybridization characteristics, and summing multiple probe measurements into one expression measure. While none of different preprocessing and normalization methods is clearly inferior, there is evidence to suggest that RMA may provide more reproducible results [154] especially when expression level is low [155, 156]. Another approach for removing the noise and unreliable data in microarrays is data filtration, either through an arbitrary cutoff of expression level, this is, gene expression below a certain level was excluded [135, 137], or through its association with end points of interest, e.g., survival [127, 138, 139]. Recent publications have reported a mean of data filtering by using coexpression and its association with hallmark genes such as TP53, MET, RB1, EGF, EGFR, KRAS, E2F1, E2F2, E2F3, E2F4, and E2F5 [136, 141].

For the RT-qPCR platform, preprocessing methods are also critical for converting raw fluorescence data to a gene measurement. These processes are not typically accessed or modified by users; instead rely mostly on the algorithms that have been incorporated with the qPCR instrument by each manufacturer. Efforts have been dedicated to determine the ideal normalization strategies for RT-qPCR expression data. It has been a common practice to use a single gene; however, a growing number of studies have used panels of 4–10 genes and used the geometric mean to normalize data between samples. In cancer, the commonly used "housekeeping genes" themselves may be aberrantly expressed or repressed depending on the tumor type. Therefore, normalizing against multiple housekeepers may provide more stable expression levels of the target genes. The selection of normalizer genes, whether single or in a panel, remains largely empirical.

4.4 Gene Expression Signature Selection and Risk Score

There is an urgent demand for the development of bioinformatics and biostatistical methods to handle the massive data from microarray. The main challenge is the number of variables (which can be termed markers, probe sets, genes, or features) is large in comparison to the number of subjects tested and thus making classical statistical analyses inadequate to deal with the datasets. The new methods being developed have two general goals: (a) reduce the data dimensions and (b) find a way of combining these markers into an algorithm for easier implementation in the clinical setting.

The common primary outcome used in the studies was survival, defined as the time from diagnosis to death or last follow-up. Recognizing that longer duration of follow-up may result in non-cancer related mortality, most of the studies truncate the survival at 5-years for overall survival analysis, since after this time patients with NSCLC frequently die of non-lung cancer-related causes, particularly among the elderly [137–139, 157, 158]. Some studies have also considered time to relapse or cause specific survival as alternative endpoints. One advantage of categorizing patients by survival time is the option of more deliberately defining the outcome using specific criteria. Some groups have chosen to compare groups by taking patients with extreme survival outcomes (early death compared to long term survival). While this approach may identify genes that have a significant impact on outcome, the results will likely not be applicable to the majority of patients who have intermediate outcomes [127].

There are a number of methods used to identify prognostic signatures. Generally they can be grouped into two broad types, rank based and model based. The rank based methodology was used in the vast majority of studies. In a typical scenario for rank-based signature identification, markers were selected after being ranked based on a specific measure (p-value, Cstatistics/C-index or other non-standard quantities) and the top markers in the list were chosen. Other criteria have been

described whereby the selection of markers was based on variances where markers with small variance within replicates but large variance between samples were selected [111], signal to noise metric [115], a combination of p-values and fold change between the two outcome groups [125], combination of the markers from the different publicly available dataset [119, 125] or markers that have high correlation coefficients between microarray and qPCR assays [121].

Some investigators have taken a strategy of being more relaxed initially in their selection criteria by keeping a larger number of candidate markers to build a predictive model. "Method A" in the "Director's Challenge for Molecular Classification of Lung Adenocarcinoma" (DC) study applied clustering to the group of markers in 100 clusters, using in the analysis, the average of probe sets for each of the 100 clusters [137]. This method also applied a more sophisticated analysis, ridged Cox regression, to their 100 composite variables. The same group of researches subsequently included even more cluster initially ($n = 300$) and employed the random survival forest model for the identification of a 91 genes signature prognostic for lung cancer [135]. Using the same methodology, Lu et al. [123] were able to select significant markers when the model included clinical factors. Regardless of the method used for marker selection, for all statistical tests that calculate a p value, it is important to consider the effect of multiple testing particularly when using microarrays to interrogate thousands of data points. For an α level of 0.05 ($p < 0.05$), there is approximately a 5% chance of detecting a false positive. Therefore for 30,000 probes, using the same cut off, 1,500 discovered genes could be false positives. To reduce the number of false positives when large data sets are tested, multiple testing corrections can be used. There are several methods to achieve this, with the most stringent one being the Bonferroni correction. The Bonferroni method effectively divides the α level (the level below which a test is considered significant) by the number of tests performed. So for an overall 5% Type I error with 30,000 genes to be tested, Bonferroni correction changes the α level to 0.05/30,000. This is a highly rigorous method of correction, which conversely increases the chance of a Type II error (i.e., not detecting a true positive).

Rank based method has been shown to be effective and successful in identify gene expression signatures, however, it is not effective in modeling complex molecular interaction in disease. Genes and proteins do not function in isolation, rather they interact with one another to form modular network [159]. Molecular network analyses have shown to be useful in disease classification [160] and identification of novel therapeutic targets [161]. Nevertheless, major challenges include the modeling of genome-scale coexpression networks and the identification of driver genes, from among the enormous number of potential markers, that has the highest prognostic ability for disease outcome [162]. The model based method used heuristic algorithms to select "driver" genes in terms of optimizing mathematical model to maximally explain survival variations among patients (Method C in Shedden et al. [137]).

4.5 Prognostic Gene Expression Signatures for NSCLC

Of the 37 studies reviewed, 13 were aimed to identify gene expression signature for NSCLC [113, 116, 119–121, 123, 124, 128, 132–135, 139], 6 were for SQCC [120, 124, 127, 129, 131, 138], and 18 were for AdC [109–112, 114, 117, 118, 120, 122, 125, 126, 129, 130, 136, 137]. Except for few studies [120], early investigations were characterized mostly by lack of independent validation of signatures identified [109, 111–113, 116–119]. However, gene signatures reported since 2005 have included validation in independent data sets as a common practice. Some studies validated their signature in multiple publicly available microarray data sets [125–128, 132–134, 137–139]. In addition, cross platform validation using RT-qPCR was also performed for some studies [132, 133, 138, 139].

Subsequently a 15-gene signature that was prognostic and predictive for adjuvant chemotherapy for NSCLC [139] and a 12-gene signature prognostic for SQCC [138] was identified by the Maximizing R Square Analysis (MARSA), a model based approach for the identification of gene expression signatures. These two signatures were validated extensively in multiple independent microarray data sets as well as cross-platform validated using qPCR. An experimental verification of their biological significance of members of these signatures in tumor growth and metastasis as well as in vitro proliferation and apoptosis is undergoing [163]. In addition, an 11-gene signature derived from the common genes from the in vitro cultured carcinoma-associated fibroblasts and tissues adjacent from primary tumor obtained by laser-captured microdissection was also identified by the MARSA algorithm. The prognostic value of the signature was validated in the Directors' Challenge Consortium data set [140]. Subsequently, Wan YW et al. [141] reported their success of using model based method to identify 21 sets of expression signatures and all were validated independently.

For gene signatures to be useful at the clinical level, they must provide prognostic or predictive information that adds strength to the long established factors such as tumor stage, sex, etc. [134, 136, 138–141]. In addition, the assays need to be readily implementable in clinical laboratory improvement act (CLIA)-approved molecular diagnostic laboratories. To date, none are ready for prospective validation in clinical trials or used for routine clinical practice.

5 MicroRNA Expression in Lung Cancer

A complementary approach to analyzing gene expression signatures is to assess microRNA (miRNA) expression profiles. miRNAs are endogenous small non-coding nucleotides that negatively regulate gene expression. They are involved in a wide range of biological functions, including development, growth and apoptosis [164]. By binding to complementary sequences in the 3′UTR of target mRNAs, they

can mediate either translational repression or mRNA degradation [165]. A single miRNA may bind to and regulate several target mRNAs, and a single mRNA can also be targeted by multiple miRNAs. Consequently, the aberrant expression of a small number of miRNAs is likely to perturb normal cellular activity.

Specific miRNAs have been shown to be under- or over-expressed in particular tumour types [166–168]. *let-7* was the first miRNA reported to play a role in the development and progression of lung cancer. Reduced *let-7* expression in NSCLC patients was significantly associated with shortened postoperative survival, independent of disease stage [169]. In comparison, the overexpression of *let-7* inhibited growth of lung cancer cells *in vitro* and mediated tumour suppression in xenografts and in KRAS-induced lung cancer mouse models [169–171].

miRNA expression profiling of human tumours, including lung, ovarian and pancreatic, have identified distinct expression profiles compared with normal cells from the same tissue [172–175]. The expression of miRNAs is highly tissue specific, and their expression profiles can distinguish tumours of different developmental origin [167, 176]. The increasing evidence of miRNA dysregulation in human cancers suggests the potential for miRNA expression to inform cancer diagnosis, prognosis and response to treatment.

5.1 miRNA Expression in Lung Cancer vs. Normal Lung

Yanaihara et al. [175] were the first to suggest that miRNA profiles can discriminate cancerous from healthy lung tissues and classify tumours according to their histologies. Using microarray profiling, the expression of 43 miRNAs was significantly altered between lung cancer and the corresponding noncancerous lung tissues. This set of miRNAs correctly classified 91% of the 104 profiled cases. Many of these miRNAs are located in fragile sites or in regions that are frequently deleted or amplified in several malignancies. Subset comparisons of lung AdC or SqCC versus noncancerous tissues in the same sample cohort revealed 17 and 16 differentially expressed miRNAs, respectively. Subsequent studies have identified sets of miRNAs significantly altered specifically in SqCC compared with normal lung tissues [177–179]. From 22 differentially expressed miRNAs, Tan et al. [178] reported that a minimum of five miRNAs had the highest distinguishing power, with an accuracy of 94.1% in the training set. The classifier was validated in another set of 26 SqCC patients, displaying an accuracy of 96.2%. However, the miRNA lists across several studies demonstrated minimal overlap (Fig. 1.4). The discrepancies can be a result of the different study populations or the small sample cohorts.

5.2 miRNA Expression in AdC vs. SqCC

Yanaihara et al. [175] microarray profiled 104 NSCLC tumours to construct a six miRNA classifier to distinguish between AdC and SqCC, with an 81% accuracy

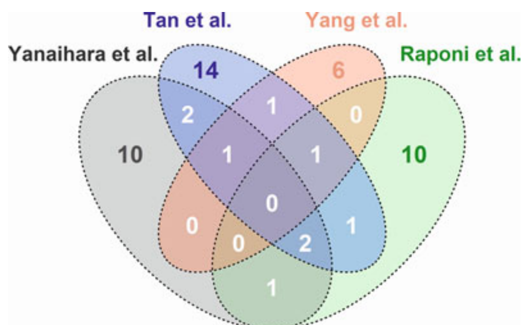

Fig. 1.4 Minimal overlap of miRNA studies. miRNAs differentially expressed between SqCC and corresponding normal lung tissues identified from four studies showing minimal overlap

in their training set. Among the six miRNAs (miR-205, miR-99b, miR-203, miR-202, miR-102, and miR-204-prec), miR-205 was identified as the most significantly differentially expressed between the two histological subtypes, being overexpressed in SqCC. This result was subsequently validated in 20 independent FFPE tissues. A qRT-PCR diagnostic assay was developed for miR-205 and validated using an independent cohort of 79 NSCLC FFPE samples, achieving a sensitivity and specificity of 96% and 90%, respectively [180]. In poorly differentiated preoperative biopsy specimen, miR-205 correctly classified (based on the integration of microscopic and immunohistochemical analysis) 20 out of 21 samples that lacked microscopic evidence of differentiation [181]. Functional studies suggest that miR-205 can function as a tumour suppressor: its inhibition caused breast and pancreatic cancer cells to adopt a mesenchymal morphology, whereas its ectopic expression forced reversal of epithelial mesenchymal transition and decreased cell proliferation, migration, and invasion [182–184]. Overexpression of miR-205 also suppressed tumour growth in an *in vivo* model of renal cancer [185]. However, in another study, miR-205 was not identified as differentially expressed among the 440 human miRNAs that were profiled in 165 AdC and 125 SqCC, even though 34 miRNAs were differentially expressed between AdC and SqCC in male smokers [186].

5.3 Prognostic Significance of miRNA in Lung Cancer

To date, several studies have investigated the prognostic relevance of miRNAs in lung cancer (Table 1.6) [175, 177, 178, 186–188]. Yanaihara et al. reported that high miR-155 or low let-7a-2 expression was an independent predictor of poor prognosis in lung AdC [175]. Several other groups have also reported the association of members of the let-7 family with survival outcome in lung cancer [169, 186, 187]. Interestingly, target prediction and functional studies indicate that multiple let-7-complementary sites are present in the 3′UTR of the RAS genes, specifically mediating let-7-dependent regulation [189]. However, upon validation in an independent set of 32 patients, only miR-155 remained significant, while let-7a-2 only approached significance [175]. In SqCC, both Raponi et al. [177] and Tan et al. [178] identified single miRNAs as having prognostic value (Table 1.6).

Table 1.6 MicroRNA signatures in lung cancer

Study	Histology	Stage	Survival outcome	Number of patients		Platform (method)	Classifier development	miRNAs in classifier	Training set HR (95% CI)	Validation set HR (95% CI)
				Testing	Validation					
Yanaihara et al. [175]	AdC	I–IV	OS	65	32	Custom array (oligo)	miR-155 expression ratio	1	3.42 (1.42–8.19)	2.52 (1.10–7.45)
Raponi et al. [177]	SqCC	I–IIIB	OS	65	NA	Ambion mirVana Bioarray	miR-146b expression	1	2.7 (1.4–5.7)	NA
Tan et al. [178]	SqCC	I–III	OS	60	88	CaptialBio (oligo)	miR-31 expression	1	2.83 (1.27–6.28)	2.40 (1.22–4.72)
Landi et al. [186]	SqCC	I–IIIA	OS	107	NA	Custom array (oligo)	Cross-validated supervised PCA	5	NA	NA
Yu et al. [187]	NSCLC	I–III	OS	56	118	qPCR	Risk score	5	10.31 (2.33–45.56)	2.81 (1.13–7.01)
			DFS						3.29 (1.24–8.71)	2.39 (1.12–5.10)
Patnaik et al. [188]	NSCLC	I	Recurrence	77	NA	Exiqon (LNA oligo)	Llinear SVM, TSP	6; 2	3.6 (1.8–7.1); 2.9 (1.4–6.4)	NA
			OS						9.0 (4.4.–18.2); 5.2 (2.4–11.4)	

AdC Adenocarcinoma, *SqCC* Squamous cell carcinoma, *NSCLC* Non-small cell lung carcinoma, *OS* Overall survival, *DFS* Disease free survival, *NA* Not applicable, *HR* Hazard ratio, *CI* Confidence interval, *PCA* Principal Component Analysis, *SVM* Support Vector Machines, *TSP* Top-Scoring gene Pair

Whereas Tan et al. reported that miR-31 was prognostic, Raponi et al. did not find this miRNA in their list of 20 miRNAs associated with patient survival [177, 178]. Of the 20 miRNAs, miR-146b alone was the most robust classifier for predicting overall survival at 78%, but dropped and stabilized at ~68% when three or more miRNAs were added to miR-146b in a linear fashion [177]. miR-155 was also associated with overall survival in this SqCC sample cohort: HR of 2.3 [95% CI: 1.4–5.7].

Using RT-PCR, Yu et al. identified a five-miRNA signature to predict survival and relapse in a training set of 56 patients, and validated in an independent cohort of 62 patients [187]. The signature consisted of two "protective" miRNAs (miR-221 and let-7a) and three "risky" miRNAs (miR-137, miR-372 and miR-182*). Patients with high-risk scores had shorter median overall and relapse-free survivals than patients with low-risk scores. Tumours with high-risk scores tend to express risky miRNAs, while tumours with low-risk scores expressed protective miRNAs. The signature was found to be independent from stage or histology, with HRs of 2.81 [95% CI: 1.13–7.10] for overall survival and 2.39 [95% CI 1.12–5.10] for relapse-free survival. In a separate study of stage I NSCLC, a six- and two-miRNA based classifier predicted recurrence with an accuracy of 70% and 83%, and hazard ratios of 3.6 [95% CI 1.8–7.1] and 9.0 [95% CI 4.4–18.2] [188]. Although a number of differentially expressed miRNAs between recurrence versus non-recurrence groups were similar to those identified by Yanaihara et al. and Yu et al. [175, 187], the miRNAs in the final classifiers had no overlap with the prognostic signatures from these previous studies. Multiple other studies have also reported the correlation of miRNA expression and prognosis, but many of them had methodological shortcomings, such as small sample cohorts, and lack independent validation [186, 190].

As in the case of mRNA gene expression profiling, miRNA signatures identified by different groups are almost non-overlapping. Inter-platform correlations between miRNA profiling methodologies are only moderate, and this may play a role in the difficulty of cross-platform validation. Furthermore, the lack of housekeeping miRNAs for qRT-PCR validation may hinder proper validation [188]. These issues are highlighted in several studies that investigated the prognostic value of several miRNAs in predicting clinical outcome in NSCLC patients. Three potential oncogenic miRNAs (miR-17, miR-21 and miR-155) identified by Yanaihara et al. [175] were evaluated in three additional cohorts of NSCLC patients with AdC histology [191]. High expression of the three miRNAs was significantly associated with cancer-specific mortality in the Maryland cohort ($n = 89$), whereas only miR-21 was associated with cancer specific mortality in the Norwegian cohort ($n = 37$) and relapse-free survival in the Japanese cohort ($n = 191$). Markou et al. [192] also showed that the overexpression of miR-21 is of prognostic significance in NSCLC patients. In a large scale miRnome analysis of various tumour types, miR-21 was upregulated in all profiled tumours, including lung, breast, stomach, prostate, colon and pancreatic tumours [176]. Functional studies of this particular miRNA have shown that it post-transcriptionally down regulates the expression of multiple tumour suppressors, including PTEN [193] PDCD4 [194–196], and

TPM1 [197]. In a KRAS^{v12}-expressing mouse model, miR-21 was expressed in higher levels compared with normal lung, suggesting that miR-21 overexpression in neoplastic transformation could be induced by the activation of RAS [198]. Furthermore, the inhibition of miR-21 in lung cancer cells markedly reduced cell growth and invasiveness [193], while its overexpression enhanced tumourigenesis *in vivo* [194]. However, when Voortman et al. analyzed the expression of seven miRNAs, including miR-155, let-7a and miR-21, by qRT-PCR in 639 FFPE tissues from patients who participated in the International Adjuvant Lung Cancer Trial (IALT), none of the miRNAs had a prognostic or predictive impact, despite the large sample size [199]. Duncavage et al. [200] came to the same conclusion of the lack of prognostic significance of miR-21 when its expression was assessed in 46 FFPE tissues. These contradictory results could have been caused by variations in sample handling, sample type (frozen vs. FFPE), normalization strategies or poor platform correlations.

5.4 miRNA as Predictive Markers in Lung Cancer

Apart from risk stratification, miRNAs can potentially be used for predicting sensitivity to chemotherapy; however, reports are very limited and validation is lacking. Weiss et al. [201] searched for candidate miRNAs that regulate EFGR and are located in chromosomal regions frequently lost in lung cancer. miR-128b was chosen for follow up because of its location on chromosome 3p, where allelic loss is a consistent deletion in all major types of lung cancers, and in up to 78% of preneoplastric or preinvasive lung epithelial samples [202, 203]. In a retrospective cohort of 58 samples (49 ADQ and 9 SqCC), miR-128b loss of heterozygosity was frequent (55%) and significantly associated with improved disease control and survival in patients treated with gefitinib [201].

5.5 miRNA vs. mRNA Expression Signatures

The growing number of studies examining the global changes in miRNA and those focusing on the role of specific individual miRNAs in lung tumours suggest that these molecules may play a role in the pathogenesis of lung cancer and serve as biomarkers for diagnosis and prognosis of this disease. miRNAs have many characteristics that make them suitable biomarker candidates. First, they can be profiled using similar technologies currently available for mRNA profiling. In addition, they are remarkably stable in archived formalin-fixed paraffin-embedded tissues and in human plasma and other body fluids, enhancing their utility as biomarkers for the diagnosis and prognosis of lung cancer via non-invasive methods [204–206]. Several groups have already reported the potential diagnostic and prognostic value

of miRNA in the plasma, serum and sputum of NSCLC patients [207–213]. In contrast to mRNAs, miRNAs have a markedly lower complexity – the current database of miRNAs encompasses around 1,400 human miRNAs compared to more than 30,000 readouts for coding mRNAs. Recent studies have even suggested that miRNAs may be superior to mRNA in classifying tumours [167]. In SqCC, Raponi et al. [124] identified a 50-gene mRNA signature that predicts prognosis with an overall accuracy of 68%. When a subset of these samples was profiled for miRNA expression, 20 out of 328 profiled miRNAs were associated with survival [177]. Whereas the miRNA-classifier provided a stable predictive accuracy of 68% when the classifier contained four or more miRNAs, no significant stratification of the prognostic groups was found when 20 or less genes were used in the gene classifier. The use of these small non-coding molecules as markers for early lung cancer detection appears promising; however, issues regarding assay reliability and normalization need to be resolved before they can be translated into clinical use.

6 Conclusion

Molecular alterations in lung cancer have so far been a largely academic exercise; with the exception of EGFR and ALK, molecular alterations in lung cancer have not reached clinical application. However, the rapidly expanding menu of targeted therapies, and accompanying predictive associations, are starting to drive us towards routine genomic profiling of all cancer types, including lung cancer. Evolving technologies including next-generation sequencing, high-density microarrays, and high-throughput expression profiling platforms are now starting to bring genomic profiling to the clinic at a reasonable cost. We are currently headed toward a rapid and significant shift towards the use of genomic information in lung cancer diagnosis and management over the next 10 years.

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Chapter 2
Understanding Melanoma Progression by Gene Expression Signatures

J. Tímár, T. Barbai, B. Győrffy, and E. Rásó

Abstract Malignant melanoma is the most aggressive cancer in humans and understanding this unique biological behavior may help to design better prognosticators and more efficient therapies. However, malignant melanoma is a heterogenous tumor etiologically (UV-induced or not), morphologically and genetically driven by various oncogens (B-RAF, N-RAS, KIT) and suppressor genes (CDKN2A, p53, PTEN). There are a significant number of studies in which prognostic gene and protein signatures were defined based on either analysis of the primary tumors (metastasis initiating gene set) or melanoma metastases (metastasis maintenance gene set) affecting progression of the disease or survival of the patient. These studies provided prognostic signatures of minimal overlap. Here we demonstrate consensus prognostic gene and protein sets derived from primary and metastatic tumor tissues. It is of note that although there were rare overlaps concerning the composing individual genes in these sets, network analysis defined the common pathways driving melanoma progression: cell proliferation, apoptosis, motility, and immune mechanisms. Malignant melanoma is chemoresistant, the genetic background of which has been unknown for a long time, but new genomic analyses have identified complex genetic alterations responsible for this phenotype involving DNA repair genes and oncogene signaling pathways. The advent of immunotherapy of melanoma placed the previously defined immune signature-associated genomic prognosticators into a new perspective, suggesting that it might also be a powerful predictor. Target therapy of malignant melanoma has changed the standard therapy based on IFN

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and dacarbazine. Target therapy of B-RAF and KIT mutated melanomas is based on careful selection of tumors with activating/sensitizing mutations, but has immediately raised the issue of genetic basis of constitutive or acquired resistances.

1 Introduction

Malignant melanoma represents the most aggressive human cancer, which cannot be small enough to not threaten the life of the patient, since even the smallest primary tumor in range of 0.5 mm in diameter has a significant potential for distant metastatization. Unfortunately, this cancer type is also characterized by chemo- as well as radio-resistance partially based on the immanent genetic make-up of melanocytes designed to protect surrounding keratinocytes from UV-induced damages. For a long time this cancer type was considered a rare tumor, but due to changes in lifestyle over the past decades, its incidence has steadily increased among caucasians. In addition, by reason of effective new treatments for common cancer types, the untreatable melanoma is ranked among the leading ten causes of cancer death in the various geographic areas of the world. Malignant melanoma is an orphan cancer since its incidence is of no interest to the industries and it is not a focus of basic research, hence our knowledge of this disease has only shown moderate development during the past years. Recently, however, malignant melanoma has entered the limelight by virtue of our increasing knowledge on its genetics, resulting development of the first successful therapies. The aim of this review is to summarize our knowledge on malignant melanoma and its contemporary histological and molecular classification, based on which a more effective prognostication and therapy can be developed. Further, it will be demonstrated that only an integrative approach could lead to success, where classical pathology is combined with gene- and protein based "molecular" characterizations.

2 Melanoma Classification

2.1 Histological Classification

Malignant melanocytic tumors can histologically be classified into the following six main categories: superficial spreading, nodular, lentigo maligna, acral, mucosal and uveal melanomas (SSM, NM, LMM, AM, MuM and UM, respectively) [1]. However, there are also rare histological variants such as desmoplastic, nevoid, blue nevus-associated, giant congenital nevus-associated and childhood melanomas (Table 2.1). Based on etiology, malignant melanomas can be classified into ultraviolet type and non-ultraviolet type, the latter comprising ALM, MuM and UM [2]. A novel classification is to separate two forms of UV-induced melanomas based on the extent of UV exposure and damage: melanomas arising from skin showing signs of

chronic sun-induced damage (CSD) and those caused by intermittent UV exposure, reflecting critical differences in etiology rather than in actual histology [3]. Similarly to other cancer types, it is becoming more and more evident that morphological subtypes of a given tumor correspond to diverse etiology and molecular variants. Accordingly, malignant melanomas can now be classified based on characteristic predominant genetic alterations. However, despite recent major developments, clear connection between a histological subtype and a molecular class cannot currently be established.

2.2 Molecular Classification of Malignant Melanoma

Human solid cancers are characterized by several hundred specific genetic aberrations comprising mutations and copy number alterations in oncogenes and suppressor genes. However, only a few deserve the designation as driver mutations. In the past decade systematic genetic analyses of malignant melanomas revealed the most frequent driver mutations, as listed in Table 2.1. In case of non-UV melanomas a clear connection can be established between the histological variant and molecular subtype: uveal melanomas harbor GNAQ and GNA11 mutations, mucosal and ALM melanomas frequently contain KIT mutations [4, 5]. On the other hand, in case of UV-induced melanomas the genetic picture is more complex and it is difficult to connect histology directly to molecular variants. The most frequent oncogene alteration in UV-induced melanomas is B-RAF mutation, which is associated to nevi and melanomas derived from pre-existing nevi, both connected to chronic sun damage of the skin (CSD). Considered the second most frequent genetic alteration in malignant melanoma for a long time, N-RAS mutation was not consistently connected to the UV irradiation type or any specific histological type, though NM was suspected to have some connection. Today it is clear that oncosuppressor gene defects are more frequent in malignant melanomas mostly associated with the UV-induced forms, but not connected to specific histological types: these include by rank of incidence CNKN2A, PTEN and p53. Concerning UV-associated oncogenes, a recent study revealed that GRIN2A mutation is among the most frequent genetic alterations in UV-induced melanomas followed by KIT, MITF (mostly amplification), BLC2, PI3K, AKT and CDK4 [2]. In summary, it can be stated that certain genetic alterations in malignant melanomas are connected to UV-exposure, such as B-RAF and N-RAS, but others equally occur in UV-induced and non-UV-induced melanomas, such as KIT, PTEN or p53.

A system biology approach to the genetics of malignant melanoma reveals five major molecular forms of malignant melanoma (Table 2.1 and Fig. 2.1) [2]. The most frequent form is the growth factor receptor signaling one (associated with KIT, MET and EGFR defects, 1a), where genetic defects frequently occur in either the N-RAS-B-RAF-MAPK axis (1b) or in the PI3K-AKT-mTOR axis (1c). The other emerging receptor signaling pathway related to malignant melanoma is the G-protein-coupled receptor pathway (MC1R and GRIN2A), where the mutant

Table 2.1 Histological and molecular classification of malignant melanoma

Categories										
Histological	SSM	NM	LMM	AM	dpm	nem	bnm	cgnm	MuM	UM
Molecular (mutated)	BRAF (50%)	N-RAS (20%)	KIT (20%)	MITF (20%)	GRIN2A (30%)	PTEN (30%) PI3K AKT	CDKN2A (50%) CDK4	p53 (20%)	KIT BCL2	GNAQ GNA11
Pathway	1. Growth factor receptor	2. G-protein coupled receptor	3. MITF	4. Cell cycle	5. apoptosis					
	1a. KIT (MET, EGFR)	2a. GRIN2A		4a. CDKN2A	5a. p53					
	1b. MAPK	2b. GNAQ,GNA11		4b. CDK	5b. BCL2					
	1c. PI3K									

SSM superficial spreading melanoma, *NM* nodular M, *LMM* lentigo maligna M, *AM* acral M, *dpm* desmoplastic M, *nemm* nevoid M, *bnm* blue nevus associated M, *cgnm* congenital nevus associated M, *MuM* mucosal M, *UM* uveal M

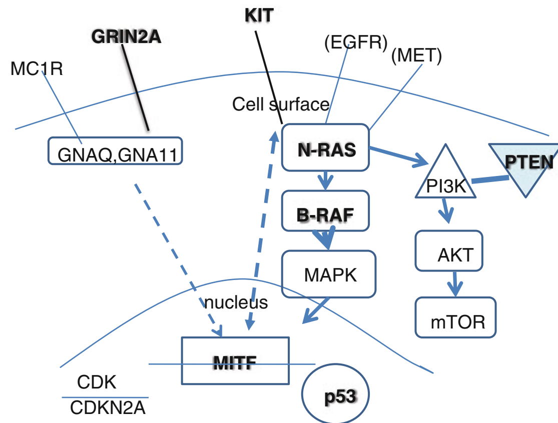

Fig. 2.1 Molecular pathways of human melanoma

receptor is GRIN2A (2a) [6], otherwise the mutant G-proteins GNAQ or GNA11 are the drivers (2b). A third signaling pathway driving a fraction of malignant melanomas is the MITF pathway [7], where genetic alteration in both MITF and its targets may occur. The fourth molecular category of malignant melanoma is associated with genetic defect(s) of the cell cycle pathway regulators, CDKN2A (4a) and CDK (4b). Finally, the fifth pathway – the genetic alterations of which characterize malignant melanoma – is the "apoptotic machinery" associated melanoma, involving p53 (5a) and BCL2 (5b) mutations. Such molecular classification is very practical from the viewpoint of planning novel target therapies or designing clinical trials for either existing or new drugs.

2.3 Melanoma Markers

In daily routine diagnostics malignant melanomas are defined by their characteristic pigment production. The melanin producing apparatus is under the genetic control of MITF regulated by the MC1R signaling pathway [7]. In malignant melanomas the melanin producing apparatus is frequently maintained completely or fragmentally offering an efficient tool for differential diagnosis (discriminating melanocytic tumors from others). However, these markers are not melanoma specific since they are expressed in all benign melanocytic lesions. MITF is responsible for stimulation of the expression of genes, the protein products of which are members of the melanosome including gp100/pmel17, tyrosinase and TRP1, DCT and melane-A/MART1 (Fig. 2.2). Melanosomes are derived from ER, Golgi and lysosomal membranes and undergo a maturation process through stage 1 to stage 4. Tyrosinase and DCT appear in stage 2 melanosomes, while melanin pigment is present in stage 4 melanosomes. Structural proteins of the melanosomes are gp100 and MART1. Melanocytes and melanoma cells express neurogenic protein S100, specifically

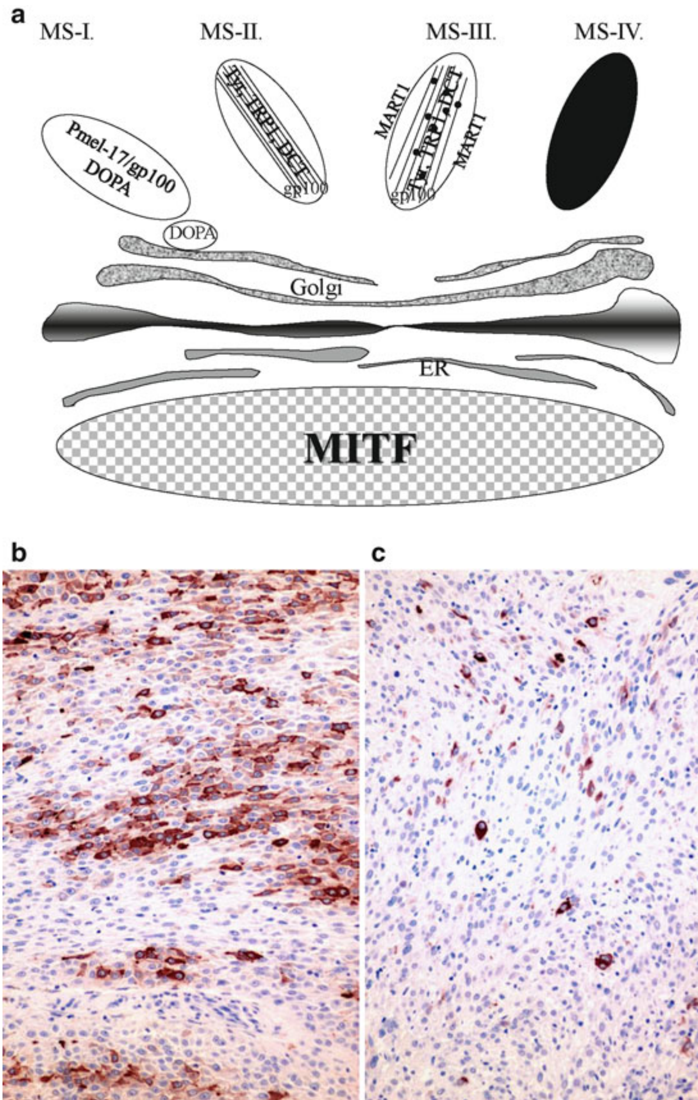

Fig. 2.2 Melanoma and melanosomal markers. (**a**) Schematic representation of the maturation of melanomosomes (MS) from stage 1–4. *MITF* microphthalmia transcription factor, *DOPA* dihydroxyphenilalanine, *DCT* opachrometautomerase, *TYR* tyrosinase, *TRP1* tyrosinase related protein, *gp100* HMB45 antigen, *MART1* Melan-A, (**b**) S-100B immunoreactivity in skin melanoma tissue (*brown color*), (**c**) Mart-1 immunoreactivity in skin melanoma tissue (*brown color*)

the β-isoform. Although it is routinely used in diagnostics, its expression is not melanoma specific, similarly to NSE or the TA90 antigen. Among the few melanoma specific proteins is the NG2 proteoglycan, which is sensitivity to fixation procedures and is therefore inappropriate for routine differential diagnostics.

There were several attempts in the past to identify melanoma specific genes, but the majority failed since the candidates were mostly present in premalignant lesions. LOH of apoptotic protease-activating factor-1(APAF1) gene was shown to be a sensitive marker for malignant transformation of melanocytes [8]. Recently a genomic approach revealed a few potential candidate marker genes, such as p107 and RyR2 [9]. A meta-analysis of array data defined a 6-gene signature of melanoma cells containing RAB33A, EGFR3, ADRB2, MERTK, SNF1 and ITPKB [10]. Similarly, instead of using a single gene or set of genes, genetic approach seems to be efficient in identifying the array of chromosomal alterations which can discriminate malignant melanomas from dysplastic melanocytic lesions. The resulting multiple FISH test can be applied to paraffin embedded samples.

3 Progression of Malignant Melanoma

3.1 Progression Stages and Variations

The initial phase of malignant melanoma progression is local invasion, which involves the potential of melanoma cells to invade the covering epidermis or underlying dermal structures. Local invasion is also characterized by formation of dermal satellite lesions, which can further propagate in several ways as will be outlined later. Today one of the most powerful prognostic markers for assessing the future biological behavior of a primary melanoma is ulceration, behind which the molecular/biological pathomechanism is still unknown (Fig. 2.3). A further strong prognostic factor that predicts outcome of the disease is thickness of the primary tumor, today ranging 0.5–4 mm and above [1]. As compared with any other human solid malignancy, this is an extremely narrow size range, with a 4 mm thick lesion having a high risk of developing distant metastasis within 10 years (Fig. 2.3) [11]. On the other hand, there is no such thing as a safe minimal melanoma, since even at a thickness of 0.5 mm the risk of developing distant organ metastasis is quite significant [12]. Accordingly, thickness is a rather efficient predictor of the future biological behavior of melanomas. Curious though it may seem, there is no 100% risk range, since even at the most advanced primary stages the metastasis risk never reaches 100%, indicating that a significant proportion of primary tumors has no or only limited metastatic potential (Fig. 2.4). This is the main reason why there is continuous search for genetic or protein markers capable of reliably predicting the individual prognosis of a given patient.

Systemic dissemination of skin melanomas can occur in the form of lymphatic or blood vessel dissemination (Fig. 2.5). The prerequisite for this type of progression – besides biological/genetic – is the availability of nearby local lymphatic and blood capillaries. Unfortunately, dermal skin provides a rich network of these capillary systems which can also be further increased by cytokines produced by primary melanoma (VEGF-C or VEGF-A, respectively) [13]. Unlike in most other

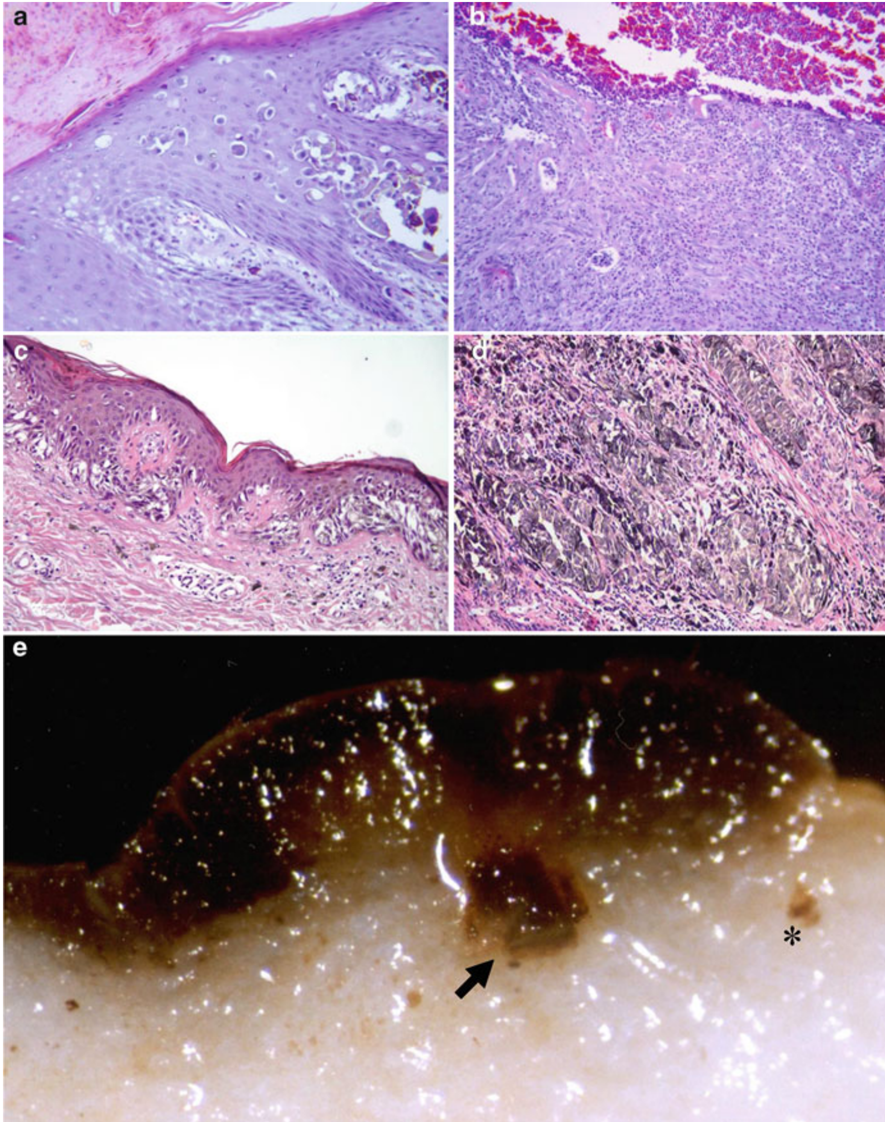

Fig. 2.3 Microscopic morphology of local invasion of skin melanoma. (**a**) Epidermal invasion, (**b**) Ulceration of the epidermis by melanoma, (**c**) Superficial spreading melanoma, (**d**) Vertical growth phase, (**e**) Dermal invasion (*arrow*)

cancer types, vascularization of malignant melanoma is provided by cooption of preexisting blood vessels and lymphatics and not by neoangiogenesis. After lymphatic intravasation melanoma cells can form "in transit" dermal metastases in the lymphatics even before reaching the regional lymph nodes [14]. One of

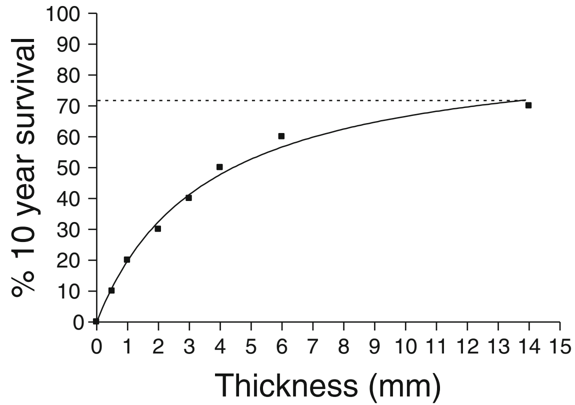

Fig. 2.4 Graphical representation of the connection between skin melanoma thickness and 10 year survival probability

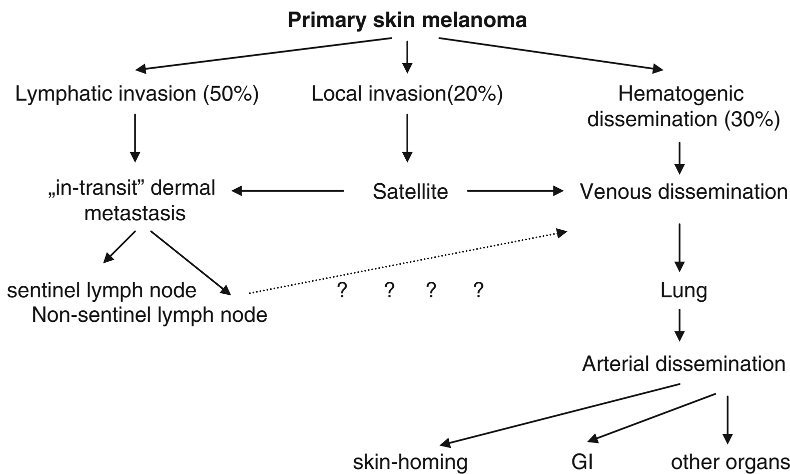

Fig. 2.5 Invasion and metastasis forms of skin melanoma. *GI*, Gastrointestinal tract

the most frequent dissemination forms of malignant melanoma is locoregional, detectable by sentinel technology. Unfortunately, in a significant proportion of cases disseminating melanoma cells can skip locoregional lymph nodes identifiable by macrophage tracers and settle in so called non sentinel nodes around or beyond the locoregional ones [1, 11, 12].

The most important systemic dissemination of malignant melanomas is vascular dissemination (Fig. 2.5) by means of dermal venous capillaries, which can be identified either by simple microscopical analysis, or specifically by IHC identification of blood capillaries (CD34). It is of note that satellite dermal nodules can also be a source for the systemic dissemination of melanomas, both lymphatic and vascular, suggesting that these features must be incorporated into future prognostication techniques. Melanoma cells from the venous circulation will reach the lung as the first filter organ after survival, a privilege for only a tiny proportion of tumor

cells. However, the lung is not the most frequently involved organ in melanoma metastatization. The simple explanation for this phenomenon is that for a significant proportion of melanoma cells the lung tissue/environment is not an ideal milieu for survival and proliferation [1, 12]. An alternative is that certain melanoma cells actively search for new territories and the arterio-venous communications in the lung provide opportunity for these cells to reach the arterial circulation and other visceral organs. Besides the viscera, malignant melanoma of the skin is characterized by a skin-homing potential, therefore skin metastases are frequently formed from the arterial circulation in progressing tumors. Melanomas, however have the potential to give metastasis to any of the visceral organs, a unique potential compared to other solid malignancies. From the perspective of the patients, skin and lung metastases are much less life-threatening features, unlike other metastases such as the brain, liver, bone etc.

A strong clinicopathological predictor of melanoma aggressiveness is the presence of locoregional lymphatic metastases. However, eradication of these metastases does not prevent development of distant organ metastases, suggesting that these locoregional lymphatic metastases are not the source of the systemic disease. On the other hand, even at high N stages (several locoregional lymph node metastases) there is no 100% chance of having distant organ metastases, which emphasizes the fact that there are metastasis-incompetent primary melanomas [1]. Meanwhile, according to relevant information from the literature there is continuing debate in this respect, with data still missing to be able to specifically answer these questions.

3.2 Progression Drivers in Preclinical Models

3.2.1 Host Factors

Clinically, skin melanoma progression is not only determined by genetic factors residing in melanoma cells, but also equally important are the host factors, immune mechanisms in particular. Animal models as well as clinical data suggest that malignant melanoma is immunogenic, and efficacy of both the specific and non-specific (innate) immunity contributes to the defense mechanism of the host in which activated cytotoxic T cells, through the help of dendritic cells, are the major contributors, although novel data point to the importance of the B cell-mediated immunity as well. Oddly enough, macrophages play a controversial role in this process [15–17].

An interesting issue is how the gender of the host affects melanoma progression. Etiological data suggest that melanoma progression is less efficient in premenopausal women and human melanoma may express sex hormone receptors. These observations have been confirmed in preclinical melanoma metastasis models, suggesting that efficacy of at least the liver metastatization strictly depends on the gender of the host [18].

3.2.2 Melanoma Metastasis Genes

The genetic factors that can fundamentally influence the invasive/organ metastatic behaviour of melanoma cells are considered metastasis genes. Unlike in other cancer types, expression of these "metastasis-associated" genes is much less known. Although the expression of CD44 in melanoma is evident, the specific role of its biological potential is highly controversial. In preclinical models, CD44 and its v3 splice variant were shown to be important in determining motile/invasive behavior of melanoma cells, but studies on clinical samples demonstrated highly controversial/contradictory data [19, 20]. The TWIST transcription factor is a prominent metastasis regulator in epithelial cancers, responsible for epithelial-mesenchymal transitions (EMT). However, its role in melanoma is questionable due to the equally questionable role of EMT in melanoma invasiveness [21]. On the other hand, experimental and clinicopathological data suggest controlled downregulation of cell adhesion molecules (i.e. E-Cadherin) and upregulation of N-cadherin during dissemination of melanoma cells, paralleled by an intermediate filament switch (vimentin/cytokeratin). Negative regulators of melanoma metastatization may exist, but only few data are available on their actual role. The expression and function of NME1/NDP kinase in melanoma are highly controversial [22]. A frequent genetic event in experimental melanoma models is loss of the gene region coding for KISS-1/metastin, which is the ligand of GPR45. Furthermore, the malfunction of this ligand-receptor axis can also be due to the loss of the transcriptional co-activator, DRIP130, in melanoma [23].

Studies on the understanding of melanoma metastatization have repeatedly indicated the importance of integrins and their signaling pathways. The predominant integrin expressed by animal and human melanomas is αvβ3 [24], which has a significant role in melanoma migration and invasion, where effector kinases FAK and ILK play prominent roles [25]. In association with these observations, it is important that a novel melanoma metastasis gene, NEDD9, was identified in animal models, which is a regulator of the FAK activity [26]. Importance of αvβ3 in melanoma invasiveness is also supported by its role in regulating MMP activity, especially that of MMP2. Similarly to other cancer types, the motile potential of melanoma is the rate limiting factor of its metastatic potential. Studies have indicated that the HGF-MET paracrine- and the AMF (CXC chemokine)-AMFR autocrine axes are equally important in shaping the invasiveness and motile potential of animal and human melanomas [27, 28].

3.2.3 Stemness

Metastatic colonization and tumorigenicity of cancers are influenced by cancer stem cells or a subpopulation of cancer cells expressing stem cell genes [21]. Studies on melanoma stem cells have identified a subpopulation characterized by CD20/CD133/CD271 surface markers expressing ABCB5 membrane transporter. This subpopulation might be regulated by a morphogen NODAL (a TGFβ-family

member), resulting in multilineage differentiation potentials. NODAL acts through its activin type receptors, forming an autocrine loop, which is also regulated by NOTCH signaling. One of the hallmarks of stemness in melanoma is its vasculogenic mimicry defined by expression of endothelial genes (VE-cadherin/CD144, EphA2, TIE-1 or even CD34), resulting in formation of vascular channels in melanoma [29]. Another example of the plasticity of melanoma is platelet-mimicry, which is characterized by expression of megakaryocytic genes integrin αIIβ/CD41, thrombin receptor(s), platelet 12-LOX and PAFR and producing thrombin, FBG or PAF [30]. Both vasculogenic mimicry and platelet-mimicry have been demonstrated to be important determinants of the metastatic potential of melanoma. Recently, an interesting novel regulatory mechanism emerged in melanomas in association with aggressiveness and stemness: loss of the expression of AP2α transcription factor by upregulation of the CREB transcription factor. These genetic alterations lead to increased expressions of MUC18, BCL2, several pro-inflammatory- and pro-angiogenic molecules. Activation and upregulation of CREB in melanomas seem to be associated with the activity of PAFR and PAR1, further supporting the importance of the platelet mimicry [31].

4 Prognostic Signatures of Malignant Melanoma

4.1 Pattern of Metastasis Initiating Genes

In the past decade genomic analyses of human melanomas have been performed, considering the disease as a homogenous cancer entity. As previously discussed, malignant melanoma is histologically, etiologically and genetically a heterogeneous tumor, which is to be taken into consideration during such analyses. Another point in question is that genetic studies on primary tumors for the determination of prognostic signatures will identify the genes most likely to be metastasis initiators. Although nine such studies can be found in the literature involving 139 genes (Table 2.2) [32–40], unfortunately not all of them evaluated primary tumors, several assessed in-transit skin metastases. Another factor of heterogeneity is that the endpoints in these studies were also heterogeneous: progression-free survival, overall survival, lymphatic or visceral metastasis. A previous meta-analysis of the majority of these studies was performed, identifying a significant overlap among these signatures. However, this overlap was due to the studies in which immune-response signatures were defined and the vast majority of overlapping genes were associated with host immune response [41]. We shall analyse these associations separately in the following chapter when considering immunotherapy. Here we focus our attention on the melanoma-gene sets.

Data collection was completed in a literature survey of gene expression data related to aggressiveness of human MM. A search in PubMed (http://www.pubmed.com) was conducted focusing on studies written in the English language till

Table 2.2 Comparison of metastasis initiating gene signatures of human melanoma (Modified and updated from Tímár et al. [50])

Bittner et al. [32]	Mandruzzato et al. [33]	Winnepenninckx et al. [34]	John et al. [35]	Conway et al. [36]	Bogunovic et al. [37]	Jönsson et al. [38]	Scott et al. [39]	Lugassy et al. [40]
Poor prognosis	Poor prognosis	Poor prognosis	Poor prognosis	Poor prognosis	Good prognosis	Poor prognosis	Poor prognosis	Poor prognosis
ENDRB	GJB2	**MCM3**	PLXNB2	OPN	ZNF385D	***Pigment sign***.	ACP5	KIF14
SThM	CX26	MCM4	ARFRP1	***HMMR***	GABBR1	DCT	FSCN1	DBF4
Synuclein A	CSPG4	MCM6	IGKC	RAD54R	VCAM1	**MART1**	NDC80	FNBP12
MART1A	ADAMTS5	KPNA2	P62B	E2F5	CCIN	SILV		ECT2
CD63	**MCM3**	KLK7	KCNIP2	**BIRC5**	NEFM	CAPN3		GLS2
SRD5A1	DCT	GMNN	HLA-E	RAD51	ITGA7	D4S234E		TCOF1
TEGT	SOX8	NME1	GTPBP2	FHIT	ZFP57	TRIM63		***HMMR***
A2M	FDFT1	FX	MFGE8	BLM	**SLC45A2**	MREG		NEI23
CD20	PRKCE	PCNA	TXNDC5	TK1	CPB2	**SLC45A2**		FGD3
PGAM1	CHEK1	PTGDS	PILRA	TOP2A	FAM40B	TUBB4		AGAP2
Syntenin	DHFR	RFL4	**NFKBIB**	RECQL		TYR		KCTD11
PAI2		CENPA	MTCH2	DEK		STK32A		F10
PLOD2		CDC6	CHST4	ING1		GMPR		CEBPA
Tropomyosin		CDC2	MRPS5			TSPAN10		AHNAK
AXL		F10	IDH1			GPR143		AQP3
HIF1a		EPHB6	ITPA			PPM1H		
ITGB1		ECT1				PTPLA		
IL-8		NEK2				MITF		
SMAD1		**BIRC5**				***Proliferation sign***.		
WNT5A		HAUSP				TNSF10		
Exostoses1		IL-6				CCND2		
stromelysin		WNT11				RIPK3		
		KLK4				RSPO3		
		KLK11				CCDC69		
						GIMAP1, 5–8		
						SLC9A9		

2012 using the keywords "melanoma", "array", "microarray", "metastasis" and "progression" and limiting the search to human entries. All retrieved abstracts were reviewed and a related article search was performed on appropriate abstracts. Articles and supplemental material were downloaded, making a gene set available with clear descriptions of applied analytical steps and detailed results. Studies related to single genes or arbitrarily selected genes were discarded. No threshold was defined according to which certain genes defined as "differentially expressed" could have shown only marginal differences. Gene symbols and Affymetrix probe set IDs were used to identify single genes using annotation databases provided by Affymetrix (http://www.affymetrix.com) and using the EMBL approved gene nomenclature (http://www.genenames.org) for gene symbols. The mapping of gene sets and the identification of overlapping genes were identified using Microsoft Access software package. It was of no great surprise that the defined prognostic gene sets showed very little and minimal overlap (2x) of 46 genes, where only three genes were present in three prognostic signatures: HMMR, PTGDS and RASGRP2 (Table 2.3). Pathway analysis of the consensus prognostic gene signature using Ingenuity software revealed top networks of DNA replication (33/46 component genes) and cell death (30/46 component genes) built around CDKs and p53.

4.2 Pattern of Metastasis Initiating Proteins

The literature on melanoma is very rich, including studies in which a myriad of proteins were analyzed in clinical settings to establish their prognostic role. In one of these studies a 38 protein prognostic signature of human melanoma was prospectively tested and validated. The study defined a 5-protein good prognosis set containing p16/INK4A, p21/WAF1, β-catenin, FN and ATF2, the prognostic power of which was maintained in a multivariate analysis. Recently, two independent meta-analyses were performed resulting in two partially overlapping sets of metastasis initiator/prognostic protein signatures (Table 2.4) [42–44]. In one study even hazard ratio (HR) was calculated for the individual proteins composing the signature which revealed two log differences in their prognostic value, suggesting heterogeneous influence of the individual proteins in this list. This 43 protein signature contained 17-protein overlap with another defined melanoma protein signature of 31. Although the individual protein of the previously validated 5-protein set could be found in the meta-sets, it was not present in the consensus list. The overlapping genes belonged to the regulation of proliferation of melanoma cells, to their differentiation and genetic background. It is very interesting that the metastasis initiating gene signature and the relevant protein signature had an overlap of two genes and their proteins, *MART1*, an MITF-regulated gene and *CDK2*, were strongly suggestive of their prognostic significance. A more careful analysis of the available protein signatures revealed that *BIRC5*/*survivin* could also be found in both gene and protein sets. Pathway analysis of this consensus protein signature using Ingenuity software

Table 2.3 Consensus gene list of melanoma metastasis initiators [32–40]

Gene	Identified by		
HMMR	Lugassy	Winnepenninckx	Conway
PTGDS	Jönsson	Winnepenninckx	Bogunovic
RASGRP2	Bogunovic	Jönsson	Winnepenninckx
AADAT	Bogunovic	Jönsson	
ANLN	Winnepenninckx	Bogunovic	
ARHGAP30	Jönsson	Bogunovic	
ATAD2	Bogunovic	Winnepenninckx	
BIRC5	Winnepenninckx	Conway	
C5orf22	Bogunovic	Jönsson	
CCL19	Winnepenninckx	Bogunovic	
CDK2	Jönsson	Bogunovic	
CEBPA	Lugassy	Winnepenninckx	
CLIC3	Jönsson	Winnepenninckx	
CRIP1	Bogunovic	Winnepenninckx	
CTNNBIP1	Winnepenninckx	Jönsson	
DLX1	Jönsson	Bogunovic	
ECT2	Lugassy	Winnepenninckx	
EXO1	Bogunovic	Winnepenninckx	
F10	Winnepenninckx	Lugassy	
FGD3	Lugassy	Winnepenninckx	
H2AFZ	Winnepenninckx	Jönsson	
HLA-DPB1	Bogunovic	Jönsson	
HLA-DQB1	Jönsson	Winnepenninckx	
HOP	Winnepenninckx	Jönsson	
ICOS	Jönsson	Bogunovic	
IKZF1	Bogunovic	Jönsson	
ITPA	John	Jönsson	
KCTD11	Lugassy	Winnepenninckx	
LAMA1	Bogunovic	Winnepenninckx	
LCK	Bogunovic	Jönsson	
LTB	Bogunovic	Winnepenninckx	
MCM4	Winnepenninckx	Bogunovic	
MRPS5	Winnepenninckx	John	
PROM2	Jönsson	Winnepenninckx	
PTGER2	Bogunovic	Jönsson	
SLC45A2	Jönsson	Bogunovic	
SPINT2	Winnepenninckx	Jönsson	
TAPBP	Winnepenninckx	Jönsson	
TCOF1	Winnepenninckx	Lugassy	
TK1	Winnepenninckx	Conway	
TOP2A	Winnepenninckx	Conway	
TXNIP	Winnepenninckx	Bogunovic	
VNN2	Bogunovic	Jönsson	
WDHD1	Winnepenninckx	Bogunovic	
MART1	Bitter	Jönsson	
MCM3	Mandruzzato	Winnepenninckx	

Table 2.4 Consensus metastasis initiator protein signature

Gould Rothberg et al. [42, 43]	HR	Consensus	Schramm et al. [44]
MCAM/MUC18†	16.34		Tyrosinase
Bcl-xL†	8.07		ID1
Matrix metalloproteinase-2†	4.5		p120
Bcl-6†	3.98		E-cadherin
Bcl-2	3.42		N-cadherin
pRb	**3.4**	**pRb**	**pRb**
p27/KIP1†	3.08		Osteonectin
RING1B	2.89		RhoC
Cyclin E†	2.89		MMP1
Ki-67†	2.66		MMP9
Double minute-2	**2.49**	**DM-2**	**DM-2**
gp100	2.29		ILK
PCNA†	**2.27**	**PCNA**	**PCNA**
p53	2.19		LDH5
CXCR4†	2.07		Glypican-3
p21/WAF1	1.98		HES-1
Tissue plasminogen activator†	1.9		
Cyclin-dependent kinase-6	1.86		
Mum-1/IRF4	1.64		
Survivin	1.62		
MelanA/MART-1	**1.57**	**MART1**	**MART1**
PH1	**1.5**	**PH1**	**PH1**
MHC class II (HLA-DR, -DP, -DQ)	1.47		
Mel-18	**1.31**	**MEL-18**	**MEL-18**
Cyclin D3	**1.14**	**CCND3**	**CCND3**
Cyclin D1	**1.07**	**CCND1**	**CCND1**
Skp2	**1.06**	**SKP2**	**SKP2**
p16/INK4a†	0.29		
Cyclin-dependent kinase-2	**0.38**	**CDK2**	**CDK2**
P-cadherin	**0.44**	**P-cadherin**	**P-cadherin**
CD44 (variant 3)	**0.53**	**CD44**	**CD44**
STAT-1	**0.64**	**STAT-1**	**STAT-1**
c-Kit	**0.65**	**c-KIT**	**c-KIT**
Protein kinase C-β	**0.7**	**PKCb**	**PKCb**
Cyclin B1	0.73		
Caveolin	**0.73**	**Caveolin**	**Caveolin**
Topoisomerase II	0.78		
Cyclin-dependent kinase-1	**0.83**	**CDK1**	**CDK1**
Ku70†	0.87		
Ku80†	0.87		
nm23	0.87		
Cyclin A	0.89		
BMI-1	0.92		

HR hazard ratio of death
† $p < 0.05$

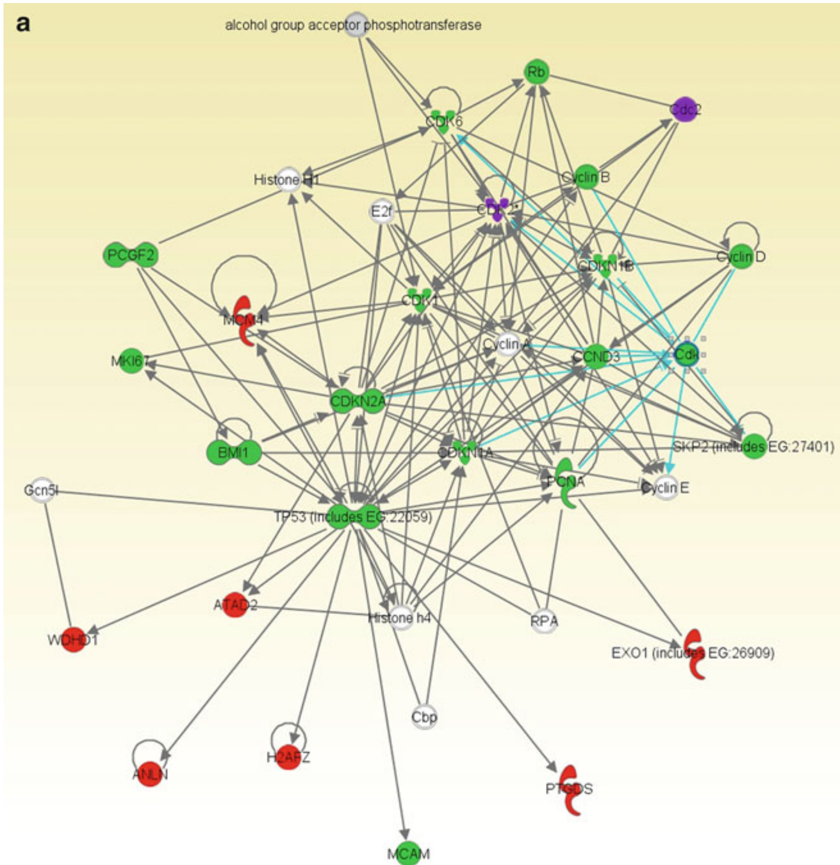

Fig. 2.6 Integrated network analysis of metastasis initiating consensus gene- and protein signatures (Tables 2.3 and 2.4). (**a**) Network 1. was built around cyclins and p53

revealed again p53/cell death- (23/43) and cell cycle networks (22/43), as major components of this signature.

Integrated network analysis of consensus metastasis initiating genes and proteins resulted in two major networks (Fig. 2.6), one built around cyclins and p53 (Fig. 2.6a) as well as a KIT-BCL2-RB-CCND1 axis (Fig. 2.6b) from 30% of the involved genes and proteins. Accordingly it can be concluded that the metastasis initiating genes and proteins in the consensus signatures were barely overlapped, the network analysis revealed that cell cycle regulation and cell death networks involving p53 and cyclins were common components of the two signatures. These data support the notion that gene defects of p53 and cyclins are important genetic factors determining metastatic potential of malignant melanoma.

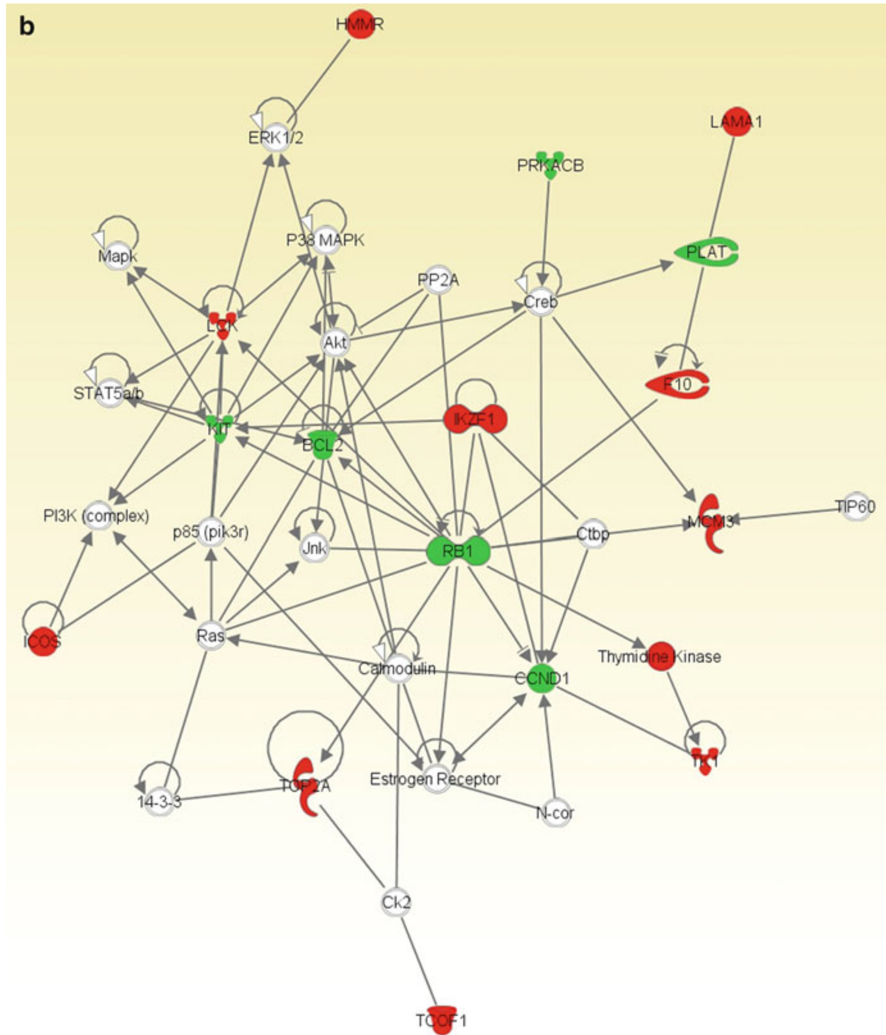

Fig. 2.6 (continued) (**b**) Network 2. was based on a KIT-BCL2-RB1-CCND1 axis. Analysis was performed by Ingenuity software

4.3 Pattern of Metastasis Maintenance Genes

Five genomic studies were also found in the literature, which defined prognostic signature by comparing melanoma metastasis to the primary tumors (Table 2.5) [45–49]. This approach can define the so called metastasis maintenance genes which are responsible for the development of the metastatic tissue. Since almost all studies compared lymphatic metastases to the primary, it can be concluded that such a gene set most probably defines the lymph node metastasis-maintenance

Table 2.5 Metastasis maintenance gene signatures of human melanoma (Modified and updated from Tímár et al. [50])

Becker et al. [45] Upregulated	Haqq et al. [46] Upregulated	Jaeger et al. [47] Upregulated	Riker et al. [48] Upregulated	Jewel et al. [49] Upregulated
Syntaxin	REH	**AQP3**	MAGEA1/2	CSF3
RNPL3		DSC1	MMP14	ERBB4
UBE21			CSAG2	FGF3
eIF2g				PLG
				PLA2G2A
Downregulated	***Downregulated***	***Downregulated***	***Downregulated***	MOS
TRP2	IGFBP1	**LGALS7**	SPRR1A/B	FGF8
MDA-7	HLA-DQ	TACSTD2	KRT6/15/16/17	TFF1
Desmin	HLA-B1/2	KRT10/14	**AQP3**	FGF6
	S100A2	**SFN**	CD24	FGF15
	RBP1	FGFBP1	FLG	
	GPD1		IVL	
	LUM		KLK7	***Downregulated***
	HPS1		**LGALS7**	MMP2
	TMP21		LOR	ETV6
	COL3A1		RAB25	**PDGFRB**
	COL5A3		**SFN**	KIT
	ZNFN1A5		ICEBERG	FYN
	ALOX5		HAS3	EMS1
	KITLG		TP73L	PRCC
	PDGFRA		RORA	CREBBP
	FBLN2		POU2F3	MX1
			TMPRSS4	GAS7

gene set. Similar to metastasis-initiating genes, these studies barely overlap with a few genes in the signature: *AQP3*, *LGALS7*, *SFN and PDGFR*. A thorough meta-analysis of the publicly available data sets was performed using robust bioinformatic technology. The analysis identified 350 genes with a central core of 17 genes present in three signatures (Table 2.6) [50]. This signature contained several well established prognostic genes of malignant melanoma including *osteopontin*, *BCL2*, *WNT5a and EGFR*. Pathway analysis of this signature by Ingenuity software indicated that significant pathways equally involved were cell cycle, cell death as well as cell movement. Interestingly, network analysis provided a single network from more than 80% of the signature built around p53, PPARG and SPP1/OPN.

4.4 Pattern of Metastasis Maintenance Proteins

A recent meta-analysis was performed to define the metastasis maintenance protein set of malignant melanoma with prognostic potential (Table 2.7) [51]. This analysis

Table 2.6 Consensus matestasis maintenance gene signature of Tímár et al. [50]

Symbol	Gene description
CKS2	CDC28 protein kinase regulatory subunit 2
DSC3	Desmocollin 3
EGFR	Epidermal growth factor receptor
CDC6	Cell division cycle 6 homolog
CTNNBIP1	Catenin, beta interacting protein 1
H2AFV	H2A histone family, member V
CXCL14	Chemokine (C-X-C motif) ligand 14
CSAG2	CSAG family, member 2///CSAG family, member 3B
WNT5A	Wingless-type MMTV integration site family, member 5A
SPP1	Secreted phosphoprotein 1
CLIC3	Chloride intracellular channel 3
PLP1	Proteolipid protein 1
AP1S2	Adaptor-related protein complex 1, sigma 2 subunit
BCL2A1	BCL2-related protein A1
AHNAK	AHNAK nucleoprotein
S100A2	S100 calcium binding protein A2
KRT15	Keratin 15

found a 28-protein signature containing several host factor derived growth factors and cytokines and only a few clearly melanoma-specific proteins, such as RARα, MAGE1/4, IGFBP4. Pathway analysis revealed that these proteins belonged to cell proliferation, cell death and cell movement pathways as well as to a unique IFN-signaling pathway. Network analysis further supported this finding revealing that almost half of the proteins of this signature were members of an IFN-signaling network.

An integrated network analysis was then performed on the metastasis maintenance gene and protein signatures. A single network was composed from 50% of the composite genes and proteins built around major nodes as IFN- and integrin signaling (Fig. 2.7) further supported the notion that melanoma progression, at least from established metastatic foci, is fundamentally influenced by immunological factors involving IFN signaling.

4.5 Consensus Prognostic Signature

From a practical point of view, a prognostic signature of a cancer can be derived from either the primary tumor or the metastasis, depending on the relative contribution of metastasis-initiating or maintenance genes or proteins. Our analysis identified two prognostic gene sets from these two gene pools which barely overlap (*CTNNBIP1*, *CLIC3 and H2AFZ*), suggesting that both types of genes are critical in metastasis formation of malignant melanoma, therefore prognostic signatures

Table 2.7 Consensus metastasis maintenance protein signature derived from Gould Rothberg and Rimm [51]

Securin/pituitary tumor transforming gene
PRSS11/HTRA1
Transforming growth factor-β (all isoforms)
Insulin-like growth factor binding protein-4
Interferon-inducible protein kinase
Platelet-derived growth factor receptor-β
Rap1-GAP
Retinoic acid receptor-α
Bak
Bok
Myeloid leukemia-1 (Mcl-1)
Ezrin
Galectin-1
Heparanase-1
Integrin-α4
Integrin-α5
Monocyte chemoattractant protein-1
Ferritin light chain
Calnexin
Interferon regulatory factor-1
Interferon regulatory factor-2
Interleukin-1α
Interleukin-24
MAGE-1
MAGE-4
Neutral endopeptidase/CD10
Tumor necrosis factor-β/lymphotoxin-A
α-Melanocyte-stimulating hormone

can be derived from both of them. A similar conclusion could be drawn from the protein based prognostic signatures, where no overlap was found between the metastasis initiating and maintenance proteins. However, comparison between the gene- and protein sets identified *BCL2 and OPN* in the metastasis initiating protein sets to be present in the metastasis maintenance gene set as well (although in differing degrees), supporting their prognostic significance and biological importance. A pathway analysis by Ingenuity software was used to compare the two consensus prognostic gene sets obtained from primary tumors or metastatic tissues (presented on Tables 2.3 and 2.6). It was possible to build two networks from 50% of the genes involved, where the major network contained 30% of the genes (Fig. 2.8) involving cyclins and CDKs, supporting the notion that cell cycle regulation is a major factor in melanoma metastasis. A similar informatic analysis performed on the two consensus protein signatures also resulted in two networks built from 50% of the protein components. Interestingly, the major network of the protein signatures corresponded to the cell cycle regulation network as well, further supporting the data obtained from the gene signature analysis.

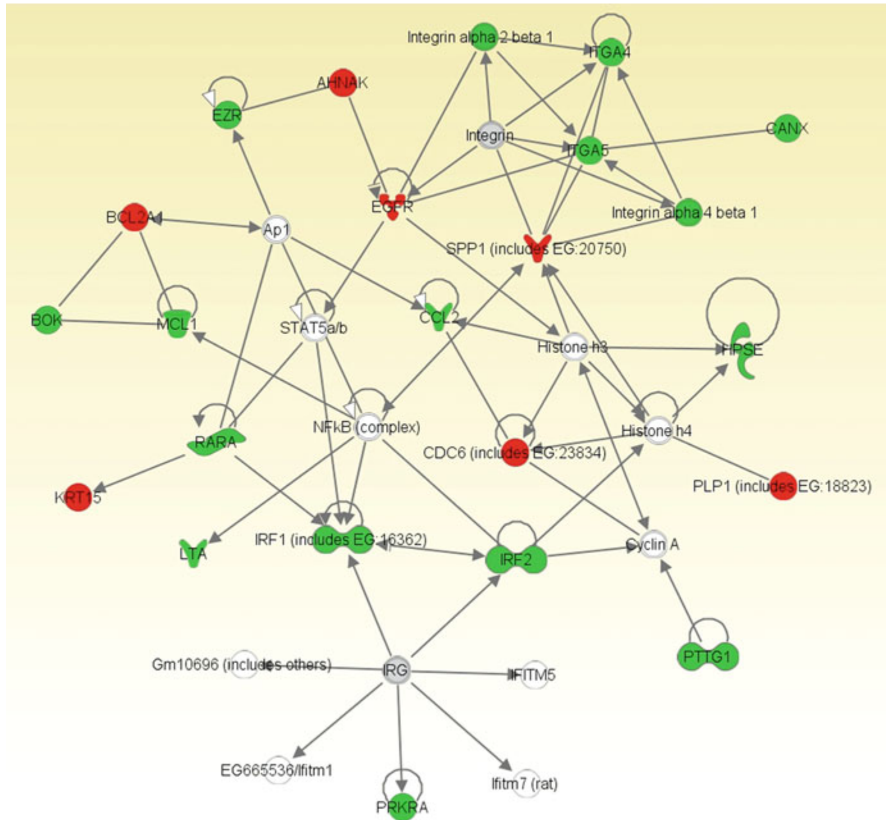

Fig. 2.7 Integrated network analysis of consensus metastasis maintenance gene- and protein signatures (Tables 2.6 and 2.7). The network contains 50% of composite elements and is built around IFN- and integrin signaling pathways. Analysis was performed by Ingenuity software

Experimental/preclinical studies provided ample data on the metastasis genes of human melanomas. It can be interpreted as a critical comment that almost none of the genes and proteins analyzed above were found in the signatures. The reasons for such an intriguing discrepancy are that preclinical data have not been further tested systematically on human materials, and/or such data are too specific for the melanoma models used.

In summary, there are interesting attempts in the literature to find relatively small gene- or protein signatures of malignant melanoma, which could be used to improve prognostication of the disease. However, selection of such genes/proteins must be based on careful unbiased evaluation and prospective validation. As an additional difficulty, any further study must be based on the molecular subclassification of the once considered "homogeneous" malignant melanoma and the signatures must be subclassified accordingly. Otherwise a virtually blind rally will be continued in the literature where subsequent studies will produce never-repeatable results.

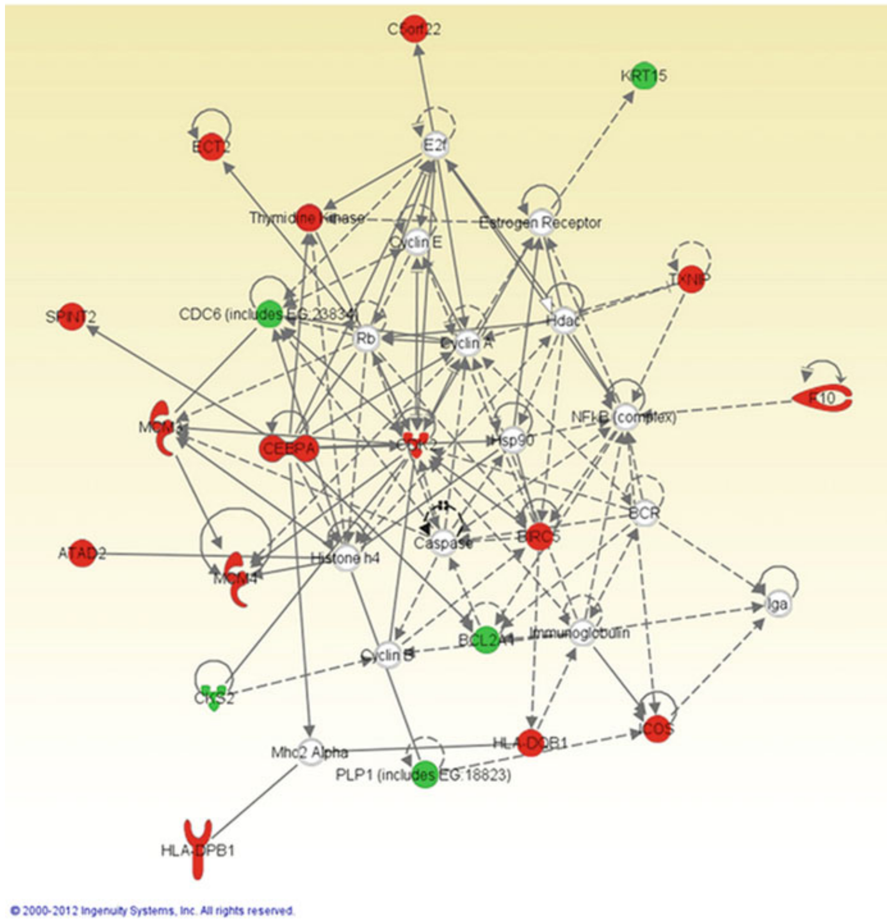

Fig. 2.8 Integrated network analysis of consensus prognostic melanoma gene signatures (Tables 2.3 and 2.6). Network 1. was built from 30% of components around cell cycle regulators. Analysis was performed by Ingenuity software

5 Genetic Prediction of Therapeutic Sensitivity

5.1 Chemotherapy

Malignant melanoma is considered a chemotherapy resistant cancer, the exact genetic background of which is still unknown. The typical apoptotic resistance of melanocytes is inherited to transformed melanocytes where defects in apoptotic genes characterize only a subset of tumors which carry p53 or BCL2 mutations. Melanoma stem cells represent a small subpopulation, which express the ABCB5

multidrug transporter., Chemotherapy of malignant melanoma relies almost exclusively on dacarbazine/DTIC treatment, which is the only registered chemotherapy since decades in this cancer type, characterized by a very low response rate (below 10%) and even lower efficacy. Sensitivity of melanoma and other cancer types to dacarbazine is considered to be in correlation with expression and activity of the DNA repair protein MGMT [52]. Novel studies indicate that increased constitutive expression of MGMT is correlated with poor response to dacarbazine, or its novel variant temozolomide [53]. On the other hand, these studies also revealed that besides MGMT, p16/INK4A levels might also affect responsiveness to DTIC/TMZ. In an elegant study it was proved that overexpression of p16 and the mutant B-RAF status are responsible for the melphalan and actinomycin-D resistance of human melanomas [54].

The most complex genomic analysis of the chemoresistance of malignant melanoma patients (472 tumors) was performed recently [55], defining RAD51 and TOPO2A as significant predictors of chemotherapy/DTIC response. However, it has to be mentioned that the overexpression of these genes in resistant tumors was in the range of 1.22 and 1.12, respectively, which raises the issue of how to detect such a small alteration of gene expression reliably in a clinical situation. In a small subset of these patients a comprehensive analysis of chemosensitivity genes was performed, which discovered a much more profound alteration of expressions in critical genes including several DNA repair genes with overexpression in a range of 2–4 fold (MSH6/2, XRCC1/5, ERCC1, MGMT). These repair genes included a wide variety of homologue- mismatch- and nucleotide excision repair genes. Furthermore, it was interesting that the AKT signaling pathway (PI3K and mTOR), Ki67, TS, HSP90 and SOD1 were among the most over- or underexpressed genes in chemoresistant tumors. This is the first comprehensive picture of DNA repair associated genes in malignant melanoma, which may shed light on the previously mentioned resistance to various chemotherapies.

5.2 Immunotherapy

One of the most critical host derived prognostic factors influencing progression of malignant melanoma is activity of the immune system. This conclusion is based on two types of approaches, direct detection and evaluation of TIL composition in melanoma and gene expression signatures (Table 2.8). Three independent genomic analyses performed on human melanomas revealed partially overlapping immune-signatures representing genes associated with T cells and their antitumoral activity [33, 37, 38]. Survival analysis indicated that patients with tumors characterized by immune-signature have significantly better survival rates [38]. On the other hand, another study found that in a significant proportion of melanoma patients (30%) peripheral T cells are defective in signaling, suggesting a tumor-induced immunosuppressive effect [17]. Taken together, one can divide malignant melanoma patients into three categories based on the activity of the antitumoral immune

Table 2.8 Comparison of good prognosis immune-signatures of human melanoma

Mandruzzato et al. [33]	Bogunovic et al. [37]	Jönsson et al. [38]	Jönsson et al. [38]
Good prognosis	Good prognosis	Good prognosis (10-gene set)	Good prognosis (30-gene set)
HLA-DR	CXCL13	ME1	ADA
HLA-B4	TLR10	NR5A2	BCAR1
TRA	CCL19	CCL16	C3AR1
LTB	**CD3D**	**CLEC4GP1**	CD19
TNFAIP3	FCAMR	LYVE1	**CD3E**
IL-4R	CCR7	F13A1	**CD79A**
IGLL1	**LCK**	CCL13	FYN
CD1D	CD69	CCL23	IKBKG
CD2	IL2RG	CD209	KLRK1
ITK	TNFRSF17	FOLR2	LAT2
SOD2	**CD2**		LAX2
DAF	CD27		**LCK**
GZMK	CD48		LYN
CD53	ZAP70		MALT1
CST1	**LTB**		MAP3K7
JUNB	**CD79A**		MAPK1
NFKBIZ	IRF8		MICA
LYZ	CSF2RB		MICB
UBD	GBP2		NFAM1
TMSB4X	IRF1		PLCG2
DUSP5	NLRC3		PSEN1, 2
	CLEC4G		PTPRC
			RIPK2
			SKAP1
			SPG21
			SYK
			TRAF6
			UBE2N

mechanisms (active, passive and defective), which could be the basis of tailored immunotherapy of malignant melanomas.

Up until now, the most effective therapy for malignant melanomas was cytokine therapy using IL-2 or IFNα2. Meta-analyses indicated that both higher and lower doses of IFN have the most beneficial effects in case of a small, but significant proportion (10–20%) of melanoma patients [56]. Studies on the possible predictive factors for IFN therapy revealed that the STAT1/STAT3 ratio might be a prognosticator in both melanomas and lymphocytes (56). Unfortunately, the previously mentioned antitumoral immune-activity stratified evaluation of IFN sensitivity has not yet been performed in case of melanoma patients. In this context, it is interesting that patients with ulcerated melanoma (a high risk group of poor outcome) benefit the most from IFN therapies. In the past decade there were attempts to define the IFN-resistance of cancers including malignant melanoma

Table 2.9 Interferone modulated gene signature of human melanoma (Krepler et al. [58])

	Upregulated	Downregulated
IRE-negative genes (fold difference >6)	b-cam	YKT6
	AchR-E	PRL-1
	LamR	PDK1
	RPP1	CL-100
	SB-IIAgA	STAM2
	GPCRHG38	AML1b
	Ig-LCh	GalK
	JM1	HSP70B
	HLA-III	INGL1
	ALP	VEGF
	p38PI3K	IGFRS1
		MGSA
		TGFb
IRE-positive genes (fold difference >2)	RIG-E	
	IF9-27	
	MxB	
	p27/KIP1	

IRE interferon responsive element in promoter

by expression profiling [57]. Unfortunately, these studies were mostly based on in vitro obtained signatures and were not evaluated in melanoma patients. The IFN sensitivity/resistance signature contained IFN-regulated transcription factors, HLA antigens and several IRE-containing and IRE-negative genes (Table 2.9) [58]. Based on these studies an IFN response gene array was produced (www.superarray.com). It is of note that the majority of genes associated with IFN sensitivity were IRE-negative, but mostly disregulated genes. Also of note is that among the upregulated genes PI3K could be found, whereas HSP70, VEGF and TGFβ were present among the downregulated genes. Unfortunately, neither this, nor a similar signature was used in recent clinical trials in which IFN-efficacy was determined in malignant melanomas.

Most recently, the first immunotherapy of cancers was registered in malignant melanomas, which can extend survival in about 10% of the patients. This target therapy uses anti-CTLA4 antibody, Ipilimumab, to suspend the immunosuppressive effect of T cells. Initially, this antibody therapy was found to be active in HLA-A0201 positive patients [59], but in a subsequent trial this type of selection was not used [60]. Ipilimumab target Treg cells can be found in primary and metastatic melanoma lesions. However, the prognostic role of Treg density in skin melanoma was not demonstrated convincingly. It is of note that the previously demonstrated immune-gene signatures do not contain CTLA4 or FOXP3, markers of Tregs. Unfortunately, in Ipilimumab trials no analyses were performed in order to demonstrate association with Treg cell density or CTLA4 expression levels. Another anti-CTLA4 antibody, Tremelimumab, was also used in trials related to

advanced melanoma cases, in which decreased Treg cell density was demonstrated in treated tumor samples [17]. Meanwhile the question is still valid, how can melanoma patients be stratified for more effective anti-CTLA4 therapies? This is an important question, since one of the most frequent side effects of anti-CTLA4 therapy is induction of severe autoimmune responses accordingly, a more tailored administration of this treatment regime is necessary.

5.3 Target Therapy

In Part 1 we showed that malignant melanoma can be classified based on predominating gene defects indicating a genetically heterogeneous tumor type. The most frequently mutated gene in malignant melanoma is B-RAF, which characterizes the majority of tumors. Another frequently mutated oncogene in melanoma is c-KIT, which unlike B-RAF, is present in both UV-induced and non-UV induced (rare) variants. These two mutations recently became successful targets for molecular therapy, fundamentally changing the management of malignant melanoma patients.

Vemurafenib is a highly selective inhibitor of mutated B-RAF and clinical trials have been highly successful in treating V600E mutated melanoma patients in monotherapy, demonstrating almost 50% response rates and significant extension of survival [61, 62]. The success of this target therapy is based on the selection of patients for V600E-mutated B-RAF expressing tumors as positive predictor of efficacy. Even in this situation the extent of antitumoral effect of Vemurafenib is limited in the majority of patients, with an occurrence of relapse sooner or later during the treatment. Therefore it is of high importance to define negative prognosticators or genetic constellations of constitutive resistance to B-RAF inhibitions. Till now, there have been no data on the constitutive mechanisms of resistance to Vemurafenib, though the response rate indicates that such mechanisms are frequently present in malignant melanomas. A recent pilot study suggested that PTEN-loss could be one of those genetic determinants, which are present in a significant proportion of skin melanomas. Genetic analysis of tumors of Vemurafenib-relapsed patients indicated several acquired resistance mechanisms. These include emergence of N-RAS mutated tumor cell population [63], development of MEK1C121S mutation [64] and overexpression of signaling pathway members B-RAF, C-RAF, and MAP3K8/COT [65]. It was also noted that overexpression of previously overseen growth factor pathways of melanoma could lead to Vemurafenib resistance involving HER2, AXL and PDGFRβ receptors. It is of note that certain prognostic signatures of melanoma contain AXL and/or PDGFR, suggesting that these resistance mechanisms could be constitutive rather than acquired in a proportion of malignant melanomas. Studies revealed other frequently acquired genetic alterations in Vemurafenib treated melanomas affecting ERBB4, FLT1, PTPRD, RET, TERT and RUNX1T1, association of which with mutant B-RAF inhibition failure is under investigation [64].

Target therapy of KIT-mutated human melanoma was also tested in two clinical trials using KIT-inhibitor TKI, Gleevec. Patient selection was based on detection

of KIT mutations. In the two trials the overall response rate was in the range of 16–23% [66, 67]. The most common mutations were similar to those found in GIST involving exons 9, 11, 13, 17 and 18. The copy number of KIT did not prove to be affecting Gleevec response in melanoma. On the other hand, exon 11 and 13 mutations seemed to be sensitizing KIT mutations in melanoma as compared with exons 9, 17 or 18. Genetic analysis also raised the issue of relative proportion of mutant KIT to wt allele, since a ratio higher than 1 was shown to be a significant Gleevec-sensitizing genetic factor. These phase-II trials did not provide a more comprehensive insight into the genetic factors affecting KIT-inhibitor therapy of malignant melanoma, but indicated several melanoma-specific factors which are different from KIT mutated GIST. Further molecular analyses are urgently needed to resolve these issues.

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Chapter 3
Prognostic Testing in Uveal Melanoma

Michael Zeschnigk and Dietmar R. Lohmann

Abstract Uveal melanoma (UM) is the most frequent intraocular tumor in adults with an annual incidence of about 6/1.000.000. Despite successful treatment of the primary tumor in most cases, approximately 50% of patients die from metastases within 15 years after treatment. This proportion has remained constant during the last decades.

Various clinical and histopathological parameters have been found to be associated with metastatic progression of uveal melanoma. Cytogenetic analysis has revealed that loss of one copy of chromosome 3 (monosomy 3), which is present in about half of all UMs is associated with poor prognosis. In fact, this is one of the most reliable prognostic markers and is superior to all clinical markers. Consequently, monosomy 3 testing is widely used in clinical routine in patients with uveal melanoma.

In recent years a UM classification model has gained acceptance, according to which UM is not a uniform entity but can be divided in at least two major classes. The strongest evidence for this model comes from global gene expression studies. Unsupervised data analysis of global expression data supports a highly robust class assignment of tumors. These classes are congruent with the chromosome 3 status and the metastatic potential of the tumor. Therefore, tumor classification by gene expression profiling is an alternative approach for predictive testing of patients. In this chapter we will describe the prognostic markers and the different diagnostic settings in more detail. Finally we will elaborate on the strength and weaknesses of these methods in the setting of routine testing of patients.

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1 Uveal Melanoma

1.1 Clinical and Epidemiological Aspects

UM, a tumor of the eye, arises from melanocytes that reside within the uveal tract. Median age at diagnosis of primary tumor is in the mid 50th [1]. Unlike cutaneous melanoma, sunlight or other environmental factors have not been identified as risk factors for UM. The majority of tumors are treated by eye conserving therapies such as brachytherapy [2]. Removal of the eye (enucleation) is considered if the tumor is large or radiation therapy is not promising due to the location of the tumor. Although the treatment of the primary tumor is usually successful, approximately 50% of patients develop metastases within 15 years after treatment. This proportion has remained constant over the last decades [3]. It is assumed that tumor cells spread primarily hematogenously as there are no lymphatic vessels in the eye. Metastases may occur even many years after successful treatment of primary tumors and the most common site of clinical manifest disease is the liver [4]. Lung or skin metastases are also observed but are usually secondary to liver metastases. The average survival time after diagnosis of metastases is only 4–12 months and to date there is no effective treatment for metastatic UM [5, 6].

1.2 Genetic Aspects

In the early 1990th, conventional cytogenetic analyses revealed non-random chromosomal aberrations in UM including chromosomes 3, 6, and 8 [7–10]. The landmark study by Prescher et al., in which comparative genomic hybridization (CGH) results were compared with patients survival, revealed a strong association of loss of one chromosome 3 (monosomy 3) in the tumor with metastatic death of the patients [11]. Monosomy 3 is rarely observed in other cancers but is a characteristic cytogenetic abnormality in UM and is found in about half of all these tumors. In recent years, significant progress has been made in the identification of non random somatic mutations that point to key regulatory pathways relevant for UM tumorigenesis and progression. Almost 90% of all UM show a mutation in either *GNAQ* or *GNA11* with the most common mutation affecting codon Q209 in either gene. Both genes code for two closely related G-proteins and act as dominant-acting oncogenes. Therefore, activation of the MAP-kinase pathway involving these two genes is assumed to be a major contributor to the development of UM [12]. This may have therapeutic implications as these mutations may sensitize cells to drugs targeting this pathway [13]. *BAP1* is another frequently mutated gene in UM. This gene is regarded as a tumor suppressor gene in tumors with monosomy 3 only because inactivation of this gene is largely restricted to these tumors. Interestingly, some patients have *BAP1* germ line mutations. These mutations are associated with a rare hereditary tumor syndrome with incomplete penetrance for UM [14, 15].

1.3 Classification Model

More than two decades ago UM was regarded as a uniform tumor entity, although an association between morphology of uveal melanoma cells with outcome was already established [16]. The presence of different UM classes was suggested first by Parrella et al. in 1999 [17] who proposed a bifurcated tumor progression model was introduced that was based on the mutual exclusivity of monosomy 3 and 6p alterations identified in 50 UM. However, more recent studies on larger cohorts of patients do not support such a bifurcate progression model because 6p alterations are frequently found together with monosomy 3 in the same tumor and show weak association with metastasis-free survival [18, 19].

The strongest evidence for the UM classification model comes from global gene expression profiling (GEP) studies [20, 21] first performed in 2003. Analysis of global gene expression data by unsupervised cluster analysis or by principal component analysis revealed two distinct expression patterns that divide UMs in two major classes. The first study was published by Tschentscher et al. [21]. They found that the classification was almost perfectly associated with the chromosome 3 status. In a subsequent study by Onken et al. [20], classification was found to be congruent with tumor related death of patients'. Based on these results it was proposed that UM are composed of at least two major classes, class 1 and class 2 [20]. A typical class 2 tumor as determined by GEP is characterized by monosomy 3 and high metastatic potential. On the other hand class 1 tumors usually have two normal chromosomes 3 (disomy 3) and rarely develop metastases (Fig. 3.1).

The recent discovery that inactivation of *BAP1,* a tumor suppressor gene located at chromosome 3p21, is almost exclusively found in class 2 tumors with high metastatic potential further supports this classification model [35]. It is conceivable that inactivation of BAP1 is a crucial step in the formation of class 2 tumors. If this is the case, loss of one chromosome 3 unmasks *BAP1* mutations on the other allele. According to such a model, monosomy 3 is one step towards complete *BAP1* inactivation. To explain the molecular basis underlying the UM classification model, it has been speculated that the different UM classes are derived from different melanocytic precursor cells [21]. This hypothesis was later on substantiated by the observation that different epigenetic patterns are associated with the chromosome 3 status and the metastatic progression of the tumors [22].

Although, in recent years the UM classification model has increasingly attracted notice of scientists and clinicians, a progression model must not be dismissed. According to such a progression model all UM arise from a melanocytic precursor by keeping both copies of chromosome 3. Then, during further progression from early to later stages, a subset of tumor cells loses one copy of chromosome 3 and this confers high metastatic potential to the tumor. Such a progression model is supported by the observation that some rare UMs show two delimited regions, each with a different chromosome 3 status [23]. It is likely that at least these rare tumors follow such a progression model. Further evidence comes from the observation that small tumors more often show disomy 3 whereas large tumors more frequently

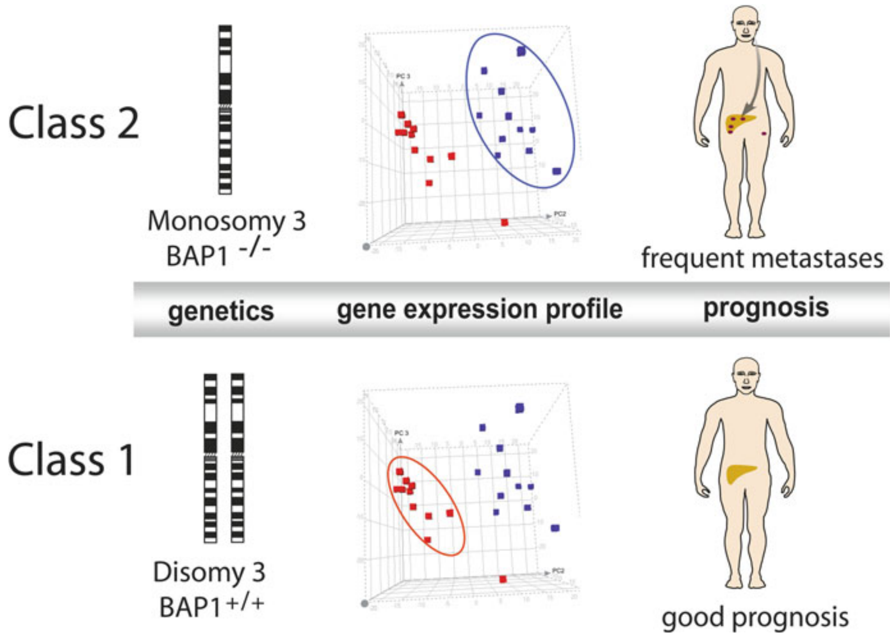

Fig. 3.1 Uveal melanoma classification model. UM can be divided in two different classes based on the chromosome 3 status, expression profile and metastatic potential. *BAP1* inactivation is associated with prognosis and tumor classification

have monosomy 3 (unpublished observation). However, it is controversial if the progression from disomy 3 to monosomy 3 is a model that applies to all UMs with monosomy 3.

2 Prognostic Biomarkers

In order to gain information on the likely course of the disease in a given cancer patient prognostic testing is frequently performed in various cancers. Some of these tests make use of biomarkers that allow to distinguish between tumors with high or low metastatic risk. Clinical application of prognostic testing may emerge from the availability of adjuvant therapy protocols for high-risk patients. At present, however, protocols for effective adjuvant treatment of UM patients do not exist. Therefore, the benefit of prognostic testing is largely restricted to those patients who want to know about their metastatic risk. Interestingly, it became evident from independent studies that the majority of patients choose to receive prognostic information [19, 24]. To identify markers suitable for prognostic testing, various clinical, histopathological and genetic features have been evaluated for association with metastatic death of

patients with UM. However, most of these markers have not found their way into routine prognostic testing and, therefore, will be mentioned here only briefly.

Clinical features are easily accessible as they are assessed during routine examination of patients. A significant association with metastatic progression was primarily found for large tumors and tumors showing ciliary body involvement [4, 25]. Extra ocular growth of the tumor and fast tumor regression after radiotherapy are also associated with patients' prognosis, however, to a lesser extent [26, 27]. Among these markers, tumor size is the most relevant marker and has been rediscovered as prognostic marker recently [28, 29].

Among *histological and cytological factors*, which are routinely assessed by pathological examination of enucleated tumors, the presence of cells with epithelioid morphology and increased infiltration of immune cells is associated with higher risk of metastatic disease. The prognostic significance of these factors in UM was reviewed in depth by Singh et al. [26].

In the early 1990th the morphology of tumor blood vessels (*vascular patterns*) in primary UM was described and evaluated for prognostic significance. Vascular patterns become visible after periodic acid-Schiff (PAS)-staining without counter staining. Of the nine different patterns that were described the presence of "vascular networks" was most strongly associated with death of metastatic melanoma [30, 31]. However, this prognostic marker has not found widespread acceptance possibly because it cannot be assessed in a standardized way.

An alternative approach to detect metastatic UM which does not require tumor tissue is based on the non invasive measurement of *serum markers*. Increased levels of some markers were found to indicate the presence of hepatic metastases: Serum levels of melanoma inhibitory activity (MIA), osteopontin (OPN), and S-100β were found to be significantly higher in metastatic UM patients compared to patients that did not show any sign of disease for at least 10 years after treatment of the primary tumor [32]. However as the study was performed on only a small number of metastatic patients more data are needed to confirm the utility of these markers.

So far, none of the above mentioned markers has reached the level of prognostic significance that is established for the *genetic marker* monosomy 3. The strong association between loss of an entire chromosome 3 in the primary tumor and metastatic death of patients was discovered by Prescher et al. in 1996 [11]. In this pivotal study, the chromosome 3 status of 54 tumors was analysed by CGH or conventional cytogenetics and was associated with metastatic death. Among patients with monosomy 3 in the tumor, 57% relapsed with metastatic disease. In contrast, none of the 24 patients with disomy 3 tumors developed metastases (Fig. 3.2). The prognostic value of monosomy 3 was later confirmed by other studies and was observed regardless of the genotyping technique used to determine chromosomal aberrations [9, 18, 33, 34]. Metastatic progression of monosomy 3 tumors is further modulated by gain of chromosome 8q: prognosis is even worse when chromosome 8 alterations occur. Gain of 8q is also observed in tumors with disomy 3 but a significant association with poor prognosis has not been identified.

It has been suggested that screening for *BAP1* mutations might be more specific for prognostic testing than chromosome 3 typing or GEP [35]. This was based on

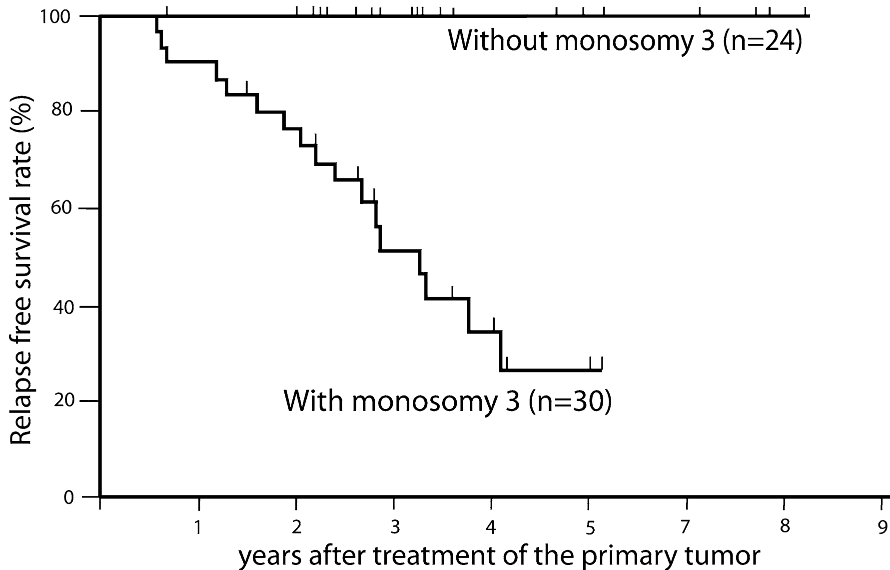

Fig. 3.2 Kaplan Meier presentation showing relapse free survival of 54 uveal melanoma patients according to the chromosome 3 status of their tumors. The figure is modified from Prescher et al. [11]

the observation that *BAP1* mutations are almost exclusively found in class II tumors with poor prognosis. However, at present there are no long term follow up studies and therefore, *BAP1* inactivation in UM must not be regarded as an established prognostic factor for poor prognosis of patients.

The gene *expression profile* (GEP) of a tumor is another feature which is associated with disease outcome. It is distinct from the above mentioned markers as it is not a single measure but a snapshot of the complex transcriptional state of the tumor tissue analysed. The profile comprises the expression values of multiple genes that in essence determine the tumor phenotype. In the first gene expression study of UM by Tschentscher et al. the expression levels of about 12,000 transcripts for each tumor were determined [21]. The gene expression profile provided a characteristic signature for each tumor and unsupervised cluster analysis revealed that, based on these signatures, tumors fall into one of two different groups (Fig. 3.5). These groups were almost perfectly congruent with the chromosome 3 status. The strong association of the GEP based classification with patients' prognosis was first published by Onken et al. and later on confirmed by other groups [20, 36]. With the broad availability of high-density microarrays the expression profiling of tumors has become easily accessible but is still rather expensive for routine diagnostic testing.

More recently, a GEP assay comprising only 12 discriminating genes and 3 control genes, which is performed on a microfluidics platform, has been introduced by Onken et al. [37, 38]. Due to the lower costs compared to array based techniques this assay might be more suited for routine clinical testing.

3 Techniques Used for Prognostic Testing

Current methods for molecular prognostic testing in UM patients require samples of tumor tissue and are largely based on analysis of two different markers: genetic alterations or gene expression profile. Genetic markers are usually determined by conventional cytogenetics, CGH, FISH and more recently by MLPA. MSA or SNP analysis are used to determine the allele ratio at several loci of the target chromosomes from which the chromosome dosage is concluded. In recent years cytogenetic methodologies have increasingly been replaced by molecular methods such as MLPA and MSA. GEP is distinct as it requires RNA isolated from the tumor tissue. Here we provide details only on those methods which have proven their suitability for prognostic testing in follow up studies on large cohorts of UM patients (Table 3.1).

3.1 Cytogenetic Analysis

In the early 1990th cytogenetic analysis were among the first techniques to identify genetic aberrations in short term cultures from primary UMs [7, 39]. Tumor cells must be maintained in culture for karyotyping, which is time consuming and costly in routine diagnostic settings. In recent years conventional cytogenetic was increasingly replaced by more convenient molecular genetic techniques.

Table 3.1 Prognostic follow up studies of patients with uveal melanoma

Follow up studies of uveal melanoma patients				
Study center	# Patients	Marker	Method	References
Essen, Germany	54	Chr.3 copy number/dosage	CGH, cytogenetics	Prescher et al. [11]
Liverpool, UK	356	Chr.3 copy number	FISH	Damato et al. [43]
Liverpool, UK	452	Chr.3 dosage	MLPA	Damato et al. [18]
Liverpool, UK	105	Loss of heterozygosity	MSA	Scholes et al. [33]
St. Louis, USA	53	Loss of heterozygosity	SNP	Onken et al. [38]
Philadelphia, USA	500	Loss of heterozygosity	MSA	Shields et al. [46]
Essen, Germany	374	Loss of heterozygosity	MSA	Thomas et al. [28]
St. Louis, USA	50	Expression profile	Microarray analysis	Onken et al. [20]
St. Louis, USA	172	Expression profile	Microfluidics platform	Onken et al. [35]

Different genetic markers were evaluated with appropriate methodologies. For each method representative studies are listed

Array based techniques for genome analysis such as Array CGH are powerful tools to identify genome alterations and have been applied to UMs for research purposes [40, 41]. These techniques allow detection of copy number changes at high-resolution and culturing of cells is dispensable. Although, these techniques are routinely used to detect chromosome imbalances in patients with genetic diseases, application in routine prognostic testing of UM is not evident from the literature. Consequently, comprehensive follow up studies based on cytogenetic or array based CGH karyotyping of tumors do not exist.

3.2 FISH

Fluorescence in situ Hybridization (FISH) is a molecular cytogenetic technique that can detect chromosomal abnormalities, particularly copy number variations. This technique can be performed on a variety of sample preparations such as touch preparations of tumor tissue, cell smears and cytospins of short term cultured cells as well as paraffin embedded tissue sections. Usually, fresh tumor samples are obtained by enucleation or, more recently, by biopsy sampling. As cultivation of live tumor cells is not needed, FISH has an advantage over conventional cytogenetics. FISH has been routinely used by some large referral centres for the analysis of the chromosome 3, 6 and 8 in UM [19, 42, 43].

Usually, a fluorescently labeled probe is hybridized to interphase nuclei on a slide. The chromosome 3 copy number is best determined by the use of a centromeric FISH probe, whereas prognostic relevant gain of chromosome 8q is evaluated by use of a locus specific probe (e.g. c-MYC at 8q28, [43]). After washing and counterstaining, the chromosome copy number in individual cells is determined by counting the fluorescent signals per nucleus in a fluorescent microscope. In contrast to all other methods described below, FISH visualizes the chromosome copy number on a single cell level. However, typically only about 80–90% of normal cells show two hybridisation signals and classifying a sample based on the results of a single cell or even a small number of cells might be misleading. Therefore it is required to count the signals of more than hundred nuclei per sample followed by statistical analysis of the results [44]. Detailed protocols for FISH analysis on UM have been published by Sisley et al. and Damato et al. [34, 43].

3.3 LOH Analysis

In UM, loss of heterozygosity, which is the loss of one allele at a polymorphic locus that is heterozygous in constitutional (normal) cells, has been determined by microsatellite analysis (MSA) or by analysis of single nucleotide polymorphisms (SNPs). Microsatellites are a class of highly polymorphic, genetic markers that are

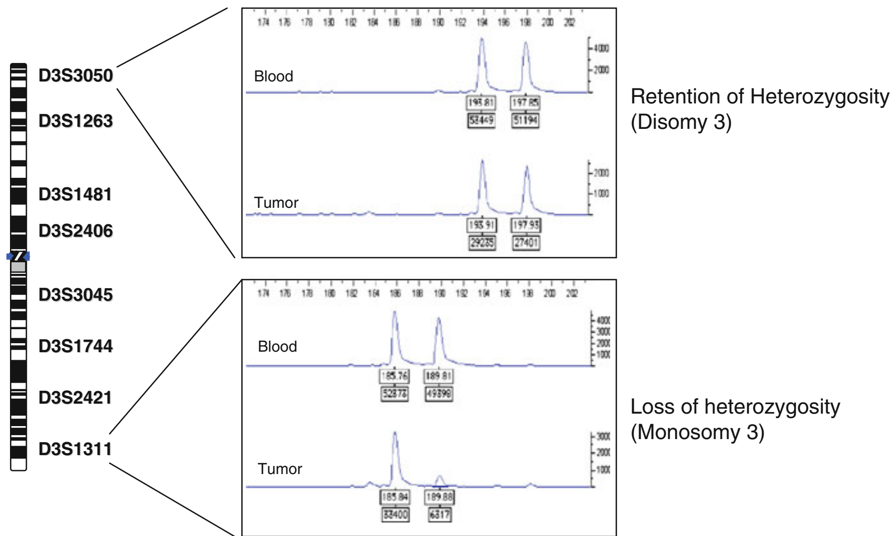

Fig. 3.3 Ideogram of chromosome 3 with the location of MSA markers as established for LOH analysis of UM by Tschentscher et al. [45]. Representative electropherograms of the marker D3S3050 and D3S1311 are shown for both blood and tumor DNA samples. D3S3050 shows retention of heterozygosity consistent with disomy 3. Marker D3S1311 shows LOH consistent with monosomy 3

widely used for mapping, linkage analysis and forensic DNA profiling. They are distributed over the whole genome. Typically, a microsatellite marker is a tandemly repeated DNA sequence with the length of the repeating unit being 1–4 nucleotides.

In practice, six to ten different microsatellite markers per chromosome are amplified by PCR and the length of the PCR products is determined by automated capillary electrophoresis. In heterozygous state, two PCR products of different lengths are amplified from each marker allele (Fig. 3.3). If the two alleles have the same number of repeat units the resulting PCR products are of the same length. This homozygous state is referred to as “not informative”. Loss of heterozygosity (LOH) is indicated if one of the two allele signals is lost or strongly reduced. To distinguish between “not informative” and “LOH” it is important to compare the marker alleles in the tumor with the corresponding alleles in non tumor DNA (e.g. DNA from blood) from the same patient. In UM testing, loss of heterozygosity (LOH) at all informative chromosome 3 loci is consistent with monosomy 3. MSA for UM typing was first introduced by Tschentscher et al. [45]. Other laboratories have also shown that tumors with high metatstatic potential can be reliably identified by microsatellite analysis [33, 46].

Tschentscher et al. established a protocol for MSA of 23 markers, 3–4 on each arm of chromosomes 3, 6, and 8. For validation purposes, MSA results were compared to the results from a CGH study on the same samples [45]. For

chromosome 3, the results of CGH and MSA were concordant, thus indicating that monosomy 3 can reliably be determined by MSA. However, a typical drawback of MSA is that, despite quantitative evaluation of allele ratios, it is not always possible to discern losses and gains reliably. However, this is of relevance for chromosome 8 analysis only as allelic imbalance of chromosome 8 markers is usually not due to loss of but gain of one allele and must therefore not referred to as LOH.

Likewise, the analysis of single nucleotide polymorphisms (SNP) can be utilized to determine LOH in tumor samples. However, the polymorphic information content of microsatellite markers is remarkably higher than that of SNPs. Consequently, there is no obvious advantage of SNP analysis over MSA for LOH detection. In spite of the availability of array based technologies for genome wide SNP analysis, which are well established for analysing gains and losses of even small chromosomal regions, this technique is not used for prognostic testing of UM.

3.4 Multiplex Ligation-Dependent Probe Amplification (MLPA)

MLPA is a multiplex PCR method that provides copy number information (gene dosage) of up to 50 different DNA loci in a single experiment at reasonable costs [47]. Like MSA, MLPA is performed on purified genomic tumor DNA and requires laboratory equipment such as thermocycler and capillary electrophoresis. A strong advantage is the availability of an UM probe set as a commercial kit.

Each of the about 40 probe sets consists of two oligonucleotides that are ligated if bound to their specific target sites on the sample DNA. Only the bound oligonucleotides are ligated and subsequently amplified by PCR using a common primer pair for all probes. As each probe has a unique length they can be separated and individually quantified by automated capillary gel electrophoresis in a single lane. The amount of the PCR product normalized against the amount of reference probes provides a measure for the gene dosage of the target sequence. This assay design facilitates relative quantification of multiple target sites in a given sample. A typical result of an MLPA analysis of a tumor with monosomy 3 is shown in Fig. 3.4. Usually, MLPA has to be performed in duplicate or triplicate to control for technical variations. The assay requires about 200–400 ng of genomic tumor DNA, which might not always be available when biopsy samples are analyzed. In contrast to MSA and SNP analysis, normal DNA is dispensable. Poor DNA quality impairs ligation and amplification reaction thus leading to ambiguous results. As DNA quality is critical for MLPA analysis the drop out rate is significantly higher compared to MSA.

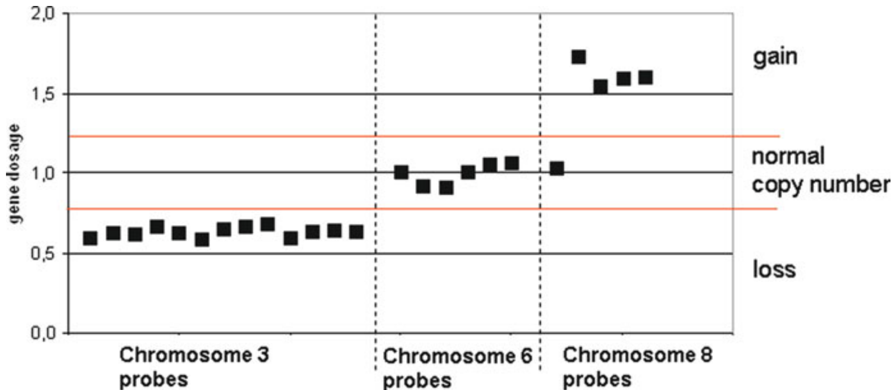

Fig. 3.4 MLPA analysis of DNA from a uveal melanoma with monosomy 3 performed with the SALSA MLPA KIT P027 (MRC-Holland). Each square displays the gene dosage of a target sequence. Control probes are not shown. Probes are grouped according to their chromosomal location. The analysis revealed a decreased gene dosage for the chromosome 3 probes. Chromosome 6 probes show a gene dosage in normal range consistent with two copies of chromosome 6. Chromosome 8 probes indicate gain of 8q material. *Red lines* delineate the normal range for gene dosage

3.5 Gene Expression Profiling

Gene expression profiling is mostly performed by use of gene expression microarrays which measure the relative abundance of several thousand transcripts in a given samples simultaneously. In contrast to all other methods described so far microarray analysis is highly demanding and requires expensive laboratory equipment. Finally, as sample classification is based on expression values of multiple genes, bioinformatic analysis of the resulting data is needed. Microarray platforms are offered by various companies the most prominent provider being Affymetrix. A microarray is a glass slide spotted with DNA fragments that represent specific genes. Each gene is represented by one or more spots, each containing DNA fragments of the same sequence. The RNA sample to be analysed is then fluorescently labelled and hybridized to the slide. After washing, the fluorescent signals of all spots are automatically quantified by laser scanning and are a measure for the expression level of the respective genes. For downstream analysis of comprehensive data sets, different mathematical algorithms are available. Hierarchical cluster analysis can be used to group the tumor samples according to the their expression profile [48]. Principal component analysis (PCA) is another tool for extracting the relevant information from large expression data sets. PCA reduces the dimensionality of the data while retaining most of the variation. The grouping of the samples can be nicely visualized by two or three-dimensional presentation of the PCA results. This algorithm places samples with similar expression profile close to each other, consequently, samples with dissimilar patterns are placed at larger distance (Fig. 3.5) [49].

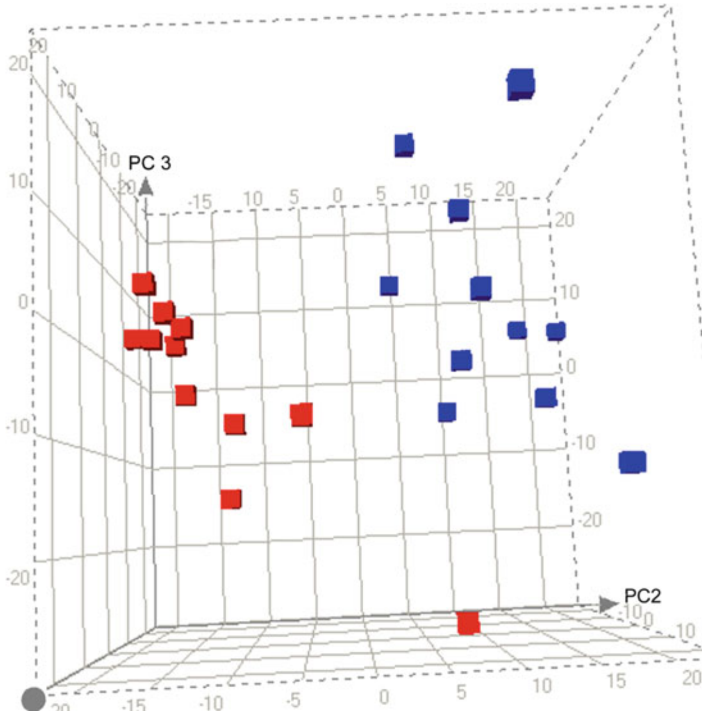

Fig. 3.5 Classification of tumors by microarray analysis. Comprehensive expression data sets of 20 tumor samples are analysed by principal component analysis (PCA). A three-dimensional presentation of the results PCA is shown. The expression profile of each sample is represented by a *square*. By this algorithm tumors with disomy 3 (*red squares*) and monosomy 3 (*blue squares*) are divided into different groups. (Data from [21])

Expression profiling on UM samples has been performed for research purposes in several laboratories but routine prognostic testing is mainly offered by a commercial company (Castle Biosciences, incorporated) using a proprietary microarray assay. This assay is based on the microarray study by Onken et al. and was validated technically and clinically during the last decade [20]. The RNA can be isolated from fresh tumor tissue obtained by enucleation or biopsy sampling or alternatively, from the formalin fixed paraffin embedded tissue.

An alternative approach to GEP based tumor classification is based on the expression analysis of a classifier set of genes. Such classifier sets were identified by various groups with the smallest set containing only 12 discriminating genes [37]. The small number of genes allows expression analysis of individual genes by real time PCR thus avoiding demanding and costly array platforms. However, this assay has been established only recently and the prognostic reliability of the test to prognosticate metastatic disease remains to be shown in long term prospective follow up studies.

4 Pitfalls of Prognostic Testing

4.1 Metastatic Tumors with Disomy 3

A link between a genetic marker and outcome as strong as that between monosomy 3 and metastatic disease in patients with UM (Fig. 3.1) is rarely seen in other solid tumors and might leave the impression that the metastatic risk of a UM patient exclusively depends on the chromosome 3 status of the tumor. However, it became apparent from long term follow up studies comprising several hundred UM patients, that some UMs with disomy 3 in fact do develop metastases (D3met) [18, 28, 46]. The proportion of these D3met tumors varies from study to study but was close to 10% in two large follow up studies in which genotyping was performed by either MLPA or MSA [19, 28]. Mis-sampling of normal instead of tumor tissue has been suggested as possible explanation for D3met tumors. However, this can not apply to all these tumors as chromosomal alterations other than monosomy 3 were present in many samples. Another conceivable explanation is tumor heterogeneity, specifically, a heterogeneous distribution of cells with and without monosomy 3 within the tumor (see below). Assuming that metastatic progression originates from cells with monosomy 3, chromosome 3 testing must fail to predict correct prognosis if, by chance, the majority of cells sampled from such a tumor have disomy 3. Furthermore, some tumors can not be clearly classified into either high or low risk group based on chromosome 3 typing. This includes tumors that show loss of only parts of chromosome 3, referred to as partial M3. Reports about the frequency of partial M3 tumors and prognosis of the patients are inconsistent [18, 28, 46, 50]. UMs with isodisomy 3 (two identical chromosomes) may also pose a problems but these tumors are rare. These tumors will present as disomy 3 by FISH, CGH, conventional cytogenetics or MLPA. In contrast, using MSA or SNP analysis the same tumors show LOH and are thus classified as monosomy 3. Due to the rarity of isodisomy 3 in UM, comprehensive survival data of patients with these tumors are not available, but it may be assumed that isodisomy 3 is functionally equivalent to monosomy 3. Accordingly, the metastatic potential of these tumors might be better captured by MSA.

Large follow up studies have shown that prognostication is improved by considering genetic abnormalities together with histologic or clinical features such as largest basal diameter (LBD) or cell type [18, 43]. Recently, Thomas et al. found a statistically significant association of metastatic progression with the largest basal tumor diameter (LBD) only when confining the analysis to tumors with disomy 3 [28]. In order to predict survival of patients based on genetic and clinical data a neural network has been designed by Damato et al. [51]. This tool estimates patients' prognosis by taking sex, clinical stage, genetic type, and histologic grade into account.

Given the current discussion in the field, it is worth pointing out that classification by GEP also fails to predict the correct prognosis in some patients. Specifically, the proportion of patients that, despite having class 1 tumors developed metastases

is similar to the proportion of patients with D3met tumors found in studies based on chromosome 3 typing [36, 52, 53]. Of note, GEP was introduced several years after chromosome 3 typing, therefore the observation period of patients included in prospective GEP follow up studies is much shorter. It remains to be shown if and to what proportion class 1 tumors will develop metastases in long term follow up studies.

4.2 Tumor Heterogeneity

Some UMs are composed of different subpopulations of tumor cells that are genetically distinct. The genetic difference usually refers to the chromosome 3 status, because this is the most intensively monitored prognostic marker in UM. Such tumor heterogeneity has been found in several studies and the degree of heterogeneity varied depending on the method used for chromosome 3 typing [54–56]. There have been reports of isolated UMs that show two discrete regions, each with a different chromosome 3 status ([57] and V. White et al. 2012, personal communication). On the other hand, heterogeneity was found as a more or less random mixture of cells with and without monosomy 3. In the later case, it is reasonable to assume that mostly the cells with disomy 3 are in fact normal cells such as macrophages or epithelial cells. Depending on the degree of infiltrating (normal) cells which can be found to more or less extent in most tumors with monosomy 3 the average chromosome 3 copy number measured over all sampled cells can reach any value between one and two copies and might thus impede tumor classification. To correctly identify tumors with monosomy 3 that are infiltrated by an overwhelming number of cells with disomy 3, it is crucial to perform quantitative analyse of gene dosage and to define a threshold above which a sample is classified as disomy 3. This threshold is best determined based on a large cohort of samples by associating the gene dosage with tumor related survival of the patients and reassessing it in large prospective follow up studies [18, 28, 45].

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Chapter 4
Capturing and Deciphering the Molecular Signatures of Head and Neck Cancer

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Abstract Head and neck cancer, while relatively uncommon in the United States remains a prevalent condition throughout the world and potentially has substantial quality of life issues for these patients.

As a disease classification, head and neck cancer, represents a diverse population of cancers which are linked anatomically, extending from the clavicles to the skull base. There are many histologic types of cancer within this grouping, although the vast majority are squamous cell carcinomas. The most significant disease sites are the oral cavity, oropharynx, larynx and hypopharynx, while other sites, such as the sinuses, are much less common and more heterogeneous. Treatment for head and neck cancer consists of surgery, radiation therapy and chemotherapy, alone or in combination. The decision to use a particular therapeutic regimen has been related to TNM stage, primary site, and comorbidity of the patient. More recently, molecular studies of head and neck cancer, such as global gene expression, methylation status, microRNA and proteomic changes, continue to identify important alterations in the genetic and molecular make-up of these tumors, all of which will be expanded upon in this chapter.

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1 Introduction

Head and neck cancer, while relatively uncommon in the United States remains a prevalent condition throughout the world and potentially has substantial quality of life issues for these patients. As a disease classification, head and neck cancer, represents a diverse population of cancers which are linked anatomically, extending from the clavicles to the skull base. There are many histologic types of cancer within this grouping, although the vast majority are squamous cell carcinomas. The most significant disease sites are the oral cavity, oropharynx, larynx and hypopharynx, while other sites, such as the sinuses, are much less common and more heterogeneous. In some parts of the world, such as eastern Asia, nasopharynx cancer is very prevalent as well. Although tobacco and alcohol have been the traditional etiologic factors for head and neck cancer, there has been a change more recently as the human papilloma virus (HPV) has been linked to oropharyngeal cancer in particular [1–3].

It is important to distinguish between the different sites of head and neck cancer, as they have different genetic signatures [4], different biomarker signatures [5] and carry different prognoses. Each of the sites has its own risk of T stage related nodal metastases [6], stage related survival, and treatment paradigm. Thus far, treatment and prognosis have been based upon gross tumor characteristics using the TNM stage, with little integration of cellular or molecular characteristics. While this has been the best practice available, it has likely led to the over treatment of some patients, with unnecessary toxicity, and the under treatment of others, with unnecessary recurrence. Therefore, there has been a continual push to identify molecular signatures for these patients, in the hope of better stratifying treatment for an individual patient.

Treatment for head and neck cancer consists of surgery, radiation therapy and chemotherapy, alone or in combination. The decision to use a particular therapeutic regimen has been related to TNM stage, primary site, and comorbidity of the patient. There has been a shift in treatment over the past century, moving from primary surgery to surgery and radiation, and more recently to chemotherapy and radiation reserving surgery for patients with recurrence or very advanced disease [7]. However, this has not translated to an increase in survival in all areas of the head and neck [8]. As treatment has changed, so too have the consequences, leading to an interest in biological therapy, such as epidermal growth factor receptor (EGFR) inhibitors [9, 10] and minimally invasive surgery [11, 12]. This has come during a time when the disease itself has evolved, with HPV becoming more prevalent in pharynx cancer, and carrying an improved prognosis [13, 14]. This has likely supported the move to less invasive therapies, facilitating their development and providing stable, or improved, survival while deintensifying therapy. It has also likely had a part in improved prognosis of pharynx cancers over the past decade, despite an increase in overall stage.

Prior to the identification of HPV as a reliable biomarkers, other efforts have been made to isolate biomarkers in hopes of tailoring treatment to improve survival

and minimize toxicity. There has been a focus on p53 [15], EGFR [16], ezrin [17], bcl [15, 18], and many others in the past, but they have not be consistent biomarkers involved in treatment decision making. HPV, on the other hand, has been clearly related to treatment prognosis, showing improved survival in the presence of HPV [13, 14]. This has been the case across multiple studies, and has led to the development of clinical trials used to validate the concept of treatment deintesification for HPV positive oropharyngeal cancers. HPV related head and neck cancers have different molecular signatures, when compared to tobacco and alcohol related head and neck cancer [19], and these factors are likely related to the prognostic differences. A correlate to HPV in earlier studies has been the expression of p16, a downstream product of HPV integration [1, 20]. Initially, this was thought to be a marker for HPV infection, however, additional studies, have demonstrated an independent favorable prognostic significance [21, 22], although the exact mechanism remains unknown. Many other molecular studies of head and neck cancer, such as global gene expression, methylation status, microRNA and proteomic changes, continue to identify important alterations in the genetic and molecular make-up of these tumors, all of which will be expanded upon in the chapter.

2 Global Gene Expression Patterns in Head and Neck Cancer

In recent years, the completion of sequencing of the human genome and the emergence of high-throughput genomics technologies has revolutionized the field of cancer research by allowing the examination of the transcription of thousands of genes at once and in parallel. Much of the earliest work utilized solid supports such as glass slides, in combination with precision instrumentation to print libraries of cDNA clones or oligonucleotides, to measure expression of several thousand genes simultaneously (Fig. 4.1). Many of the initial head and neck studies involving cDNA microarrays have focused on the identification of genes whose expression has changed in HNSCC tissue samples compared to normal tissue. But many of the studies were small in scope. For example, initial studies used arrays containing 985 clones, examined 16 HNSCC cases and identified 9 over-expressed genes, including keratin 17 and 19, laminin-5, connexin-26, and VEGF [23]. A similar study, this time utilizing laser capture microdissection of HNSCC cells to measure the expression of 588 known cancer-related genes, demonstrated increased expression of genes related to the wnt and notch signaling pathways, as well as a decrease in expression of differentiation markers such as cytokeratins [24]. Other expression profiling studies using laser capture microdissection of normal and malignant oral epithelium (five patients) with a higher density oligonucleotide array platform identified about 600 differentially-expressed genes [25]. Studies using higher-density cDNA microarrays also revealed over 200 gene expression

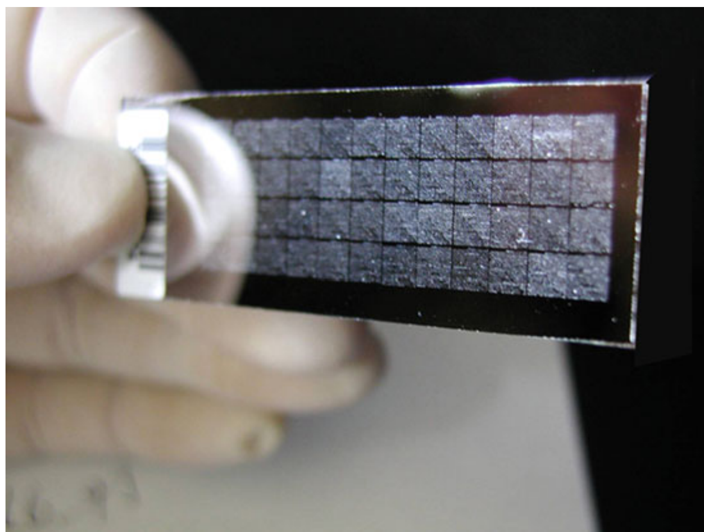

Fig. 4.1 Sample of the original microarrays containing approximate 28,000 cDNA clones printed by the Albert Einstein College of Medicine Microarray Facility

changes in head and neck cancer cell lines when compared to matched normal non-cancerous epithelial cell lines from the same patients [26]. The lists included genes associated with many cellular processes, including signal transduction, cell structure, cell cycle, transcription, apoptosis, and cell-cell adhesion.

Later studies examined not only differential gene expression, but changes in gene expression associated with progression of this disease. Our group used a cDNA microarray platform containing 17,840 clones to examine gene expression changes in primary HNSCC tissue compared to normal surgical margins, as well as gene expression in HNSCC primary tumor tissue compared to metastatic lymph node. A combination of these data sets was then used to identify genes that show a consistent pattern of expression during progression from normal to primary tumor, and ultimately to metastatic lymph node [27]. We identified 94 genes, including four tumor suppressor genes, which consistently decrease in expression during tumor progression, and 140 genes that consistently increased their expression. One of these was the ERM protein moesin, a member of the ezrin family of membrane-cytoskeletal linkers that regulate cell adhesion. In fact, both membrane and cytoplasmic expression of moesin significantly increased when comparing normal epithelium to dysplastic epithelium to tumor samples (Mann–Whitney, $p < 0.0005$). Many of the genes identified by microarray platform also revealed prognostically significant molecular targets. For example, Warner et al. identified 23 differentially expressed genes in oral cancer, that correlated with tumor stage (III–IV) and metastasis [28]. An approach similar to ours by Liu et al. identified differential gene expression associated with the progression of disease from primary to metastatic HNSCC through the retrieval by microdissection of pure epithelial

cells from paired primary tumors and cervical lymph node metastases [29]. Again, several hundred genes were identified as differentially expressed, specifically up-regulation of CCL19, CR2, EGR2, FUCA1, RGS1, and SELL, as well as the down-regulation of IGFBP6 and KLK8 in nodal metastasis compared to primary tumors. And again, the genes identified revealed new and interesting markers of interest. For example, primary tumors with higher FUCA1 and SELL expression were associated with significantly worse patient survival. Furthermore, *in vitro* manipulation of expression of the genes (eg. expression of exogenous SELL, knock-down of FUCA1 and RGS1) resulted in changes to phenotypic parameters such as invasion and anchorage-independent growth. Overall, these studies of differential gene expression could successfully classify genes according to their patterns of expression during progression of the disease, thus opening new mechanistic avenues for exploration in this disease.

Beyond differential gene expression, global microarray platforms have made it possible to sub-classify tumors into distinct groups based on observed global gene expression patterns. In our initial study, we categorized patients with HNSCC by comparison of the global gene expression patterns using cDNA microarrays containing 9,216 genes [30]. It was possible to sub-classify these tumors into two distinct groups by unsupervised clustering analysis. Patients in Group I had both lower cause-specific and overall survivals relative to those in Group II. The results provided preliminary evidence that patient segregation by gene expression profiling might be a better predictor of outcome than established clinicopathological variables. From here, cDNA microarrays have been used to link gene expression profiling of HNSCC to radiation response. For example, Hanna et al. were able to identify 60 tumor-related genes from a cDNA microarray containing 1,187 genes that could successfully predict the radiation response of tumor samples [31]. With higher density commercial microarray platforms came the ability to identify gene expression signatures associated with prognostic parameters such as recurrence of HNSCC disease [32]. Notable here was the identification of a gene expression signature enriched for genes involved in tumor invasion and metastasis with patients experiencing locally recurrent disease, as well as the marked absence of an immune response signature suggesting that modulation of tumor-specific immune responses may play a role in local treatment failure [32]. More recently, a 75-gene high-risk signature for disease recurrence using formalin-fixed head and neck squamous cell carcinoma (HNSCC) tumors was identified and compared with an independent data set obtained from fresh frozen tumors. That study revealed that the high-risk tumors enriched for genes involved in epithelial-to-mesenchymal transition (EMT), NF-kappaB activation, and cell adhesion [33]. Even as recently as 2011, gene expression profiling has been used to predict nodal metastasis in oral cancer patients [34]. Overall, the results of these and other studies provide strong evidence that the pattern of global gene expression in a HNSCC clinical specimen contains information that can be used as a predictor of prognosis, response to treatment, and a molecular classifier of this disease [35–38].

3 Copy Number Variation, Loss of Heterozygosity and Single Nucleotide Polymorphisms as Biomarkers of Head and Neck Cancer

DNA alteration, on a qualitative and quantitative level, has been a staple of cancer research for decades. It is well established that changes such as copy number variation, chromosomal rearrangements and sequence alterations can activate oncogenes and inactivate tumor suppressor genes. Advances in genomic technology have allowed us to increase our analysis throughput from single genes or regions to entire genomes while also dramatically increasing the resolution of these changes from entire chromosomes or large chromosomal regions to single nucleotide polymorphisms. In head and neck cancer, loss of heterozygosity is a well-established DNA alteration connected with oncogenesis. Techniques such as restriction landmark genomic scanning using polymeric microsatellite markers and restriction fragment length polymorphisms, fluorescence *in situ* hybridization and comparative genome hybridization have identified frequent alterations on chromosomes 3, 9, 11 and 17 in HNSCC. However, while it is clear that regions of 3p are deleted in HNSCC with frequencies of 52–64% it is still not clear which of the potential tumor suppressor genes if any are dominant [39, 40]. The major regions involved include 3p13-21.1, 3p21.3-23 and 3p25. In 2010 Lee et al. took a slightly different approach; they noted that while novel tumor suppressor genes have been identified on the 3p locus in HNSCC biallelic inactivation of these genes was not frequently observed in other studies [41–44]. They chose to look for alterations of genes located on 3p in HNSCC tumors and cell lines that were also identified as part of a 189 cancer candidate gene signature reported by Sjoblom et al. in a study of breast and colorectal cancers [45]. They identified somatic mutations in the EPHA3, ALS2CL and CMYA1 genes on 3p which were hemizygous, resulted in amino acid change and were accompanied by LOH in HNSCC; two of the three (EPHA3, CMYA1) were also found to be tumor suppressor genes in pancreatic cancer as well as breast, colorectal and HNSCC [46].

Other chromosomal regions feature prominently in head and neck cancer studies. Chromosomal region 9p21-22 is one of the most often deleted regions in HNSCC with a frequency of 70% [47]. The p16 gene resides in this region and has been shown to be one of the most commonly deleted regions as well as being epigenetically silenced [48–50]. Losses of 9p21 or 3p also are observed in 30% of benign hyperplastic lesions which make these events some of the earliest events during HNSCC progression [48]. While p16 seems to be a major alteration on chromosome 9 it is not the only one. There are other regions of with significant LOH on chromosome 9 such as 9q22.1-32, 9q31-34 which contains putative tumor suppressor genes like TGFBR1 which is associated with Ferguson-Smith syndrome and PTCH1. Chromosome 11q13 is another hot spot for alterations in HNSCC appearing in 60% of HNSCC. A small cluster of genes (CCND1, FGF3 & 4 and EMS-1) in this region have been implicated, but the gene most often observed to be amplified is the CCND1 gene. The Hras gene on 11p15 has also been observed to undergo LOH in HNSCC; however this locus seems to show mutations mostly

in oral cancer in India and other developing countries with only about 5% of the HNSCC cancers in the west having codon 12 or 61 mutations [51]. And finally, chromosome 17p13 contains the p53 tumor suppressor gene. Due to high turnover p53 expression is very hard to detect in normal tissue but loss of its function has been reported in most forms of cancer either through genomic deletions or up regulation of negative regulators. Mutant forms of p53 can be highly stable and studies have shown that positive TP53 staining can be observed in 60% of HNSCCs along with high rates of LOH [52, 53].

Another area of recent interest has been in the use of array platforms to look for associations between single nucleotide polymorphisms (SNPs) and disease risk. When paired with normal tissue (ideally from the same source however many studies use reference sources) these platforms can be used for CNV and LOH assays as well. Linkage analysis and positional cloning have revealed high penetrance susceptibility genes for other cancers like APC and BRCA 1&2, however high penetrance factors do not appear to account for any significant amount of cases in HNSCC. While there have been many small scale SNP studies looking for associations with protein variants involved in HNSCC risk factors like tobacco and alcohol which have been reviewed by Hung et al., there have been relatively few large scale genomes wide association studies (GWAS) [54]. In 2009 Wu et al. used a custom SNP array containing 9,645 SNPs covering 998 genes that were associated in the literature with cancer to identify SNPs associated with occurrence of second primary tumors or recurrence in aerodigestive tract cancers [55]. Their study looked for germline susceptibility SNPs using blood lymphocyte DNA from 150 recurrence and 300 control cases that were a subset of a study to look at the efficiency of low dose 13-cis-retinoic acid to prevent recurrence. They reported a list of 13 gene related SNPs and one mtSNP that exhibited cumulative unfavorable outcome effect as one goes from having less than 4 of them (4.29-fold; 95% CI, 2.52–7.29; $P = 7.59 \times 10^{-8}$) to having more than 8 (26.72-fold; 95% CI, 14.00–50.99; $P < 1 \times 10^{-20}$). Founded in 2004, the International Head and Neck Cancer Epidemiology (INHANCE) Consortium is using GWAS SNP arrays to analyze low penetrance factors and their associations with behavioral risk factors like alcohol consumption and smoking. In 2011, the INHANCE Consortium published its own GWAS for susceptibility to upper aerodigestive tract cancers [56]. They reported five variants with significant association with cancer risk, three of the variants (rs1573496, rs1229984, rs698), had been observed before, within the alcohol dehydrogenase genes (ADH1B, ADH1C, and ADH7) [57]. A novel variant (rs4767364) was observed near another key gene in alcohol metabolism the Aldehyde dehydrogenase 2 on chromosome 12. The 5th variant reported (rs1494961) was located on 4q21 near the DNA repair helicase HEL308 and the BRCA1-A complex subunit FAM175A (or Abraxas).

Future directions hope to examine how such genetic events can be utilized as biomarkers in guiding treatment. The understanding that genes do not work in a vacuum and are, in fact, parts of complex regulatory networks is now the focus of an increasing number of studies. These networks do not only exist within the tumor itself but these interactions involve the surrounding cells. Regional metastasis

was correlated only with mutation of TP53 in the stromal compartment in a study of invasive breast carcinoma [58]. In 2011 Bebek et al. took this approach to perform a meta-analysis integrating their LOH analysis from microsatellite marker genotyping of 122 HNSCC specimens with publicly available GWAS and mRNA expression datasets [59]. They separated tumor tissue and stroma using laser capture microdissection and looked for hot spots (regions that have significantly higher frequencies of LOH compared with other markers along the same chromosome) and cold spots (significantly lower) in each compartment. They observed that in samples containing stroma and tumor, the observed numbers of hot and cold spots were equal; however, when they separated stroma from tumor they noted that more hot or cold spots could be identified and stroma had three times the number of hot or cold spots as the epithelium. By comparing the list of 273 genes that resided within these regions of LOH to known signaling pathways they were able to reduce the gene list to ~50 genes. They noted that some of these genes had been previously identified in HNSCC as tumor suppressors, proto-oncogenes and metastasis-related genes, biomarkers and fragile sites. By analyzing data using combinations of DNA alterations and clinical data these biomarkers could allow earlier detection and better outcomes. In a recent study Graveland et al. looked at several previously reported DNA alterations such as LOH at chromosomes 3p,9p,17p, mutant TP53 and Ki-67 staining (as a proliferation marker) as possible markers for local relapse [60]. They observed that while a relatively large (greater than 5%) p53 positive field gave the best specificity for local relapse, the combination of LOH at 9p and p53 staining had had greater sensitivity and provided the most predictive potential. It was interesting to note that they did not observe the previously reported trend of an association with LOH at 3p; they acknowledged that it could be the result of their experimental design which used equal numbers of patients with and without local relapse while previous studies tended to have larger nonprogressing cohorts [61–66].

While there has been progress in the identification of common DNA alterations associated with HNSCC there remains several hurdles that must be overcome. First, studies should include some description of the percent of tumor or in lieu of that use microdissected material, since there appears to be defined interactions between tumor and stroma, mixtures of these groups will only dilute the significance of possible markers that are located within either these compartments. Likewise since there exists significant diversity between the regions which can be broadly defined as HNSCC, care must be taken to frame whether the hypotheses being explored rely on the commonalities across or the uniqueness within anatomic sites. Sample size is also a problem in many studies. SNP analysis using either high density microarrays or genomic sequencing results in an enormous amount of data and without proper safeguards spurious associations are to be expected. Future studies should employ robust statistical methods, which are necessary to correct for multiple testing problems, eliminate SNPs which associate strongly with specific populations' traits with methods like Principle component analysis (PCA) and better define CNV boundaries using advanced segmentation algorithms like circular binary segmentation [67]. Finally since the functional effect of a given gene is regulated on many levels it will ultimately be beneficial to integrate markers from

multiple analytical venues like genomics, epigenetics, functional microarrays, and proteomics to achieve reliable, predictive and prognostic biomarkers to facilitate better outcomes for HNSCC patients.

4 DNA Methylation and Epigenetic Signatures of Head and Neck Cancer

It is now widely believed that so-called "epigenetic" changes, in addition to the genetic mutations and deletions/rearrangements described to date, contribute significantly to the onset of human malignancies. By far, the most common epigenetic event in the human genome is the addition of a methyl group to the carbon-5 position of cytosine nucleotides. This covalent DNA modification occurs almost exclusively in cytosines immediately preceding guanine nucleotides (CpG dinucleotides). In 98% of the human genome, CpG dinucleotides are present approximately once for every 80 dinucleotides, a fraction of the expected frequency [68]. However, the remaining 2% of the human genome is composed of CpG-rich sequences, known as "CpG islands", which range in length from several hundred to several thousand nucleotides. In certain diseases, including head and neck cancer, hypermethylation of CpG islands is associated with the inappropriate silencing of critical genes.

In HNSCC, promoter methylation of tumor suppressor genes appears to be a common mechanism of transcriptional silencing. Numerous studies have identified promoter methylation of CDKN2A (p16), DAP kinase (DAPK), and DNA repair genes MGMT and MLH1 [69, 70]. In many cases, these epigenetic markers were also of prognostic value. Methylation of the promoter region of MGMT was associated with decreased expression of MGMT, as well as increased tumor recurrence and decreased patient survival independent of other factors [71]. In studies by Ogi and co-workers with oral SCC, methylation of the DCC gene was significantly associated with bone invasion by gingival tumors, aggressive invasiveness of tumors of the tongue, and reduced survival [72]. Methylation of two CpG islands (MINT1 and MINT31) also correlated with poor prognosis in these patients, whereas methylation of p14ARF actually correlated with a good prognosis. Invasion and metastasis of oral SCC cells have recently been shown to be dependent on methylation of the E-cadherin promoter with associated reduction of E-cadherin expression [73]. There is evidence of a statistically significant association between DNA hypermethylation of the ADAM23 gene and progression the laryngeal cancer [74]. A highly significant difference in DNA hypermethylation of the DAPK gene promoter was observed between laryngeal cancer patients with and without lymph node metastasis [75]. MLH1 and CDKN2A are also known to play an important role in laryngeal cancer development and progression [76]. A more complete description of some of many of the epigenetically silenced genes implicated in HNSCC is discussed by Ha and Califano [77], Shaw [78] and Pérez-Sayáns et al. [79].

It is known that there is considerable variability in promoter methylation events among a tumor population, thus making the high-throughput approaches an

intriguing means to identify prognostic epigenetic markers. There is also awareness that the global patterns of genomic DNA methylation may play a critical role in the molecular characteristics of neoplastic disease. To this end, the application of new "epigenomics" technologies to study global promoter hypermethylation events in human malignancies has now revealed a great deal of new information. In early studies, technologies such as restriction landmark genome scanning (RLGS) demonstrated that patterns of aberrant CpG island methylation are tumor-type specific [80]. Epigenomic profiling techniques have also revealed that CpG island methylation is associated with the histological grades of breast tumors [81]. Specifically, poorly differentiated tumors appear to exhibit more hypermethylated CpG islands than moderately or well-differentiated types. A similar study of late-stage ovarian carcinomas with CpG island microarrays revealed that a higher degree of CpG island methylation was significantly associated with early disease recurrence following chemotherapy [82]. This study identified a select group of CpG island loci that could be used as epigenetic biomarkers for predicting outcome in ovarian cancer patients. Such studies have laid the groundwork for population-based studies to examine DNA methylation patterns in human malignancies and to identify associations between specific epigenetic signatures and clinical parameters.

In the case of HNSCC, our group has applied a genome-wide approach that has led to the identification of hundreds of new epigenetically silenced genes in head and neck cancer. We have identified 958 CpG loci (including many previously unidentified genes) in which measurements of DNA methylation were altered in the primary oropharyngeal tumors relative to the normal mucosal samples. An abundance of these identified methylation alterations occur on chromosome 19, and are associated with genes belonging to the Krüppel family of zinc finger (ZNF) transcription factors [83]. Using a combination of the luminometric methylation assay (LUMA), pyrosequencing of LINE-1Hs and AluYb8 repetitive elements, and the methylation of more than 27,000 CpG loci with the Illumina HumanMethylation27 beadchip, Poage and colleagues [84] were able to demonstrate that global hypomethylation and gene-specific methylation processes are associated in a sequence-dependent manner, and that clinical characteristics and exposures leading to HNSCC may be influencing these processes. Similarly, studies using both beadarrays and tiling arrays with in HPV(+) and HPV(−) HNSCC cell lines demonstrated that HPV(+) cell lines have higher DNA methylation in genic and LINE-1 regions than HPV(−) cell lines [85]. They additionally observed higher promoter methylation of polycomb repressive complex 2 target genes in HPV(+) cells compared to HPV(−) cells and increased expression of DNMT3A in HPV(+) cells. Similarly, LINE hypomethylation was shown to be more pronounced in HPV-negative than in HPV-positive tumors [86]. Moreover, genomic instability, as measured by genome-wide loss-of-heterozygosity (LOH) single nucleotide polymorphism (SNP) analysis, was greater in HNSCC samples with this more pronounced LINE hypomethylation, possibly reflecting alternative oncogenic pathways in HPV-positive versus HPV-negative HNSCC tumors. Indeed, studies using bisulfite pyrosequencing were able to show that despite regional promoter hypermethylation, HNSCCs were generally more globally hypomethylated when evaluated against the minimum

level of methylation in the normal mucosal specimens, and the degree of global hypomethylation was associated with smoking history, alcohol use and tumor stage [87].

Another more complicating factor to consider here is the strong evidence for the existence of a CpG island methylator (CIMP) phenotype [88]. The CpG island methylator phenotype (CIMP) refers to a state of epigenetic instability in a tumor cell resulting in the concordant hypermethylation of a group of cancer genes (cell cycle, DNA repair etc.). These tumors appear to form a clinically distinct group; however, the criteria for evaluation of CIMP status vary among individual studies [89–92]. The CIMP phenotype has also been linked with prognosis. In colon cancer, for example, the CIMP+ phenotype defined a subgroup of this disease with three to five-fold elevated frequency of aberrant DNA methylation [93]. Colorectal tumors with widespread DNA hypermethylation have distinctive clinicopathological and molecular characteristics [90]. Studies have now shown that this CIMP+ phenotype is an independent predictor of survival benefit from 5-fluorouracil (5FU) treatment in stage III colorectal cancer [94]. Analysis of 140 cases of neuroblastoma recently demonstrated that the CIMP+ phenotype was associated with a significantly poorer survival, and suggested induction of transcriptional silencing of important genes as an underlying mechanism [89]. Aberrant methylation of several of the genes used to define the CIMP+ phenotype have been linked with prognosis in oral squamous cell carcinoma [72]. In one of these reports, the CIMP+ ve oral squamous cell carcinomas exhibited a greater host inflammatory response ($P = 0.019$) and a more positive prognosis [95].

The identification of biomarkers based on DNA methylation is particularly intriguing. Aberrant DNA methylation represents a stable tumor-specific biomarker that occurs early in tumor progression and can be easily detected by PCR-based methods. Such detection methods are usually minimally invasive to the patient and can be highly specific. Examples of such approaches include breast cancer detection by ductal lavage, lung cancer by sputum, and head and neck cancer by saliva [70, 96, 97]. Furthermore, the identification of genes specifically silenced by DNA methylation represents a powerful approach for the comprehensive identification of new tumor suppressor genes in HNSCC.

5 Micro RNA Changes and Target Genes

In every tumor type examined there are numerous alterations in microRNA (miRNA) expression patterns so it is no surprise that head and neck squamous cell carcinomas are no exception [98–100]. Just about every cellular phenotype is impacted by miRNA regulation including growth and proliferation, motility, differentiation and apoptosis. While some mechanisms that regulate the expression levels of protein products in a cell such as gross chromatin compaction, hypermethylation of a given gene or transcription factor binding can act much like and on-off switch, miRNA repression functions more like a rheostat to adjust

the expression levels of a protein over a smaller dynamic range. Each miRNA is capable of altering the expression pattern of hundreds to thousands of proteins and each protein in theory can be regulated by combinations of individual miRNAs [101, 102]. It is the context of the tissue specific composition of mRNAs in a given cell type and the level and composition of miRNAs that ultimately determine the levels of any protein product. Thus, the context of mRNAs available as miRNA targets will differ with cell type and disease states and the resulting phenotype of miRNA alterations could differ among diseases.

One of the great promises of molecular classification of tumor samples using genomic platforms will be to guide the best treatment options for individual patients based on their genetics [103]. To achieve this goal we need clinically relevant biomarkers that offer reliable assessment of risks for poor outcome or presence of metastatic disease. MiRNAs are amenable to both global expression measurements using microarrays and more recently high throughput sequencing and represent the best discovery tool for identification of relevant molecules to study and to potentially use in a clinical setting. The regulatory nature of each miRNA increases the probability that any given aberrantly regulated miRNA can serve as a prognostic or diagnostic marker when compared to mRNAs. Head and neck cancer studies have progressed steadily towards that end though most of the initial studies would catalog changes in miRNAs in tumors relative to their normal tissue counterparts and relative expression levels of individual patient tumor samples or tumor to normal ratios compared and statistical tests are used to rank them [104–109]. Candidate genes are chosen based on these ranking and they are subsequently studied in tissue culture models where their expression can be manipulated and phenotypic changes studied. There have been several studies investigating miRNA expression in HNSCC cell lines and primary tumors from which a host of over and under-expressed miRNAs have been identified [104–107, 109]. Oncogenic roles have been tentatively established for several miRNAs that are consistently overexpressed in HNSCC tumors. The overexpression of miR-21 is perhaps the most commonly observed defect in solid tumors [103]. This miRNA has an anti-apoptotic role and targets the PTEN and PDCD4 tumor suppressor genes. One study associated the higher expression of miR-21 with significantly decreased 5 year survival [110]. Other oncogenic miRNAs identified as being of potential significance in altering tumor behavior in HNSCC are miR-106 family that can regulate p21 and TGFB signaling and miR-155 which impacts inositol 5-phosphatase 1(SHIP1) which like PTEN acts on Akt signaling and cell survival [105]. Perhaps the combined effects of miR-21 and miR-155 on this pathway in specific patient samples are deserving of more study. High expression of miR-211 in oral tumors has been associated with poor prognosis nodal metastasis and vascular invasion [111]. Subsequent manipulation of miR-211 levels in oral HNSCC cell lines confirmed a role in proliferation, migration and anchorage independent colony formation. Induction of miR-210 under conditions of tumor hypoxia has been documented along with an association with locoregional recurrence and poor overall survival. Use of this gene expression marker might select patients for possible therapies associated with hypoxia [107].

Repression of miRNA expression occurs via several different mechanisms including epigenetic silencing, deletion, mutation or transcriptional silencing can result in tumor suppressor phenotypes. Our group previously demonstrated that low-level expression of miR-205 is significantly associated with local or regional recurrence in HNSCC independent of disease stage at diagnosis and treatment and that combined low levels of miR-205 and let-7d expression were associated with poor head and neck cancer survival [106]. A combined computational and experimental approach has identified low expression of miR-204 impacting networks of genes involved with EGFR, MMP9, SPARC and BMP-1 among others. Ectopic expression of miR-204 affected migration, adhesion and lung colonization in mice. This type of combined computational and experimental approach towards identification of protein networks that are perturbed in tumors via a miRNA mechanism could be a valuable tool to uncover therapeutic targets aimed at specific phenotypes [112]. It is not known if miR-204 alterations are directly associated with patient outcome or other clinical parameters. The methylation status of the miR-137 promoter is associated with overall survival of HNSCC patients but not disease specific survival [113]. It is presumed that the hypermethylation of this gene results in lower expression of miR-137 in tumors and the methylation status can be used as a prognostic marker of the disease. Potential protein targets and pathways impacted by this methylated gene are not known at this time. Repression of let7 family members in HNSCC has been observed by several groups in different populations of patients [105, 106, 108]. Let7 is among the most widely studied miRNAs and is known to suppress K-Ras oncogenic activity as well as HMGA2 another important cancer associated target [114, 115]. The most consistently down regulated miRNA in HNSCC tumors is miR-375 [104, 109, 116]. MiRNA-375 was first identified as a critical regulator of glucose homeostasis in pancreatic islets where it regulates insulin secretion and cell mass [117]; however, it has also been implicated in gastric cancer [118–120], hepatocellular carcinoma [121], breast cancer [122] as well as head and neck cancer [104, 105, 116]. MiR-375 targets a diverse set of proteins including, 14-3-3ζ [119] and JAK2 [118] as well as the transcription factor Sp1 and Hippo-signaling pathway member YAP [121, 123]. Overexpression of miR-375 using transient transfection of precursor miR-375 RNA in FaDu cells, a hypopharyngeal SCC, reduced proliferation, but not in two other laryngeal SCC cell lines (UTSCC-8 and UTSCC-42a) [108]. Our group using oral cavity derived UMSCC1 and UMSCC47 lines that are stably expressing increased levels of miR-375 are consistent with the results seen for UTSCC-8 and UTSCC-42a [116]. The relevant phenotype observed by aberrant down regulation of miR-375 is effects on cellular invasion. Coupled with the observation that miR-375 affects the invasive properties of HNSCC cells is the fact that miR-375 expression levels can predict risk of poor outcome and distant metastasis in HNSCC regardless of the tumor site [116].

A great challenge ahead of us is the goal of using genomics data to design gene expression signatures that can be used to predict tumor behavior at diagnosis and guide subsequent treatment options for maximal success. We are currently at a point where several individual miRNAs have significant clinical value but not yet likely

to be useful as a clinical test. It will be critical to move on towards assessing the predictive power of combinations of miRNAs to obtain signatures that can be used in the clinic.

6 Exploring Cancer Proteome for Its Molecular Classification

Proteomics is a relatively new 'post-genomic' science [124]. In contrast to gene expression studies, proteomics directly addresses the level of gene products, proteins, present in a given state. Progress in this field is based mostly on the technologies which allow the analysis of a large number of proteins-ideally the entire proteome- at the same time. Earlier proteomics has mostly relied on two-dimensional gel electrophoresis (2-DE) which enables analysis of a few hundred proteins on a single gel [125–127]. However, 2-DE has relatively low resolution and limited dynamic range when protein expression ranges 7–12 orders of magnitude within most tissue and serum samples [128, 129]. Hence, the method of detection for the field has recently shifted toward mass spectrometry (MS) based proteomics. MS measures mass of ions divided by their charge (m/z) along with intensities reflecting their abundance and ionization efficiency. MS detects almost anything ionizable in the samples. Thus it can analyze very complex mixtures and generate molecular information as long as components can be differentiated by their mass. The nature of this non-specific detection makes MS-based proteomics a very unique tool that does not rely on any particular probes or antibodies. Mass spectrometers measure m/z in a limited range with excellent sensitivity. Hence, the analysis strategy commonly involves specific proteolytic digestion of all proteins extracted from patient tissue or serum samples. Once digested, m/z of peptides can be measured with high accuracy and their sequence identified through fragmentation called tandem mass spectrometry (MS/MS). Combination of m/z and the identified sequence leads to the identification of proteins originally present in tissues or sera.

There are two ways to compare proteins from different disease states and classify tumors of interest employing MS-based proteomics. One involves measuring the absolute amount of proteins present in samples and comparing their molecular distributions. It requires as many proteins present in a single sample as standards, which makes this approach unrealistic for classification purposes. The other can be achieved through comparison of relative quantities between two states. In relative quantification, the amount of a protein is measured as fold changes in abundance in relation to the same protein in another sample. The latter strategy is usually employed to classify cancer samples since proteins from different samples can be easily paired using their m/z and classified through their intensities. However, pairing peptides solely based on m/z can be limiting when the samples are extremely complex with a large dynamic range in abundance, such as whole tissue extracts or sera. Mass spectrometry typically covers 3–6 orders of magnitude in dynamic range depending on the type of analyzer. This is usually not sufficient to investigate

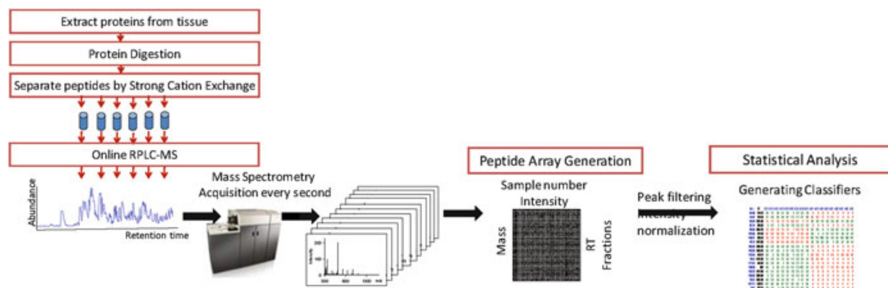

Fig. 4.2 A general work-flow of molecular classification of tumors using MS-based proteomics (*RPLC* reverse phase liquid chromatography, *RT* retention time)

the whole proteome in depth. Hence, a combination of various chromatographic separation methods is utilized to lower the complexity of samples by somewhat evenly distributing peptides into different fractions. The most common combination involves strong cation exchange and reverse phase chromatography whose orthogonal characteristics enable the most efficient fractionation. The general strategy employing mass spectrometry with chromatographic separation is illustrated in Fig. 4.2. Extracted and digested proteins are separated by strong cation exchange chromatography and split into multiple fractions. Each fraction is separated again by on-line reverse phase chromatography and the eluent is directly scanned on the mass spectrometer every second (or less, depending on the analyzer). An array of proteolytic peptides is constructed, normalized, and statistically analyzed to produce molecular classifiers. These are identified by MS/MS and can work as prognostic and/or diagnostic depending on the experimental designs. Table 4.1 summarizes potential diagnostic markers generated from the studies of patient HNSCC tissues using MS-based proteomics. The markers were verified with parallel detection method such as immunohistochemistry (IHC) or blotting method. Currently, most studies are focused on the discovery of early diagnostics by comparing protein profiles from normal and cancerous tissues. Even though experimental designs are widely different; a few common markers have been detected as is described in Table 4.1. Some of these are related to HNSCC development showing strong potential to be further developed for clinical applications for HNSCC.

MS-based proteomics to study the cancer proteome to classify tumors has been quite successful [135, 140]. Despite its success, the previous approach tends to dilute the fold changes; this is partly due to the admixture of different tissues (tumor and stroma) when extracting proteins. To overcome this disadvantage, imaging mass spectrometry (IMS) has been recently introduced to study the cancer proteome [141, 142]. IMS can be explained as a mass spectrometry *in situ* [143]. A tissue section is mounted on a mass spectrometry compatible slide. It is then split into multiple pixels and each pixel is analyzed by MS to provide protein expression profiles. These profiles acquired from IMS may be limited since the amount of proteins analyzable on the tissue section is minimal. Detected proteins are then reconstituted into hundreds of images based on the intensity of proteins in the

Table 4.1 Potential biomarkers discovered with MS-based proteomic approach using human HNSCC tissues

Potential biomarkers	References
Annexin I	[130, 131]
Cytokeratins	[132, 133]
Desmoglein-3	[132]
Desmoplakin	[132]
Receptor of activated protein kinase C 1	[134]
S100 A7	[135]
Stathmin	[131, 136]
Stratifin (14-3-3 σ)	[131, 135, 137]
Transglutaminase 3	[138]
Ubiquitin cross reactive protein	[139]
Vimentin	[132]
14-3-3 ζ, δ (YWHAZ)	[135, 137]

particular pixel. IMS therefore allows the correlation of spatial and temporal protein expression profiles with distinct morphological features without requiring target-specific reagents, such as antibodies. Data can then be analyzed based on guidance from histological staining performed after the MS analysis [144]. Unsupervised clustering of spectra can also be used to differentiate regions molecularly [145]. This approach has recently been applied to study frozen sections of head and neck tumors by Eggeling and coworkers [146]. They have demonstrated its potential in differentiating HNSCC by comparing IHC using α-defensins 1–3 and S100A8 to IMS. An example of a visualized tumor region which shows a strong expression of a particular protein is illustrated in Fig. 4.3. On the right in Fig. 4.3, H&E stained images shows clear distinction among regions such as muscle, tumor mass, and lymph node. Each has a protein expression profile with distinguishing characteristics. For example, proteins with m/z of 12,133, 10,167, and 14,044 have strong expression only in muscle, tumor mass, and lymph node respectively as shown in three figures on the left. A zoom in mass spectrum around 14,044 m/z illustrates an example of a peak and its intensity which will be reflected in a pixel of an IMS image. Classifiers in this approach can be sequenced and identified by protein extraction, digestion and MS/MS of proteolytic peptides as is described previously. IMS is a new technology of distinct clinical applications. Its outstanding molecular recognition capabilities should benefit molecular pathology with more studies to come in the very near future.

A large number of classifiers have been discovered over the last few decades with various means of detection, yet when it comes to acting as a single marker to classify tumors, most of them fall short of expectation. MS-based proteomics approaches are multiparametric in nature which should improve sensitivity and specificity. In addition, many classifiers identified through microarray and microRNA analysis can also be integrated into a proteomic panel. This can be achieved by specific targeting of proteolytic peptides originating from those classifiers. A mass spectrometric analysis called multiple reaction monitoring (MRM) [147, 148] should enable this integration through more simple, sensitive and specific detection. Absolute

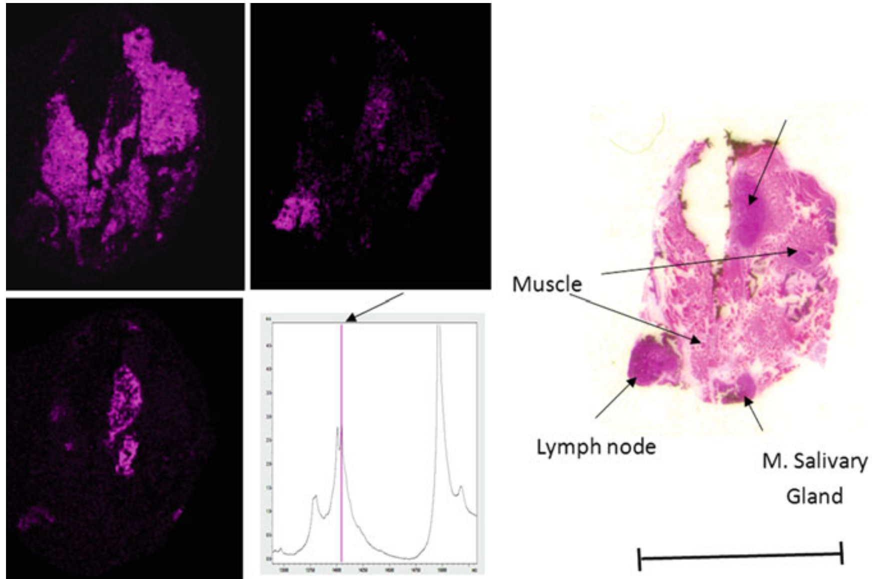

Fig. 4.3 IMS images (*left*) and the corresponding H&E image (*right*) obtained from a mouse jaw tissue with head and neck tumor growth (Zoomed in mass spectrum around m/z 14,044 displayed with MS images on the *left*)

amount of classifiers can easily be measured using MRM and compared among samples. This may eventually replace the traditional single classifier for diagnosis and prognosis of cancer.

7 Human Papillomavirus (HPV) and Head and Neck Cancer

Human papillomavirus (HPV) positive HNSCC is increasingly recognized as a distinct disease associated with improved survival and response to therapy [149–152]. HPV DNA detected in these tumors (predominantly HPV type 16, the most common high-risk type also found in cervical cancer) is frequently integrated and transcriptionally active, with the majority expressing viral oncoproteins E6 and E7 [153, 154]. In comparison to HPV-negative tumors, HPV-positive HNSCCs are more likely to be located within the oropharynx, are poorly differentiated and are diagnosed at a late stage.

The incidence of oropharyngeal cancer, the site most strongly associated with HPV infection has been rising [155]. This increase is hypothesized to result from changes in sexual norms (e.g., higher number of sexual partners) [150, 156] and cohort effects (e.g., related to early sexual activity) [155, 157] having led to a higher risk of HPV infection. However, reported detection rates of oral HPV vary

considerably by detection technique (PCR vs. other), cell sampling method, and whether superficial or basal epithelial cells are sampled. Drawing from studies that used sensitive detection and sampling techniques (i.e., PCR analysis and oral brush or biopsy), HPV prevalence in HNSCC ranges from 20% [158] to 70% [159–161]. In contrast, HPV in normal oral mucosa is much lower [162–164].

Despite the increasingly recognized importance of HPV in HNSCC, the epidemiology of oral HPV infection is still not well understood. For example, inconsistent evidence exists between oral HPV, tobacco exposure and HNSCC [157, 165–167]. These inconsistencies may be explained by the fact that HPV infection alone is not a sufficient cause of HNSCC. In addition to cell immortalization and host-cell transformation of HPV oncogenes [168], other genetic and epigenetic changes in the cell may be to blame (e.g., mutagenic and carcinogenic effects of tobacco and alcohol habits over time). Under the HPV-induced HNSCC model, HPV E6/E7 oncogene expression, uncoupled from differentiation-dependent regulation following viral integration, may initiate a number of cancer transformation processes by conferring many of the characteristics required of a malignant cell including: resistance to growth inhibition, evasion of apoptosis, immune response and immortalization as also described in cervical cancer [169], combined with a reduction in angiogenesis and independence from mitotic stimulation that may not be specific to HPV-induced HNSCC [19]. Results from molecular array studies [86, 170–174] support the hypothesis that HPV positive and negative HNSCC are clinically and biologically distinct and may represent different cancer lineages formed through separate etiologic pathways of multistage tumorigenesis [175].

There is also growing consensus that HPV status is an independent predictor of HNSCC survival. A meta-analysis of 23 case-series studies showed a 15% reduction in risk of death in HPV-positive HNSCC patients compared to their HPV-negative counterparts [152]. When restricted to the oropharynx, there was a 75% reduction in risk of overall death and 53% reduction in risk of progression, which is in line with recent reports [14, 149, 152]. Others have also demonstrated improved prognosis for HPV and/or p16 positive HNSCC treated with either radiotherapy or chemo-radiation alone, or by surgery with or without adjuvant radiotherapy [176–178]. A lower risk of secondary primary HNSCC has also been attributed to HPV-associated cervical cancer survivors [179]. Survival studies such as these have provided the basis for the development of therapeutic trials for HPV-positive HNSCC; including assessing established therapeutic approaches combined with HPV diagnostics, and designing specific therapies targeting HPV. For example, an ongoing ECOG protocol (#E1308) will compare induction chemotherapy followed by cetuximab with low dose versus standard dose IMRT for oropharyngeal SCC patients with resectable disease. Alternatively, investigators are also testing therapeutic DNA vaccines encoding the HPV-16 E7 oncoprotein administered in an adjuvant setting for patients with HPV-positive HNSCC [180], while others are developing radio-immunotherapies for virally associated cancers targeting HPV-16 E6 oncoproteins [181].

More recently, the introduction of prophylactic HPV vaccines, targeting HPV-16 among others, also provides promise of significant reductions in HNSCC [155].

However the phase III trials focused primarily on reducing incidence of HPV associated anogenital disease in men and women [182–185], and are effective if administered prior to infection and in younger individuals [182, 186]. Therefore, any anticipated benefits of vaccination on head and neck cancer incidence may not be observed before several decades. However, methods to detect HPV in tumors are being considered to improve diagnosis and identify candidates for targeted therapy using radiation/chemotherapy [187–189]. Coupled with improved understanding of the biology of HPV-associated HNSCC, and the development of novel therapies targeting these cancers, there is growing hope for the prevention and control of a substantial fraction of HNSCC.

8 Integrating Current Methodologies with Better Analysis: Teaching an Old Dog New Tricks

The future of genomic analysis can be divided into two paths: the growth of analyses that better utilize well established high throughput techniques such as microarrays, and the development of analyses that make use of the emerging high throughput sequencing technologies. For the former, the plethora of publically available datasets combined with the cheaper costs of microarray based experiments drives the development of new workflows and algorithms that can better utilize the scores of data that a single experiment can generate. In the case of massively parallel sequencing (MPS), not only are advances in analyses methods needed, but also advances in data management and manipulation continue are actively explored. The single base pair resolution that MPS provides, however, makes the investment worth it, and the costs of performing large sequencing experiments has decreased exponentially over the past few years [190]. Both paths of research can and must be integrated with clinical and biological data in order for the dream of personalized medicine to become a reality for patients with HNSCC.

Since its advent in 1987, the cDNA microarray has revolutionized modern science, allowing for investigators to probe the gene expression of hundreds to millions of genes in a relatively inexpensive manner [191]. The scope of these technologies has been widened through subsequent modifications: copy number variations, single nucleotide polymorphisms, and epigenetic modifications can now also be probed on various platforms [191, 192]. As the technology has grown and expanded, so has the level of sophistication of the analysis performed on these experiments. Some of the earliest work utilizing microarray data in matched tumor and normal epithelium utilized standard T-tests to examine the differences in expression of genes between four paired tumor and normal samples [193]. The standard T-test a very powerful tool for microarray analysis when used correctly, but can only give you information about individual markers correlating with disease. Furthermore, standard T-tests ignore interrelationships between multiple individual genes that may work in synergy to produce the observed clinical phenotype [194]. In these cases, most important genes would not be identified by this type of analysis.

More recently, newer methods for identifying genes associated with a particular clinical phenotype take advantage of methods that can overcome the hurdle of multidimensionality. We will discuss three here: support vector machines (SVMs), Bayesian networks, and Random Forests. Support vector machines utilize multi-dimensional space to separate cohorts based on defined features that could not be separated in lower dimensions [195]. SVMs operate better than most other statistical methods in handling multidimensionality, and allow for the discovery of features that work in unison or antagonistically. Most work with SVMs in HNSCCs has been utilizing them for characterization of neoplasias by mass spectroscopy [196]. In other cancers, SVMs have been utilized for multiple types of data, including methylation and gene expression arrays in breast and lung cancer [197]. Taking advantage of these algorithms might give more insight to cancers as heterogeneous as HNSCCs, where the effects of that heterogeneity have proven difficult to overcome in defining reliable molecular markers for classification. Bayesian networks also are able to overcome the conundrum of multidimensionality through the calculation of probabilities of a given outcome for features in a selected panel [196]. The use of Bayesian networks in HNSCC was initially discussed by Sebastiani et al. in 2003 [198]. Since 2003, some studies have utilized these robust machine learners to classify patient cohorts which might respond better to a particular drug or treatment regimen [199, 200]. Bayesian networks might also be very powerful in classifying HNSCC subtypes. In a 2009 study in Blood, Oehler describes the use and validation of a Bayesian model averaging algorithm to accurately classify different subtypes of CML that were indiscriminate using the current classification methodologies [201]. In HNSCC, where previous literature highlights the considerable differences in behavior and composition between tumors arising from different sites of origin, this may prove especially useful [30]. And finally, random forests are specifically optimized for handling datasets where the number of variables is considerably larger than the number of observations, an ideal model for today's high throughput screenings [202]. The random forest algorithm works by building a set of CART trees. Each individual tree contains a single feature that can make a decision about a classifier. The individual trees can then be combined together to generate a forest that has the greatest accuracy for classifying on your chosen criteria. This algorithm is uninhibited by dimensionality and has been utilized to infer novel regulatory networks from expression data from *E. coli*. [203]. Thus far, random forests have not been used to infer gene networks in HNSCC, and their use has been very limited in other cancers thus far.

Since the sequencing of the first human genome in 1998, it has been apparent that the future of medicine would be personalized to an individual's genome. With the cost of sequencing an entire genome decreasing significantly, this dream is much closer to becoming a practical reality [190]. Undoubtedly, large scale projects utilizing massively parallel sequencing hold the answers to deciphering many of the complexities of HNSCC. Despite the advances in sequencing technology, the management and analysis of such a large amount of data continues to be a problem for researchers and clinicians alike. A collaborative study examining sequencing data from HNSCC was published in Science this year [204]. By examining matched

pairs of 77 tumor and normal samples, Stransky et al. revealed several novel mutations previously unknown to be implicated in patients with HNSCC. One mutation, a truncation mutation in NOTCH1, was found to possibly play a tumor suppressive role [205]. Collaborative studies such as these are the first step in integrating genomics with modern day medicine.

Integrating high throughput genomics with other levels of gene regulation holds tremendous possibilities. The researchers of the Cancer Genome Atlas project have begun the process of laying the groundwork for conducting such large studies [206]. Integrating genomic, epigenomic, and expression data can reveal novel members of critical pathways that are routinely perturbed in different ways in patients with a particular cancer. The advancement of techniques such as CHIP-seq and RNA-seq can help to identify novel gene fusions and non-coding RNAs that were previously uncharacterized as playing a role in tumorigenesis [207]. One such example is highlighted in the Expectation Maximization of Expression Binding Profiles (EMBER) algorithm [208]. EMBER combines transcription factor binding sequencing data with expression profiles generated from either microarray or RNA-seq analysis to reveal transcription factor binding profiles. Such high level analysis will allow researchers an unprecedented insight into the biological mechanisms that drive cancer progression, and ultimately new targets for therapy.

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Chapter 5
Predictive and Prognostic Biomarkers for Colorectal Cancer

Lara Lipton, Michael Christie, and Oliver Sieber

Abstract The pathogenesis of colorectal cancer (CRC) is complex with at least two distinct pathways defined by different forms of genomic instability, and with each pathway including multiple sequential genetic and/or epigenetic changes. The treatment of CRC has evolved substantially over the past decade, due in part to a better understanding of the biology of the disease and development of new drugs including molecular-targeted agents. In this chapter we review molecular classification, prognostic markers and predictive markers in CRC. We focus on markers that have a substantial body of literature available to assess their potential role in routine clinical practice. Future strategies including gene-expression array based testing are also discussed.

1 Introduction

Colorectal cancer (CRC) is a leading cause of cancer related morbidity and mortality. It ranks as the second most common cancer in women (∼570,000 cases in 2008) and the third most common cancer in men (∼663,000 cases) worldwide. CRC incidence shows pronounced geographic variation, with the highest rates observed in Western Europe, Australia and North America, and the lowest rates reported in

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sub-Saharan Africa. In most regions of the world the incidence of CRC is increasing. About 608,000 deaths from CRC were recorded in 2008, making it the fourth most common cause of death from cancer [1].

Patients presenting with stage I (confined to the bowel wall), stage II (penetrating the bowel wall) or stage III (involvement of lymph nodes) disease can often be cured by surgery, with 5-year survival rates in the United States of approximately 90, 80 and 50%, respectively. However, following resection of the primary tumor there remains a considerable risk for tumor recurrence for patients with stage III and high-risk stage II disease (T4 stage, high grade, lymphovascular invasion, obstruction and/or perforation of the bowel at presentation), with relapse in approximately 50% of patients in the absence of further treatment. In these patients, 5-fluorouracil (5-FU)-based adjuvant chemotherapy after surgery can reduce recurrence risk by approximately 30% [2, 3], and the addition of oxaliplatin further improves outcomes and is the current standard of care (Fig. 5.1a). In clinical practice, many CRC patients receive adjuvant treatment unnecessarily, either because they were cured by surgery or because they will relapse despite treatment. It is therefore critical to identify new prognostic and predictive markers to more appropriately target adjuvant treatment to those patients who will benefit the most.

In patients with advanced (metastatic, stage IV) disease at presentation or as a result of relapse, prognosis is poor with a 5-year survival rate of only 8%. In such patients, potentially curative surgery is rarely possible. However, the development of combination therapies utilizing 5-FU together with either oxaliplatin or irinotecan has lead to progressive improvements in patient survival. Recently, these combinations have been expanded to include agents that selectively target molecular pathways that drive CRC growth. These include cetuximab and panitumumab, monoclonal antibodies against the epidermal growth factor receptor (EGFR), and bevacizumab, a monoclonal antibody against the vascular endothelial growth factor A (VEGF-A) (Fig. 5.1b). While the addition of these agents to chemotherapy in metastatic disease has led to improvements in both progression-free and overall survival, these targeted agents have not proven to be of benefit in the adjuvant treatment of stage II and III CRC [4, 5]. The introduction of targeted treatments for metastatic CRC has been associated with a very significant increase in healthcare cost and an expanded spectrum of side effects. Given these increasing constraints, novel prognostic and predictive markers are required to guide their use in advanced and early-stage disease with an intense research focus on molecular biomarkers to personalize therapy.

Recent developments in the application of anti-EGFR monoclonal antibodies are an example of how tumor molecular markers can be used to personalize treatment for CRC. In patients with metastatic CRC, response to cetuximab monotherapy in clinical trials has been repeatedly shown to be limited to *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) wild-type tumors (response rate of 13–17%) with very few responses observed in *KRAS* mutant tumors (response rate of 0–1.2%) [6, 7]. Based on these data, current American Society of Clinical Oncology (ASCO) guidelines recommend the use of anti-EGFR monoclonal antibodies only for patients with *KRAS* wild-type cancers [8].

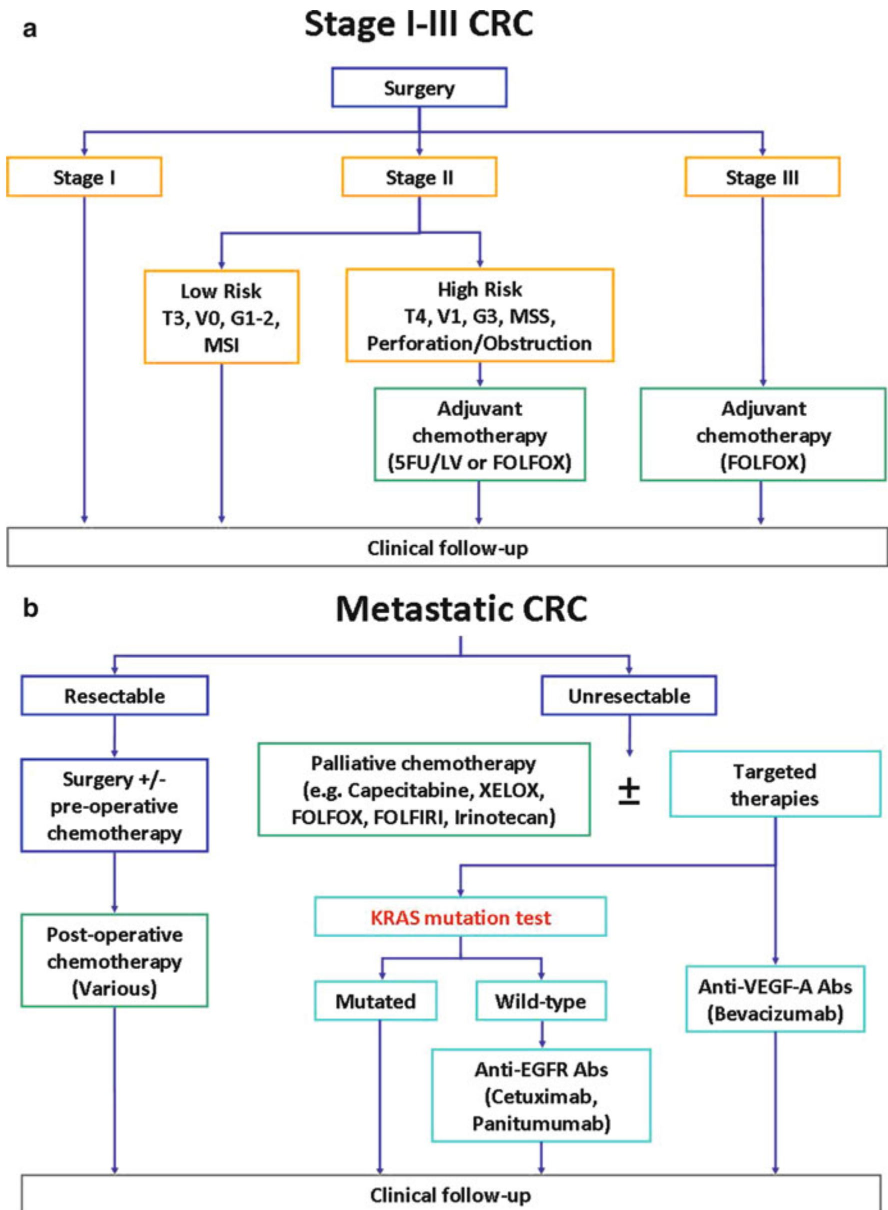

Fig. 5.1 Patient pathways for colorectal cancer (CRC) for (**a**) early-stage and (**b**) metastatic disease including common treatment regimens. The central role for *KRAS* mutation testing in guiding the use of anti-EGFR antibody therapies for the treatment of metastatic CRC is *highlighted*. Pre-operative chemoradiotherapy for rectal cancer is omitted from these pathways

2 Molecular Classification of CRC

To date the most intensely studied biomarkers in CRC are somatic (tumor acquired) changes that have been associated with cancer development, including mutations in tumor suppressor and oncogenes, CpG island methylation and global genomic instability status (microsatellite or chromosomal instability). Analyses of germline (inherited) changes have mostly focused on pathways involved in the metabolism and mechanism of action of chemotherapy agents including 5-FU, oxaliplatin and irinotecan.

Sporadic CRC is often considered to develop along two main genetic pathways, a working model which is an oversimplification. The majority of CRCs appear to follow the classical adenoma-carcinoma pathway (Fig. 5.2a), which is frequently associated with mutations of the *APC* (adenomatous polyposis coli), *KRAS*, *PIK3CA* (phosphoinositide-3-kinase, catalytic, alpha polypeptide), *SMAD4* (SMAD family member 4) and *TP53* (tumor protein p53) genes and the acquisition of chromosomal instability [9]. Less frequently, CRCs may arise via the serrated neoplasia pathway (Fig. 5.2b) characterized by mutation in the *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) gene, CpG island hypermethylation at specific sites and the loss of DNA mismatch repair function resulting in hypermutation detected as microsatellite instability (MSI-H) [10].

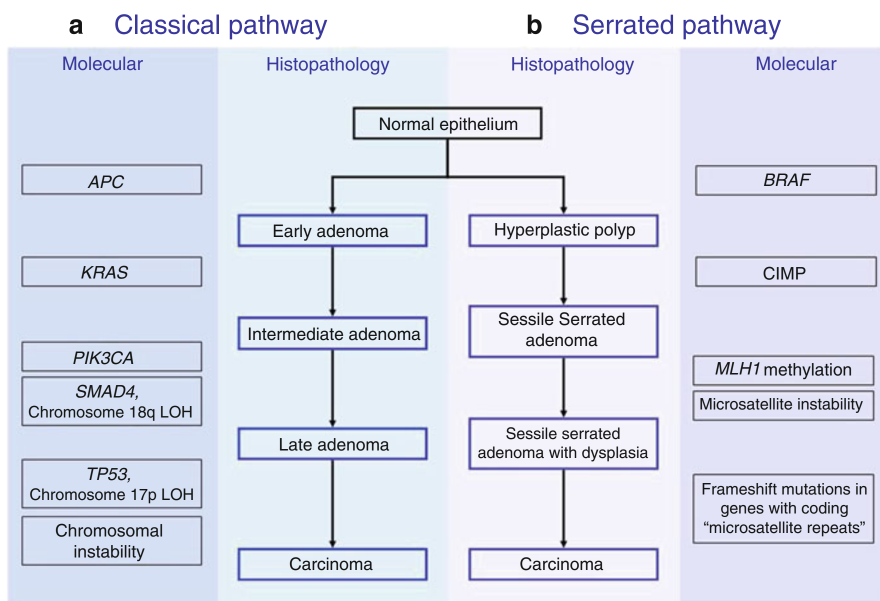

Fig. 5.2 The two main pathways of colorectal tumorigenesis. (**a**) The classical adenoma-carcinoma pathway and (**b**) the serrated pathway. Histopathological progression is driven by successive genetic and epigenetic changes in tumor suppressor genes and oncogenes and the acquisition of genomic instability

In addition, individual CRCs accumulate a plethora of low frequency genetic and epigenetic aberrations some of which are likely to influence their pathogenesis and biological behavior. Recent development of microarray and next-generation sequencing technologies has paved the way for analysis of such changes, but the evaluation of low frequency alterations as prognostic or predictive markers remains challenging, requiring the analysis of large patient cohorts with detailed clinical and long-term follow-up data and the development of standardized methodologies and standards of reporting.

In this chapter, we discuss molecular markers in CRC that have a substantial body of data evaluating their potential prognostic or predictive value. Currently, few of these markers have reached the level of evidence required for routine clinical application and for many markers there are conflicting results amongst studies. We further introduce recent developments in the application of array technologies to develop "unbiased" biomarker signatures.

3 Prognostic Biomarkers for CRC

3.1 KRAS

The *KRAS* proto-oncogene is a central component of the RAS/RAF/MEK/ERK/MAPK signaling pathway. Activating mutations in *KRAS* are common and early events in colorectal tumorigenesis, occurring in codons 12 and 13 (exon 2), 61 (exon 3) and 146 (exon 4) in approximately 37% of cases [9, 11, 12]. Mutations lead to a constitutively active GTP-bound protein that signals to BRAF triggering downstream activation of the MAPK signaling cascade.

Multiple studies have investigated the role of *KRAS* mutation as a prognostic marker in CRC with varying results. The RASCALII study, combining data on 3,439 patients with stage II to IV CRC, analysed outcome for 12 different mutations identified in codons 12 and 13. In multivariate analysis, only a glycine to valine substitution in codon 12 (present in 8.6% of all patients) was found to be associated with poorer failure-free survival (FFS) and overall survival (OS). This mutation appeared to have a stronger impact on outcome in stage III patients as compared to stage II patients [13]. In the QUASAR trial, amongst 1,583 patients with stage II CRC, presence of *KRAS* mutation was associated with a decrease in recurrence-free survival (RFS), a difference which appeared more pronounced in rectal cancers [14]. For patients with metastatic CRC (n = 711), the FOCUS trial reported *KRAS* mutation (codons 12, 13 and 61) as a poor prognostic factor for OS, although no significant relationship was observed between *KRAS* status and progression-free survival (PFS) [15]. In contrast, other large studies have found no prognostic effect of *KRAS* mutation on patient outcome. For example, *KRAS* analysis (codons 12 and 13) in 1,564 patients with resected stage II and III colon cancer from the PETACC-3 trial found no evidence for association with RFS or OS [16]. Similarly,

analysis of the best supportive care arms of several phase III studies of anti-EGFR monoclonal antibodies in metastatic CRC failed to identify a significant prognostic value of *KRAS* mutation status [6, 7, 17]. At present the combined evidence remains insufficient to support the use of *KRAS* mutation as a prognostic marker in CRC.

3.2 BRAF

The *BRAF* gene encodes a serine–threonine protein kinase that acts downstream of KRAS in the RAS/RAF/MEK/ERK/MAPK signaling pathway [18]. *BRAF* mutations occur in approximately 10% of CRCs, with the most common activating change being a valine to glutamic acid substitution at codon 600 (V600E). Presence of *BRAF* mutation is positively associated with a number of clinical and molecular features including female gender, older age at diagnosis, right-sided tumor location, MSI-H status and the CpG Island Methylator Phenotype (CIMP). *BRAF* and *KRAS* mutations tend to be mutually exclusive in tumors [10, 16].

There is emerging evidence to suggest that presence of a *BRAF* mutation is a predictor of poor prognosis in patients with metastatic CRC. In a retrospective analysis of BRAF V600E mutation status in 519 tumors from the CAIRO2 trial, *BRAF*-mutated tumors showed significantly shorter PFS and OS [19]. Similarly, the FOCUS (n = 711) and AGITG MAX (n = 315) trials detected a negative association between *BRAF* mutation and OS, although no difference was apparent for PFS [15]. In addition, the CRYSTAL study (n = 635) reported poorer PFS and OS for *BRAF*-mutated/*KRAS* wild-type tumors [15, 20–25], and comparable results have been reported for a number of retrospective non-trial cohorts [15, 20 25].

The prognostic value of *BRAF* mutation in early-stage disease, on the other hand, remains uncertain. In both the PETACC-3 (n = 1,564, stage II and III) and QUASAR (n = 1,584, stage II) trials BRAF V600E mutation was not associated with RFS, although PETACC-3 reported poorer OS for patients with MSI-low (MSI-L) and stable (MSS) tumors [14, 16]. The latter finding is consistent with results from a retrospective study on 911 stage I to IV colon cancers [26]. In contrast, the Intergroup 0135/NCCTG 91-46-53/NCIC CTG CO.9 trial (n = 533, stage II and III) found no association between *BRAF* mutation and OS for MSI-L and MSS tumors, but did find worse OS in MSI/BRAF-mutant cancers compared to MSI/BRAF-wild type cancers [14, 27]. Three other large retrospective studies have reported a negative impact of BRAF V600E mutation on outcome in early-stage patients, although this was limited to right-sided cases in one study [28–30].

Taken together, the evidence suggests that BRAF V600E mutation is a marker of poor prognosis in patients with metastatic CRC, although routine testing has not yet been endorsed by current clinical guidelines. The prognostic value of *BRAF* mutation in the early-stage disease setting – in particular with respect to prediction of recurrence risk – is less certain.

3.3 *PIK3CA*

Somatic mutation in *PIK3CA*, the p110 alpha catalytic subunit of phosphatidylinositol 3-kinase (PI3K), have been described in 10–30% of CRCs. The majority of these activating changes are localized in the helical (exon 9) and catalytic (exon 20) domains of PIK3CA [31] and are thought to constitutively activate the PI3K/AKT pathway driving cell proliferation [32].

Despite their considerable prevalence in CRC, data on the prognostic value of *PIK3CA* exon 9 and 20 mutations are relatively sparse. A retrospective study on 158 patients with stage I to IV CRC reported shorter RFS for stage II/III individuals with *PIK3CA*-mutated tumors [33], and similar results were reported in a study on 240 patients with stage I to III rectal cancer [34]. An analysis on 450 patients with stage I to III colon cancer further observed reduced cancer-specific survival (CSS), but this appeared limited to persons with *KRAS* wild-type tumors [35]. Intriguingly, differential effects on patient outcome have been observed between *PIK3CA* mutations in exons 9 and 20. In a study of 685 patients with stage I to III colon cancer, *PIK3CA* mutations in exon 20 were found to be a negative prognostic factor for DFS, CSS and OS in stage III tumors (but not in stage I and II tumors). In contrast, *PIK3CA* exon 9 mutations did not appear to affect survival [36]. Currently, the combined evidence on the prognostic value of *PIK3CA* status in early-stage disease remains insufficient. Existing data in the metastatic setting do not suggest a prognostic role for *PIK3CA* mutation [37].

3.4 *TP53*

The *TP53* tumor suppressor gene encodes a transcription factor that is activated in response to a variety of cellular stresses including DNA damage. The activated TP53 protein regulates transcription of downstream target genes to initiate programs of cell cycle arrest, DNA repair, apoptosis and/or angiogenesis. Loss of TP53 function through gene mutation, often accompanied by loss of the wild-type allele, occurs in approximately 50% of CRCs [9, 38].

Numerous studies have evaluated TP53 status as a prognostic marker in CRC with contradictory results. A particular challenge in assessing these data has been the use of different methodologies to determine TP53 status including mutation screening and immunohistochemistry (IHC) for protein expression. A meta-analysis of 168 eligible studies comprising all stages of disease found an increased risk of death for patients with abnormal TP53 based on both IHC ($n = 12{,}257$, relative risk (RR) 1.32, 95% confidence interval (CI) 1.23–1.42) and mutation analysis ($n = 6{,}645$, RR 1.31, 95% CI 1.19–1.45), although suboptimal study design of component studies, publication bias and study heterogeneity were evident. The adverse impact of abnormal TP53 appeared to be greater in patients with a lower baseline risk of dying [39]. In contrast, the TP53 CRC International Collaborative

Study, analyzing *TP53* mutation data on 3,583 stage I to IV patients, found no significant prognostic value of *TP53* status for the overall cohort, but some evidence of inferior prognosis was reported for certain types of mutations, particularly for distal colon tumors [40, 41]. Further analysis of this cohort, classifying *TP53* mutations according to functional status for transactivation based on reporter assays, suggested that such loss of function mutations were more frequent in stage IV CRC and associated with worse prognosis in this stage of disease [42]. Given these heterogeneous results, the prognostic value of *TP53* remains uncertain.

3.5 Chromosome 18q LOH/DCC Protein Loss

One of the most common cytogenetic abnormalities in CRC is deletion of the long arm of chromosome 18q present in up to 70% of cases [9, 43]. The *DCC* (deleted in colorectal carcinoma) gene was initially suggested as the primary target of 18q loss, but *SMAD4* has since emerged as the more likely candidate supported by the identification of frequent somatic mutations [44–46]. SMAD4 is a central effector of the transforming growth factor-β (TGF-β) signaling pathway. TGF-β is an important growth inhibitor of epithelial cells, and loss of sensitivity to this cytokine as a result of *SMAD4* inactivation is thought to contribute to uncontrolled cell proliferation [47].

The prognostic value of chromosome 18q deletion has been evaluated using different methodologies, either directly using DNA-based loss of heterozygosity (LOH) analysis or indirectly using the level of DCC protein expression as a surrogate. Several studies have suggested an inferior prognosis for patients with stage II and III cancers harboring 18q LOH or loss of DCC protein [43, 48–50], but others have found no association including an analysis of 955 DNA mismatch repair proficient stage II and III colon cancers from the CALGB 9581 and 89803 trials [51–55]. A meta-analysis of 17 retrospective studies (2,189 patients, stages I to IV), demonstrated worse OS for patients with 18q LOH/DCC protein loss compared to those with intact 18q/DCC protein expression (hazard ratio (HR) 2.00, 95% CI 1.49–2.69), although there was evidence of study heterogeneity and publication bias [56]. Other investigators have analyzed SMAD4 protein loss and reported a negative prognostic effect in early stage [57, 58] and metastatic CRC [59].

Currently, the prognostic value of chromosome 18q status remains to be fully elucidated. In particular, chromosome 18q deletion is strongly correlated with the presence of overall chromosomal instability (CIN), another potential prognostic marker [60]. Despite these limitations, one ongoing adjuvant study (ECOG5202/NCT00217737) is currently stratifying completely resected stage II colon cancer patients for treatment in part based on the LOH status of chromosome 18q (http://www.cancer.gov/clinicaltrials/search).

3.6 Defective DNA Mismatch Repair/Microsatellite Instability

DNA mismatch repair (MMR) is integral to the correction of base-base mismatches generated during normal DNA replication, recombination or as a result of DNA damage. Germline mutations in MMR genes underlie the syndrome of hereditary non-polyposis colorectal cancer (HNPCC), and somatic inactivation of MMR is found in approximately 15% of sporadic CRCs [61]. The most common mechanism of MMR inactivation in sporadic CRC is transcriptional silencing of the *MLH1* (human mutL homolog 1) gene by promoter methylation [62, 63]. Cells defective for MMR (dMMR) accumulate mutations at an increased rate including insertions/deletions at nucleotide repeat sequences, a phenotype called microsatellite instability (MSI). Cancer MSI status can be determined using PCR-based techniques in which the length of microsatellite repeats is compared between tumor and matched normal DNA (Fig. 5.3a). A consensus panel of five microsatellite markers is commonly used, with cancers having instability detected at two or more markers considered to have MSI-high (MSI-H) [64]. MMR deficiency may also be reliably detected by immunohistochemical analysis for the mismatch repair proteins MLH1, MSH2, MSH6 and PMS2 (Fig. 5.3b). dMMR/MSI-H is associated with right-sided cancer location, mucinous histology, poor differentiation, female gender and older age [65]. dMMR/MSI-H prevalence appears to decrease with advanced tumor stage, with low frequencies reported for metastatic CRC [66]. Strong positive associations exists with *BRAF* mutation [10, 67] and the CpG Island Methylator Phenotype (CIMP) [10].

Evidence from the majority of published studies suggests that dMMR/MSI-H status is associated with improved prognosis in CRC [14, 53, 68–71]. In a meta-analysis of 32 eligible reports (7,642 patients, stages I to IV) the combined HR estimate for overall survival associated with MSI-H was 0.65 (95% CI 0.59–0.71) [71]. In the PETACC-3 study (n = 1,564), the prognostic value of MSI status was found to be stronger in patients with stage II as compared to stage III colon cancer [72], and the QUASAR study (n = 1,584) identified both loss of MMR protein expression and T4 stage as independent prognostic factors for stage II CRC [14]. Similarly, an analysis of 1,852 stage II and III colon cancer patients from the CALGB 9581 and 89803 studies reported improved DFS and OS in patients with dMMR tumors [55].

Based on the weight of the currently available evidence supporting the prognostic value of dMMR/MSI-H status in the adjuvant setting, and data suggesting that dMMR/MSI-H cancers may not benefit from 5-FU-based chemotherapy (see below), it may be reasonable to forego adjuvant chemotherapy in moderate and high-risk stage II patients with a dMMR/MSI-H phenotype. This has been implemented as a criterion for treatment stratification in the ongoing ECOG5202/NCT00217737 trial.

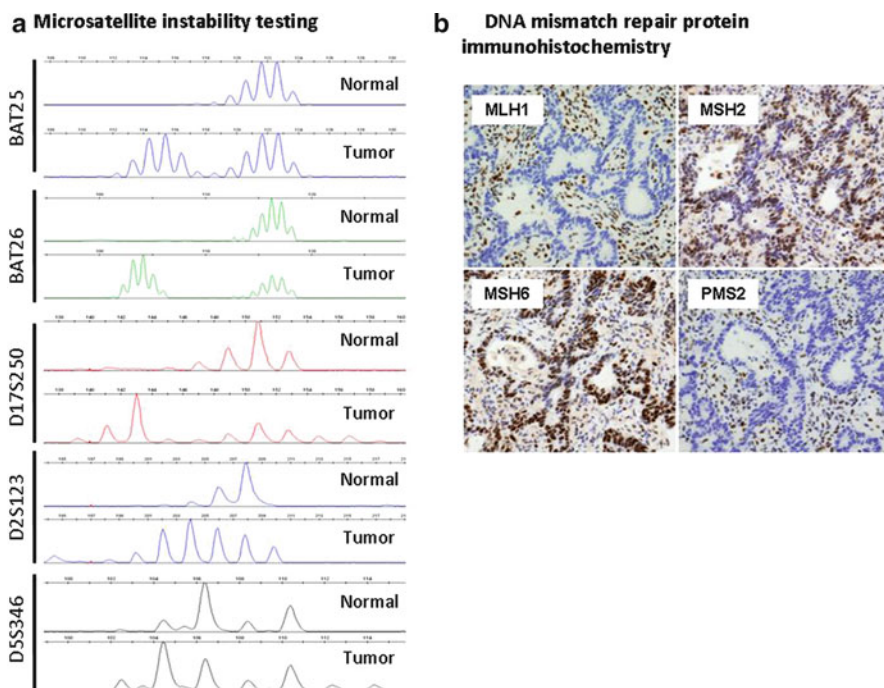

Fig. 5.3 The two main methodologies for detecting DNA mismatch repair deficiency in colorectal cancer. (**a**) Microsatellite instability testing using the Bethesda panel of markers (BAT25, BAT26, D17S250, D2S123 and D5S346). The tumor sample shows the acquisition of novel alleles of different size as compared to the matched normal sample. Cases with instability demonstrated in two or more of the five markers are considered to have microsatellite instability. (**b**) Immunohistochemistry for the DNA mismatch repair proteins MLH1, MSH2, MSH6 and PMS2. In the sporadic case shown, there is loss of MLH1 and PMS2 protein from carcinoma cells, indicating loss DNA mismatch repair function. Non-neoplastic stromal cells provide an internal positive control

3.7 CpG Island Methylator Phenotype

The term CpG Island Methylator Phenotype (CIMP) refers to a subset of CRCs that exhibit concurrent cancer-specific (or 'type C') hypermethylation at a high proportion of defined CpG islands within gene promoters, frequently associated with MSI-H, *BRAF* mutation and tumor location in the proximal colon [10, 73]. 'Type C' DNA hypermethylation affects multiple loci and several CIMP marker panels have been proposed. One of the most widely used panels is *NEUROG1*, *IGF2*, *SOCS1*, *CACNA1G*, and *RUNX3*, and cancers are termed CIMP-high (CIMP-H) if four or more of these loci are methylated in tumor DNA [10]. Given the strong association of the CIMP phenotype with MSI-H status and *BRAF* mutation, the prognostic impact of CIMP-H must be considered in the context of these variables.

Inconsistent data exist for the effect of CIMP status on CRC outcome, with the use of variable marker panels causing some difficulty in the comparison between studies. Some investigators have suggested an improved CSS for persons with CIMP-H stage I to IV colon cancer (n = 649) independent of MSI and BRAF mutation [30], whereas others have reported a detriment in DFS for proximal stage III colon cancer (n = 161), but not for distal stage III colon cancer [74, 75]. The E2290 trial on 188 patients with metastatic CRC found an association with shortened OS, but *BRAF* mutation status was not considered [74, 75]. Several authors have observed a negative prognostic impact for CIMP-H on OS or CSS in stage I to IV CRCs, but only in cases with MSS [76–79]. However, in two of these studies with available *BRAF* data, poor outcomes appeared to be largely related to the presence of *BRAF* mutation [76–79]. Taken together, the independent prognostic value of CIMP-H status in CRC remains uncertain.

3.8 Chromosomal Instability

Aneuploidy is present in 60–70% of CRCs and is often attributed to the presence of some underlying form of chromosomal instability (CIN). CIN may have multiple causes, including perturbation of processes controlling mitotic spindle or kinetochore function, mutations in genes involved in DNA double-strand break repair, or progressive erosion of telomeres triggering the breakage-bridge-fusion cycle. Alternatively, CIN may result as a by-product of inactivation of cell cycle checkpoint genes. For CRC, genes proposed to directly or indirectly cause CIN include *APC* [80–82], *TP53* [83], *BUB1* [84], *BUBR1* [85] and *FBXW1/CDC4* [86]. CIN and MSI tend to be mutually exclusive, although a small proportion of cancers exist that show evidence of both of these forms of genomic instability [87, 88].

The majority of studies evaluating the prognostic impact of CIN have used flow or image cytometric measurements of DNA content which provide a basic indication as to the presence of aneuploidy. Higher-resolution technologies, such as comparative genomic hybridisation (CGH) or single nucleotide polymorphism (SNP) arrays exist, but their application to large patient series has been limited. Data from 63 flow-cytometry studies reporting outcomes for 10,126 patients with stage I to IV CRC have recently been assessed in a meta-analysis [89]. Overall, 60% of patients had CIN+ cancers, and presence of CIN was associated with inferior prognosis (HR 1.45, 95% CI 1.35–1.55). Poorer PFS and OS could be demonstrated for patients with stage II and III disease, but data for stage I and IV patients were insufficient for conclusive evaluation.

While the combined evidence is consistent with CIN+ status as a predictor of poor prognosis in CRC, the relationship with MSI status remains unclear. To date, only one major published study on 528 patients with stage II and III CRCs has evaluated both MSI and CIN in multivariate analysis and found that the effect of MSI on survival was not independent to that of CIN [70].

4 Predictive Biomarkers for Cytotoxic Chemotherapies

4.1 5-Flourouracil (5-FU) and Capecitabine

The antimetabolite drug 5-FU is a pyrimidine analogue which primarily acts through irreversible inhibition of the enzyme thymidylate synthetase (TS or TYMS). TS normally methylates deoxyuridine monophosphate (dUMP) into thymidine monophosphate (dTMP) which is subsequently phosphorylated to thymidine triphosphate, a nucleotide required for DNA synthesis and repair. Inhibition of the action of TS results in a deficiency of dTMP, triggering apoptosis in dividing cells [90]. 5-FU is administered intravenously by bolus injection or infusion, generally with leucovorin to enhance activity. Capecitabine is a 5-FU prodrug which can be administered orally.

4.1.1 Thymidylate Synthetase

The level of intratumoral TS expression has been suggested to predict response to 5-FU-based chemotherapy. Preclinical studies in human colon cancer cell lines found that high levels of TS activity were correlated with intrinsic or acquired resistance to 5-FU [91–94], and higher levels of TS mRNA were observed to be associated with resistance to 5-FU treatment in patients with metastatic CRC [95–98]. With the development of robust antibodies against TS, an increasing number of studies have evaluated the association between intratumoral TS expression and 5-FU response using IHC. In both the adjuvant and palliative treatment setting, such studies have produced evidence that a high level of TS protein expression is associated with reduced benefit from 5-FU chemotherapy [99–102], although some investigators have reported contradictory findings [103–106]. In a meta-analysis of 13 studies on advanced CRC ($n = 997$) and seven studies on localized CRC ($n = 2{,}610$), higher TS expression was associated with inferior survival in both groups. The combined HR for OS was 1.74 (95% CI 1.34–2.26) and 1.35 (95% CI 1.07–1.80) in the advanced and adjuvant settings, respectively. However, evidence of heterogeneity and possible publication bias was observed [107].

Two main polymorphisms have been identified that influence the level of TS expression: A 6 base pair (bp) insertion and deletion variant in the 3′-untranslated region of TS that alters mRNA stability and is associated with low TS expression [108]; a 28-bp sequence within the promoter region of TS which occurs in two (2R), three (3R) or rarely more repeats that correlates with increasing TS expression, probably due to increased efficiency of mRNA translation for longer alleles [109, 110]. In addition, a SNP present within the second repeat of the 3R allele may further increase mRNA expression [111]. Several studies have analysed the predictive value of the tandem 28-bp repeat polymorphism, with conflicting results. Some studies have shown a lack of benefit from 5-FU treatment for persons with the 3R/3R genotype [112–115], while others have found no effect [116–119].

The clinical value of TS genotype, mRNA and/or protein levels for guiding the use of 5-FU-based chemotherapy remains uncertain given the current evidence.

4.1.2 Defective DNA Mismatch Repair/Microsatellite Instability

There is evidence from *in vitro* and clinical studies to suggest that persons with dMMR/MSI-H CRC do not benefit from 5-FU-based chemotherapy. In a study of 77 CRC cell lines tested for sensitivity to 5-FU, MSI-H status was found to be the strongest molecular predictor of reduced response [120]. A retrospective study on 570 patients with stage II and III colon cancer from five clinical trials of adjuvant 5-FU-based chemotherapy revealed superior OS for patients with MSI-H tumors in the no-treatment group, but no difference in outcome for patients in the chemotherapy group. Adjuvant chemotherapy significantly improved OS among patients with MSS/MSI-L tumors, but not in patients with MSI-H tumors [69]. Further data by the same group on an additional 467 patients confirmed the lack of efficacy of 5-FU-based adjuvant chemotherapy in MSI-H colon cancer [121], and similar results have been reported in a large study on non-trial patients ($n = 754$) [122] and a meta-analysis [71]. In contrast, other retrospective data and results from the QUASAR study suggest that patients with dMMR/MSI-H CRC do benefit from adjuvant 5-FU administration [14, 123]. The value of dMMR/MSI-H status as a predictive marker of adjuvant 5-FU-based therapy warrants further investigation.

4.2 Oxaliplatin

Oxaliplatin is a platinum-based cytotoxic drug that acts by preventing DNA replication through the formation of intra- and inter-strand platinum-DNA adducts. It lacks efficacy as a single agent, but is administered in combination with 5-FU in the treatment of early-stage and metastatic CRC.

4.2.1 Glutathione-S-transferase P (GSTP1)

GSTP1 is thought to be the primary enzyme for the detoxification of oxaliplatin, causing inactivation and excretion of the drug by conjugation with glutathione. Two coding polymorphisms in GSTP1 (Ile105Val and Ala114Val) show a relationship with reduced enzyme activity [124]. The Ile105Val variant was associated with differential response and survival in one retrospective study on 106 metastatic CRC patients who received second-line oxaliplatin and 5-FU treatment, with the valine allele more common in patients with better outcomes [125]. However, a number of other studies found no effect on survival in metastatic CRC patients [126–129]. Contradictory results have also been reported for the Ile105Val variant with respect

to neurotoxicity [126, 129, 130]. Similarly, limited existing data on the predictive value of GTSP1 polymorphisms in the adjuvant setting do not suggest any major effect [128, 131].

4.2.2 Nucleotide Excision Repair Genes

ERCC1 and ERCC2 (excision repair cross-complementing rodent repair deficiency, complementation group 1 and 2) encode two rate-limiting enzymes of the nucleotide excision repair pathway which act in the repair of platinum-DNA adducts. Two functional polymorphisms with these genes, ERCC1 Asn118Asn (G > A) and ERCC2 Lys751Gln (T > G), have been repeatedly studied as potential markers for response and outcome to oxaliplatin treatment. The former variant affects *ERCC1* mRNA expression [132], whereas the latter is associated with reduced ERCC2 DNA repair capacity [133]. A recent meta-analysis has summarized published studies on metastatic CRC, comprising eight studies on the ERCC1 (n = 993) and seven studies on the ERCC2 polymorphism (n = 858) [134]. Assuming a dominant model, the ERCC1 T/T genotype was not associated with objective response, PFS or OS for all patients, whereas the ERCC2 G/G genotype was associated with reduced objective response (OR 0.52, 95% CI 0.35–0.77) and inferior outcomes for PFS (HR 1.50, 95% CI 1.11–2.02) and OS (HR1.77, 95% CI 1.11–2.84). Significant study heterogeneity was evident. In a pooled analysis with metastatic gastric cancer, ethnic differences between Asian and Caucasian individuals were suggested, but a sub-analysis for CRC was not presented. One small study of stage III CRCs (n = 98) found no evidence that *ERCC1* and *ERCC2* polymorphisms predict response to oxaliplatin in the adjuvant setting [131]. Presently, the existing evidence is insufficient to support *ERCC1* and *ERCC2* genotyping as a predictive marker for oxaliplatin response, with larger prospective studies required to confirm previous findings.

4.3 Irinotecan

Irinotecan is an inhibitor of topoisomerase I, an enzyme that is essential for DNA replication. For the treatment of metastatic CRC, it may be given as a single agent or in combination with 5-FU.

4.3.1 UDP-Glucuronosyltransferase (UGT1A1)

The active metabolite of the topoisomerase I inhibitor irinotecan, SN-38, is detoxified primarily by the enzyme UGT1A1. A TA-repeat polymorphism within the TATA promoter element of the UGT1A1 gene affects the level of enzyme expression and activity [135, 136]. Persons who are heterozygous (6/7) or homozygous

(7/7 or UGT1A1*28) for the 7-repeat allele show reduced clearance of SN-38 and have an increasing risk of suffering severe toxicity in the form of grade 3 or 4 neutropenia. Reports determining the size of this effect have shown variable results, and a meta-analysis has shown that the incidence of toxicity in UGT1A1*28 patients is positively correlated with the drug dose used [137]. Genetic testing for this polymorphism to avoid life-threatening neutropenia has been approved by the US Food and Drug Administration and is recommended to guide irinotecan dosing. However, in clinical practice this test has found limited use largely because improved scheduling with lower, more frequent dosing has reduced the incidence of haemotological toxicity.

5 Predictive Biomarkers for Targeted Biological Agents

5.1 Anti-EGFR Monoclonal Antibodies

Binding of ligand to EGFR stimulates cellular signaling via the RAS/RAF/MEK/MAPK and PI3K/AKT pathways which are of central importance to colorectal tumourigenesis. Two monoclonal antibodies against EGFR, cetuximab (chimeric IgG1) and panitumumab (fully humnised IgG2), have been demonstrated to have activity in metastatic CRC in first and second line therapy when combined with chemotherapy and as a single agent in third line therapy [23, 138, 139].

5.1.1 EGFR

Studies evaluating EGFR protein expression and somatic mutations as predictive markers for the response to anti-EGFR targeted therapy have failed to demonstrate reliable clinical value in CRC [138, 140–144]. However, evidence from a number of investigators suggests that EGFR amplification may be a negative predictive marker of response with small retrospective studies using monotherapy and combination therapy showing a lack of efficacy in EGFR amplified tumors [145–149]. Some difficulty remains with assay reproducibility and there is no agreed standard threshold for reporting of increased copy-number.

5.1.2 Amphiregulin and Epiregulin

Gene expression of the stimulatory EGFR ligands amphiregulin (AREG) and epiregulin (EREG) has been suggested as a potential marker of sensitivity to anti-EGFR antibodies in a small number of reports, including two studies on primary tumor and metastatic biopsy tissues from patients with advanced CRC receiving cetuximab monotherapy [150, 151], and one study on primary tumor tissues from

patients receiving cetuximab in combination with chemotherapy [152]. However, AREG and EREG expression have not yet been studied in a large validation trial including a non-treated patient arm and the optimal cut-off for guiding use of anti-EGFR therapy has not yet been determined.

5.1.3 KRAS

Mutations in the *KRAS* gene are thought to activate the EGFR signaling pathway independently of ligand stimulation of the receptor, thus bypassing the efficacy of anti-EGFR therapy. Accordingly, multiple studies in metastatic CRC patients have demonstrated *KRAS* tumor mutations in codons 12 and 13 to be predictive of a lack of response to cetuximab and panitumumab. These include single-arm studies [150, 153, 154], and large randomized studies in the first-line setting [155, 156] and in pre-treated mCRC patients [6, 7, 23, 157, 158]. Similarly *KRAS* mutations in codons 61 and 146 may be associated with anti-EGFR therapy resistance, although data are more limited [6, 7, 23, 157, 158]. There is some evidence to suggest that not all tumors with mutated *KRAS* are resistant to anti-EGFR therapy, and one study has proposed that patients with a glycine to aspartate substitution at codon 13 (G13D) may respond to such treatment [159]. Confirmation of these latter findings will require further study. Some studies have further suggested a detrimental effect of anti-EGFR monoclonal antibodies when used to treat *KRAS* mutant cancer [160, 161].

Based on these results, ASCO, ESMO and NCCN (category 2A) presently recommend the use of monoclonal antibodies against EGFR only in metastatic CRC patients with wild-type *KRAS* status. Current NCCN testing recommendations are for *KRAS* codons 12 and 13 in CLIA-88 (Clinical Laboratories Improvement Amendments of 1988)-certified laboratories. No formal recommendations exist regarding testing for *KRAS* codons 61 and 146.

5.1.4 BRAF

The presence of BRAF V600E mutation has been postulated to be a predictive biomarker for anti-EGFR therapy response in cancers with *KRAS* wild-type status, but this has been challenging to assess given the strong prognostic impact of this mutation in metastatic CRC. Recently, a retrospective analysis of a European consortium on 773 metastatic CRC patients treated with cetuximab between 2001 and 2008 reported a lower objective RR to cetuximab in *BRAF*-mutated/*KRAS* wild-type tumors. Data on untreated individuals were not available, but it was suggested that a measure of objective response was a good estimate of treatment effect which was not confounded by the prognostic impact of the mutation [162]. In contrast, analysis of *KRAS* wild-type CRCs from the CAIRO2 trail of chemotherapy and bevacizumab with or without cetuximab did not find an association between *BRAF* mutation and PFS according to anti-EGFR therapy [163].

5.1.5 PIK3CA

A number of studies have investigated activating mutations in *PIK3CA* exons 9 and 20 as a predictive marker for anti-EGFR therapy. One study in 110 metastatic CRC patients receiving various anti-EGFR therapy regimes in first- or subsequent-line settings found a lack of response in patients with *PIK3KCA*-mutated/*KRAS* wild-type tumors [164]. In contrast, another study on 200 patients with chemotherapy refractory metastatic colorectal cancers treated with cetuximab in monotherapy or in combination with irinotecan found no evidence for a strong predictive role of *PIK3CA* status [165]. Subsequently, a European retrospective consortium analysis on 773 metastatic CRC patients treated with cetuximab observed that lack of response in the *KRAS* wild-type population was limited to patients with *PIK3CA* exon 20 mutation (ORR, PFS and OS), and proposed that this may explain the previous conflicting results [162]. Further validation of these findings in studies including a non-treated patient arm is required.

5.2 Anti-VEGF Monoclonal Antibodies

Bevacizumab is a humanized monoclonal antibody that inhibits VEGF-A, a growth factor that stimulates neo-angiogenesis in cancer. This anti-angiogenic agent is used in the treatment of metastatic CRC and increases response rates and overall survival in combination with 5-FU alone or with irinotecan or oxaliplatin plus 5-FU.

No effective and reliable biomarkers for bevacizumab response have been discovered to date. Suggested biomarkers include angiopoietin-2 levels [166], polymorphisms in VEGF [167, 168] and VEGFR-1 [167, 168], baseline levels of soluble VEGFR1, VEGF, placental-derived growth factor (PlGF), interleukin 6 (IL-6) and IL-8 during treatment [169, 170], and tumor and/or stromal expression of VEGF and thrombospondin-2 [171]. Although some of these studies show promise validation data are limited and there are currently no biomarkers for bevacizumab response in clinical use.

6 Unbiased Molecular Signatures

Besides the targeted approaches described above, high-throughput PCR-based assays and microarrays for evaluating mRNA expression, SNPs, DNA copy number and methylation are increasingly being utilized for large-scale hypothesis-driven and unbiased genome-wide marker discovery. In addition, next-generation sequencing approaches are beginning to play an important role, although their implementation for large cohort studies is currently hampered by cost and technology constraints. To date, most development effort has been invested in the area

of prognostic mRNA expression signatures with significant industry involvement. One prognostic test (Onco*type* DX Colon Cancer, Genomic Health Inc) is now commercially available for patients with stage II colon cancer, and a second test (ColoPrint, Agendia) is in the final stage of development for patients with stage II and III disease.

6.1 Prognostic Gene Expression Signatures

Multiple studies have evaluated gene expression profiles derived from RT-PCR or microarray analysis for potential prognostic value in CRC [172–179]. Although sample sizes have often been small, patient populations heterogeneous and external validation limited these studies have indicated promise for expression signatures to discriminate recurrence risk in patients with early-stage disease. A meta-analysis of studies of various gene expression assays including 271 patients from eight cohorts with stage II CRC showed a prognostic likelihood ratio of 4.7 (95% CI, 3.2–6.8) for recurrence or death within 3 years, with an average accuracy, sensitivity, and specificity of approximately 82%, 76%, and 85% [180].

Two commercial assays, Oncotype DX Colon Cancer and ColoPrint, have been developed as prognostic markers for recurrence risk in stage II and III colon cancers, with clinical validation studies ongoing. The Oncotype DX Colon Cancer test is a quantitative, multigene RT-PCR assay for use on formalin-fixed paraffin-embedded tissue. The assay has been developed based on the analysis of 761 selected candidate genes with putative significance in colon cancer in 1,851 specimens from four adjuvant trials (NSABP C-01/C-02, Cleveland Clinic Foundation, NSABP C-04, and NSABP C-06) [181]. A total of 48 genes were identified as significantly associated with recurrence risk and 66 genes as significantly associated with treatment benefit. The final assay incorporated the seven genes most strongly associated with recurrence, the six genes most strongly identified with treatment benefit, and five reference genes for standardization. The assay was evaluated in 1,436 patients with stage II colon cancer from the QUASAR clinical trial. In multivariate analyses, the classifier retained prognostic significance independent of conventional prognostic factors including mismatch repair status, tumor T stage, number of lymph nodes examined, grade, and presence of lymphovascular invasion. However, the classifier was not confirmed to be predictive of treatment benefit in the 725 patients treated with fluorouracil and leucovorin [182].

The ColoPrint assay is an 18-gene signature developed in fresh-frozen tumor specimens from 188 patients with stage I to IV CRC using high density Agilent 44K oligonucleotide arrays, with subsequent validation in 206 patients with stage I to III colon cancer. In the validation cohort, the signature classified 60% of samples as low risk and 40% as high risk, with an HR for RFS of 2.69 between groups. RFS at 5 years was 87.6% in the low-risk group as compared to 67.2% in the high-risk group. The signature was a predictor of outcome when applied separately to stage II and stage III patients, and to individuals treated with or

without adjuvant chemotherapy [183]. The PARSC trial, a prospective study for the assessment of recurrence risk in stage II colon cancer (CC) patients using ColoPrint is ongoing [184].

7 Conclusions

The successful improvements in treatment of CRC over the past decade and our increasing understanding of the molecular biology of the disease have driven substantial efforts to identify biomarkers of prognosis and therapy response. These efforts have been fraught with difficulties with many markers supported by insufficient data and failing to demonstrate clinical utility. Small sample size, limited clinical and follow-up data, differences in patient selection and therapies employed, low frequency of candidate marker alteration, heterogeneous screening methodologies and lack of standardization of reporting account for much of the conflict. Many of these deficiencies are beginning to be addressed, and a number of comprehensive biomarkers studies are currently underway. Despite these challenges, encouraging progress has recently been made with the recognition of the importance of *KRAS* mutation status for selection of EGFR-specific therapy.

With improving technology, evaluation of large panels of markers – perhaps tailored to interrogate particular pathways – or genome-wide analyses will become feasible. The ongoing commercial development of prognostic gene expression signatures utilizing microarrays is an early example of this. The development of such global biomarker signatures will require large well-planned studies including cooperative national and international consortia.

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Chapter 6
Expression Profiling of Hepatocellular Carcinoma

Rosina Maria Critelli, Elisabetta Cariani, and Erica Villa

Abstract Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths in the world.

The molecular mechanism of HCC onset involves a complex interplay of both genetic and epigenetic factors. Hepatic carcinogenesis is characterized by an increase in allelic losses, chromosomal aberrations, gene mutations, epigenetic alterations, changes of gene expression and alterations in molecular cellular pathways. The integration of genetic, epigenetic, genomic, and proteomic data provides insight into the molecular mechanisms underlying hepatocarcinogenesis and is revealing promising clinical approaches. Resulting findings offer the possibility for the identification of relevant biomarkers, useful for the detection, molecular diagnosis, prediction of recurrence and prognosis of HCC as well as for the improved identification of novel therapeutic targets. This will be of utmost importance in the near future as more and more new targeted drugs will become available.

Keywords Hepatocellular carcinoma • mRNA profiling • Genomic profiling • miRNAs • Methylation • Histone modification

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1 Introduction

Primary liver cancer ranks fifth among the most common types of cancer and third as cause of cancer-related death [1]. Hepatocellular carcinoma (HCC) accounts for 85–90% of total cases of liver cancer. The incidence and mortality of HCC are maximal in Asia and sub-saharian Africa, and have been increasing in Western countries during the last few years [1].

HCC in most of the cases develops in individuals with cirrhosis due to hepatitis B or C virus chronic infection, to chronic alcohol abuse or to metabolic disease (Fig. 6.1). Due to the scant presence of symptoms, most of the patients are diagnosed at late stages of tumor development unless they are known to have liver cirrhosis and are enrolled in active ultrasound surveillance. Due to the heterogeneous clinical outcome of HCC, patients are classified for prognosis and treatment stratification according to tumor burden, residual liver function and general status [2]. Based on this classification, patients with early stage HCC are stratified to radical therapies such as liver transplantation, resection or percutaneous ablation, whereas patients with intermediate stage disease are candidate to locoregional treatments. However, the outcome is far from optimal, as even resection and ablation lead to high rates of recurrence (about 70% after 5 years). Until recently, limited therapeutic options have been available for patients with advanced disease.

The molecular background of HCC is heterogeneous and deregulated signaling pathways include Wnt-beta Catenin, RAS-MAPK, AKT-mTOR, EGFR, IGFR, HGF-MET (Table 6.1) [3]. The activation of a specific pathway appears to be associated with HCC prognosis; namely, the presence of the MET signature has

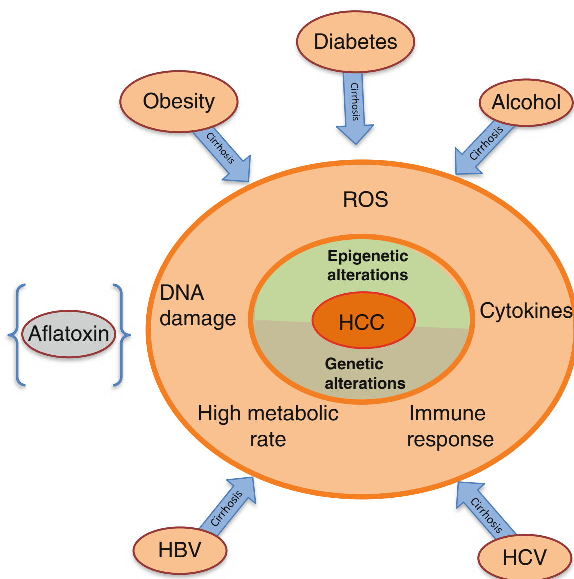

Fig. 6.1 Genetic and epigenetic events in the onset and progression of hepatocellular carcinoma. The most frequent etiologic factor are listed (Aflatoxin, although relevant, play a lesser role in western countries). Most etiologic factors act by determining an inflammatory reactions, fibrosis and eventually cirrhosis. It is important to note that very often there is a cooperation of two or even three different etiologic factors in the same subjects. This leads to more rapid progression of liver disease and to synergistic interaction between the listed events

Table 6.1 Cellular signaling pathways most often implicated in HCC

VEGF and PDGFR signaling cascades
EGFR-RAS-MAPKK pathway
AKT-mTOR signaling
Wnt-beta catenin pathway
C-MET pathway
IGF signaling

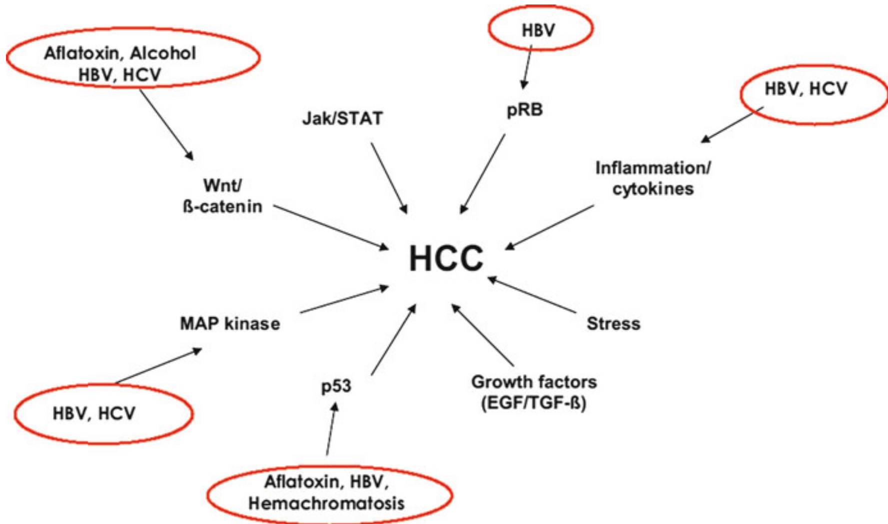

Fig. 6.2 Signaling pathways involved in *HCC* development in relation with the various etiology of chronic liver disease

been shown to correlate to increased vascular invasion rate and decreased survival [4] (Fig. 6.2). Recently, a series of molecularly-targeted drugs were made available. The most used so far is the non-specific tyrosine kinase inhibitor Sorafenib, that is active on B-RAF, VEGF receptor and PDGF receptor: Sorafenib proved effective in obtaining a 3 months increase in survival in advanced HCC [5], thus becoming the standard of care in this subgroup of patients. The other drugs that have reached clinical practice (although they are not yet released for general use) are Sunitinib, Bevacizumab, Erlotinib, Gefitinib, Cetuximab, Brivanib, Lapatinib [6]. All these drugs share a common mode of action (blocking of one or more molecular pathways). Although the results associated with some of them look promising, the best results so far have been obtained with Sorafenib [5].

Molecular profiling, including expression analysis, comparative genomics, epigenomics and proteomics, provides powerful tools to gain insight into the molecular mechanisms underlying carcinogenesis. Resulting knowledge offers the potential for better understanding of cancer biology, and for the discovery of new tools and biomarkers for detection, diagnosis, prevention and prognostic evaluation, as well as new targets for therapeutic developments (Fig. 6.3).

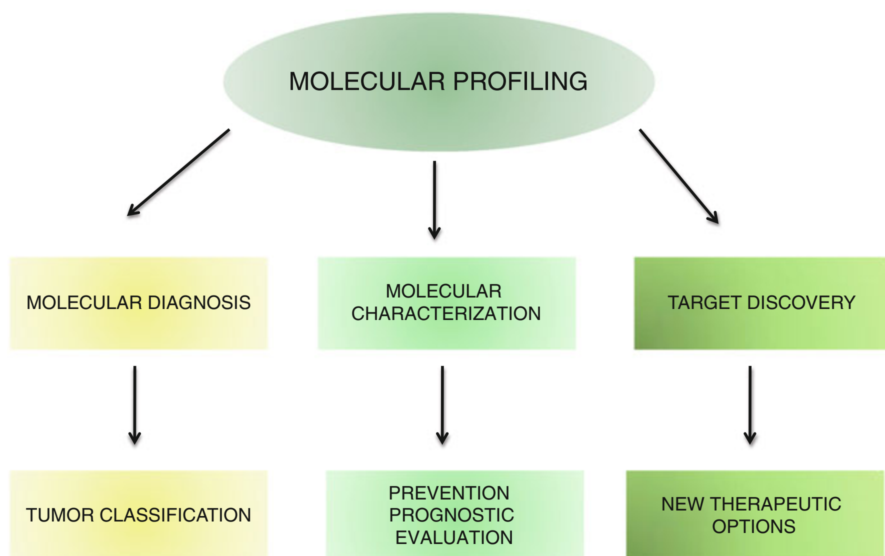

Fig. 6.3 Potential clinical application of molecular profiling in carcinogenesis

2 Approaches for Molecular Classification

2.1 *mRNA Profiling*

In the last few years microarray-based gene expression profiling on HCC has been extensively used to detect differences that can help to improve diagnosis and prognosis and predict patient response to treatment. Since molecular classification is tightly linked to pathway activation and to sensitivity and/or resistance to drugs targeting these pathways, the biomarkers identified can represent at the same time indicators of clinical aggressiveness (prognostic significance) and drug targets (therapeutic significance). Genome-wide microarray studies targeting thousands of genes have consistently detected HCC-specific changes in the hepatic messenger RNA (mRNA) expression pattern. However, little overlap was found among the mRNA profiles identified in different studies, and the application of molecular signatures as predictors of survival has been seriously hampered by the instability of the profiles identified and by the high rate of misclassification. To reduce the multiple sources of variability affecting final results, the bulk of microarray information was merged into common signatures useful to build predictive models potentially suitable to clinical use.

Considerable effort has been devoted to the identification of molecular profiles consistently linked to clinical features of HCC. Class aggregation of molecular HCC profiles related to disease progression was tried by identification of common pathway-gene families. Despite a huge amount of scattered and partly contradictory

results, by merging the enormous amount of results obtained in large-scale gene expression studies, common HCC subclasses have been identified that share some molecular features and correlations with clinical parameters [7, 8]. The HCC gene expression profiles reported in most of studies appear to cluster in three main subclasses: a first group appears to be characterized by high proliferation and chromosomal instability (involving mTOR, IGF, RAS pathway activation), and be associated with aggressive clinical behavior. The cell proliferation profile, including cluster A, subtype HB [9, 10], profile B [11], groups G1-G3 [12], proliferation profile [7], groups B and C [13], S2 class [8], consistently showed over-expression of cell proliferation, antiapoptotic and fetal genes, and was associated with poor prognosis. A second group, i.e. the cell differentiation profile, including cluster B [9, 10], groups G5-G6 [12], CTNNB1 profile [7], groups A and D [13], and S3 class [8] was characterized by better prognosis and lower expression of cell proliferation and antiapoptotic genes, accompanied by activation of Wnt pathway in some studies [7, 12]. Finally, a third group of HCCs was described characterized by expression of interferon response genes [7, 11].

Several lines of evidence indicate the relevance of the surrounding microenvironment in the pathogenesis and clinical behavior of solid tumors and namely of HCCs [14]. The tumor is surrounded both by cellular and non-cellular components (extracellular matrix, growth factors, cytokines) involved in a complex interplay with tumor cells. Infiltrating immune cells play a major role in the progression of HCC. Results obtained by microarrays, histological techniques and flow cytometry on HCC infiltrating cells consistently suggested that an inhibitory profile (tumor infiltrate rich in regulatory T cells [Tregs]) is related to poor prognosis [15, 16] whereas immune activation (Th1 gene signature, lower Treg/CTL ratio in infiltrate, expression of IFN-induced genes) is related to better prognosis [7, 11, 17, 18]. The functional deficiencies in immune surveillance detected in HCC have been explained by several mechanisms, including increased expression in tumor-infiltrating cells of the inhibitory receptor Programmed Death 1 (PD-1) and of its ligand PD-L1 or B7-H1, expressed by dendritic cells, macrophages and parenchymal cells. The PD-1/PD-L1 interaction induces the suppression of the T-cell response [19] contributing to the aggressiveness and recurrence of HCCs overexpressing PD-L1, and/or harboring infiltrating cells with enhanced PD-1 expression [20–22].

Recent results suggest that not only the molecular profile of tumor, but also the expression signature of the corresponding non-tumorous liver tissue could be useful for predicting the outcome of HCC [23, 24]. As liver cirrhosis is a pre-tumorous condition, the appearance of new HCC nodules may be due both to the intrahepatic dissemination of primary HCC, and to the development of new foci in the cirrhotic liver. The timing of appearance of HCC recurrence is believed to differentiate metatastatic spread, usually occurring within 2 years after resection, from de novo HCC developing in a chronically diseased liver. This suggests that profiling the non neoplastic liver, rather than the actual tumor, may uncover a pre-neoplastic state affecting the whole liver and therefore potentially detectable by needle biopsy [25], thus disclosing new perspectives for the application of molecular technologies in this setting. Results support the involvement of inflammatory and

immune-related gene expression in non tumorous liver for the progression and recurrence of HCC. A pro-inflammatory cytokine milieu of peritumorous liver with increased Th2 cytokines was identified as predictive of metastatic spread [23], and an expression signature including gene sets associated with inflammation and interferon, nuclear factor–κB and tumor necrosis factor α signaling was reported as predictive of late recurrence [24]. The last result is consistent with a recent report of up-regulated inflammation, oxidative stress and carcinogenesis-related pathways [26]. In addition, CYP1A2 downregulation in noncancerous tissue was shown to predict HCC recurrence and be associated with activation of oxidative stress pathways [27].

Although a relationship between mRNA-based molecular subclasses and pathway activation has been demonstrated [28], the prognostic impact of the mRNA profiles of HCC and non-tumorous liver is still controversial and the translation of these results into clinical practice yet to be achieved. Recently an integrated approach including clinical and pathological features together with molecular signatures of tumors and surrounding liver indicated that the gene expression profiles of HCC and non tumorous liver represent independent predictors of recurrence [29]. A comprehensive HCC prognostic model taking into account both clinical and molecular features might represent an important tool for effective risk evaluation and stratification to different treatment options.

2.2 *Genomic Profiling*

Tumor cells are subjected to increased turn-over resulting in enhanced genomic variability through the appearance of copy number variations and losses of heterozigosity (LOH) that may be involved in HCC pathogenesis and evolution, thus providing potential biomarkers/therapeutic targets.

The genetic alterations of transformed cells have been traditionally investigated by cytogenetic approaches. Comparative genomic hybridization (CGH), developed in the early 1990s, has represented the first tool allowing a genome-wide appraisal of copy number alterations [30]. Array-CGH (aCGH) approaches, in which arrays of genomic sequences instead of metaphase chromosomes are used as hybridization targets, represent a further improvement due to increased resolution.

The comparison between HCCs and corresponding non-tumorous liver tissues by CGH and LOH analysis allowed the detection of considerable chromosomal and microsatellite instability in tumors. Chromosomal instability, i.e. the loss or gain of chromosomal segments during cell division, is increased by cell proliferation and is possibly involved in cancer progression, whereas the instability of microsatellites (small tandem repeats scattered throughout the genome) derives from mutations in DNA mismatch repair genes during the carcinogenic process. The detection of copy number gains and losses in cancer cells may represent a useful tool for the identification of candidate oncogenes and tumor suppressor genes, respectively.

In HCCs, gains of chromosomal material were mostly detected in 8q, 1q, 6p, and 17q, whereas chromosomal deletions were most frequent in 8p, 16q, 4q, 1p, 6q, 9p, 13q, 16p, and 17p [31–34]. The same chromosomal segments (1p, 1q, 4q, 5q, 6q, 8p, 9p, 13q, 16p, 16q and 17p) were found to harbor allelic deletions identified by LOH [31, 35, 36].

The genetic heterogeneity of the tumors includes both bystander mutations and genetic alterations potentially involved in the carcinogenic process through mutational activation and inactivation of individual genes. Redundant chromosomal abnormalities repeatedly reported in HCCs involve known driver genes as c-MYC (located in the 8q24 region), p53 (located in 17p) and CTNNB1 (β-catenin) [33, 34, 37]. Recurrent chromosomal imbalances in human HCC have also been related to etiology, since losses of 4q, 8q, 13q, and 16q were more frequent in HBV-related tumors and specific alterations at 1q32.1, 4q21.2-32.33 could discriminate HBV- and HCV-associated HCCs [33, 38]. Furthermore, a relationship has been reported between specific genetic alterations and tumor progression [33, 34] or prognosis [39]. In addition to chromosomal imbalances, somatic mutations as insertions, deletions, translocations and point mutations have been reported in HCCs. A very frequent point mutation involves the codon 249 of p53 gene specifically related to exposure to aflatoxin [40].

The variable degree of risk of HCC development in relation with ethnic background has suggested since long the involvement of genetic traits in its development. In this view, genome-wide association studies (GWAS) have been used to identify inherited polymorphic traits associated with HCC development. Clifford et al. [41] identified three variants contained within the MHC class II locus strongly associated with onset of HCC compared to control subjects, and two variants (one of which lying in the PTEN homolog TPTE2) whose allele frequencies differed significantly between HCC and liver cirrhosis. A study focused on hepatitis B-related HCC patients identified a single nucleotide polymorphism (SNP) located at 1p36.22 and potentially associated with altered expression and function of candidate tumor suppressor genes [42].

A recent GWAS [43] identified a novel SNP, located in the flanking region of MICA (MHC class I polypeptide-related sequence A) gene on chromosome 6p21.33, associated with the progression from chronic hepatitis C to HCC in Japanese patients. However, since a control group of patients with liver cirrhosis was not included, this SNP may be actually related to the risk of developing liver cirrhosis rather than HCC. In a large two-stage GWAS of Japanese patients with chronic hepatitis C and HCC, a SNP located in the DEPDC5 gene on chromosome 22 [44]. It is worth noting that the last two studies, although carried out in the same ethnic group, identified different predictors, raising concerns on the study design and selection of control groups [45]. In addition, both studies reported relatively small odds ratios (<1.5 in [43], 2.2 in [44]), suggesting that these SNPs may not be useful as single predictors. This observation is consistent with the hypothesis that polygenic factors are involved in the risk of HCC development, and that the prognostic value of these genotype may be considered as part of a multi-marker panel predictive of chronic liver disease progression/HCC risk.

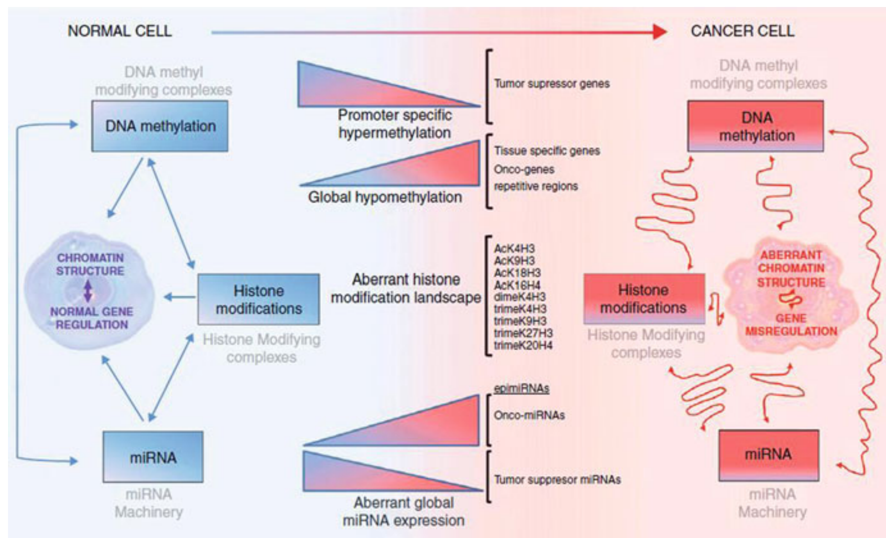

Fig. 6.4 Global depiction of epigenomic alterations during oncogenesis. In conjunction with accumulation of genetic lesions, there is an aberrant pattern for the different epigenetic effectors: DNA methylation, histone modifications, and miRNAs. In normal cells, the interplay between the epigenetic factors and the chromatin structure leads to a tuned gene regulation. However, in cancer cells tumor suppressor genes promoters become hypermethylated and with an altered global pattern of histone modifications resulting in aberrant gene silencing. Moreover, global hypomethylation leads to chromosome instability and fragility. Epigenetic changes, including DNA methylation and histone modifications are responsible for abnormal mRNA and miRNA expression producing altered activation of oncogenes and silencing of tumor suppressor genes (Reprinted from Sandoval and Esteller [49], with permission from Elsevier)

2.3 *Epigenomics*

Epigenetics refers to heritable changes in gene expression that occur without alteration in DNA sequence [46]. The key processes responsible for epigenetic regulation are DNA methylation, histone modifications and post-transcriptional gene regulation by noncoding RNAs commonly referred to as microRNAs (miRNAs) [47, 48]. These mechanisms are critical components in normal development and growth of cells and their modifications contribute to neoplastic phenotypes (Fig. 6.4). Liver cancer is no exception; indeed, it is now accepted that there is a complex interplay of genetic and epigenetic abnormalities that accumulate in precancerous tissues and culminate in the development of full-blown carcinoma [12, 50].

Epigenetic modifications in cancer and precancerous lesions might fulfill the promise of novel and more effective biomarkers for early cancer detection, prediction, prognosis and response to treatment than simple expression signatures (see Sect. 2.1). Many recent studies have identified a large number of genes and pathways that are targeted by epigenetic deregulation during the development and

progression of HCC. Profiling studies reveal that HCC tumors and pre-cancerous lesions may exhibit epigenetic signatures associated with specific risk factors and tumor progression stage.

2.3.1 miRNA

miRNAs are small non-coding RNAs 19–25 nucleotides long that regulate gene expression by directly degrading mRNA or repressing protein translation [51–53]. miRNAs are vital to normal cell physiology [54, 55]but their deregulated expression has been linked to a wide variety of human diseases, including cancer [56]. An altered miRNA expression was observed in a large variety of neoplasms [57], including HCC [58–65]. In human cancer, miRNAs can function as oncogenes or tumour suppressor genes during tumor development and progression [66, 67]. In addition, miRNA genes have been found to be frequently located in cancer-associated genomic regions, such as fragile sites, minimal regions of loss of heterozygosity and minimal regions of amplification [68, 69].

Most recently, miRNAs were found to be frequently deregulated in HCC, and some specific miRNAs were found to be associated with the specific clinico-pathological features of HCC, such as metastasis, recurrence, and prognosis [59, 61, 70]. Moreover, several studies demonstrate that miRNAs have important roles in HCC progression and directly contribute to cell proliferation and inhibition of apoptosis. Altered expression of some miRNAs has been recurrently found in different studies and this indicates that miRNAs profile may be a useful tool to classify tumors (Table 6.2). For example, miR-122 is specifically repressed in a subset of primary HCCs that are characterized by poor prognosis and has been shown to be a potential diagnostic and prognostic marker for HCC progression.miR-122 acts as a potential tumor suppressor inhibiting hepatic cell growth by targeting Cyclin G1 [78] and promoting apoptosis of hepatic cells by targeting BCl-w [79]. It has also been suggested that miR-122 suppresses HCC intrahepatic metastasis by regulation of a disintegrin and metalloprotease family proteins ADAM10 and ADAM17 [80, 81]. By contrast, overexpression of miR-221 was found to be associated with a more aggressive HCC phenotype [72]. This is because miR-221 down-regulates the expression of p27 and p57 [73], two tumor suppressor proteins whose decrease is associated with negative prognostic factors in HCC [74–76].

Reduced miR-26 expression has been linked with NF-kB and interleukin 6 signaling, shorter survival, and better response to IFN α therapy [82]. These results indicate that miR-26 status in tumors may be a useful tool in estimating prognosis in patients with hepatocellular carcinoma and in assisting in the selection of patients who are likely to benefit from adjuvant therapy with IFN α to prevent relapse [83].

The aberrant expression of miR-21 was shown to contribute to development, progression and metastatic phenotype of HCC by targeting PTEN [71]. Downregulation of miR-101 has been associated with worse survival of HCC patient; miR-101 inhibits the expression of the FOS oncogene post-transcriptionally, thereby reducing HGF-induced cell invasion and migration [84]. It may also exert a pro apoptotic function via targeting Mcl 1 [85].

Table 6.2 HCC-associated microRNA

microRNA	Differential expression	Gene target(s)	Function	References
miR21	Upregulated	PTEN	Apoptosis, growth	[71]
miR221	Upregulated	CDKN1B/p27, CDKN1C/p57	Inhibition of apoptosis, promotion of cell growth	[72–76]
miR18a	Upregulated	ERα	Proliferation	[77]
miR122	Downregulated	CCNG1, BCL-W, ADAM10, ADAM17	Tumour suppressor, promotion of apoptosis, invasion and metastasis	[78–81]
miR26	Downregulated	NK-kB and IL6 signalling	Poor overall survival	[82, 83]
miR101	Downregulated	FOS, Mcl-1	Promotion of apoptosis, inhibition of cell growth, migration and invasion	[84, 85]
miR125b	Downregulated	AKT	Inhibition of cell growth, proliferation	[62]
miR 99a	Downregulated	IGF-1R, mTOR	Inhibition of cell growth	[86]
miR224	Upregulated	API-5	Promotion of growth, proliferation, apoptosis	[65]
miR223	Downregulated	Stathmin1	Inhibition of cell growth	[63]

Some miRNAs have been linked with better course of HCC. For example, HCC patients with high expression of miR-125b have good prognosis, while those with lower expression have poor clinical outcome as miR-125b might suppress cancer cell proliferation through Akt inactivation [62]. A marked decrease of miR-99a correlates with shorter survival and its restoration suppresses HCC growth in vitro and in vivo by targeting IGF-1R and mTOR. Thus, miRNA-99a has an important role in HCC development and may be useful for prognosis prediction and for a targeted therapeutic approach [86].

An interesting finding concerns miR-18a: this miRNA is preferentially increased in females with HCC compared to males and promotes the proliferation of HCC cells by down-regulating the ESR1 gene, which encodes for Estrogen Receptor alpha, thus potentially blocking the protective effects of estrogens [77]. The discovery of the molecular mechanism regulating the increase in miR-18a expression in female HCC patients will be crucial for the future design of specific strategies to inhibit liver carcinogenesis in females.

Altered miRNA expression has been found not only in tumor tissue but also in surrounding non-tumor cirrhotic tissue, suggesting that miRNA alterations may represent early events in tumor progression [58, 60, 61, 87, 88]. The study by

Murakami et al. [58] identified eight differentially expressed miRNAs, three of which expressed mainly in HCC samples and five in the non tumorous liver. The study by Ladeiro et al. [61] identified miRNA signatures able to classify liver samples according to degree of malignancy, risk factors and gene alterations. Budhu et al. [60] reported a 20-miRNA-based signature associated with venous invasion, that may represent a simple and useful diagnostic/prognostic profiling method able to identify HCC patients likely to develop metastases and/or hepatic recurrence.

In conclusion, miRNA expression patterns, alone or in combination with other parameters, may potentially become useful markers for HCC classification and prognostic risk stratification. The full potential of mi-RNAs as diagnostic and prognostic factors awaits the results of larger prospective studies. In addition, miRNAs represent promising future therapeutic targets.

2.3.2 Methylation

DNA methylation is a major epigenetic mechanism playing an important role in maintenance of genome integrity, genomic imprinting, transcriptional regulation, and developmental processes. Methylation changes may occur early in the process of cancer development, and CpG island hypermethylation of the regulatory regions of tumor-relevant genes is a frequent event in hepatocarcinogenesis [89–91]. Some studies have revealed clear-cut differences in DNA methylation between HCC and surrounding non-HCC tissue, consisting of specific promoter hypermethylation and global hypomethylation [92, 93].In this regard, genomic hypomethylation correlated with genomic instability in HCC, whereas methylation of CpG islands was associated with poor prognosis [94]. In addition, DNA methylation status correlated with tumor recurrence after hepatectomy, cancer-free survival, and overall survival [93].

While hypomethylation of the whole genome might be associated with the occurrence, progression and metastatic spread of tumor, activation of oncogenes and genomic instability [95], hypermethylation of CpG island located in tumor suppressor genes is thought to be tightly linked with the silencing of these genes, which plays an important role in oncogenesis [96, 97]. A number of studies have indicated that promoter hypermethylation may be a key mechanism involved in the inactivation of some tumor suppressor genes in HCC [97].

DNA methylation status of some genes can be used as a potential biomarker. For example, p15, p16 and RASSF1A are suggested as potential diagnostic markers since their methylated DNA sequences can be detected in the serum of HCC patients [98]. In addition, the methylation status of other genes can be prognostic markers. As an example, the frequent promoter methylation of M-cadherin is associated with poor prognosis in HCC [99]. T-cadherin down-regulation due to promoter methylation is associated with the development and progression of HCC [100]. Different etiological factors, such as HBV and HCV infections, can induce different methylation statuses of a variety of genes: HBx protein may play an important role in the early stage of HBV-associated hepatocarcinogenesis via induction of

hypermethylation of p16INK4A promoter [101]; methylation of SOCS-1, APC, and p15 was more frequently seen in HCV/HBV-positive HCC than HCV/HBV-negative HCC [102].

Many studies indicate that alterations in gene promoter methylation is a common event in HCC [97, 103]. Moreover, the two major de novo DNA methyltransferases (DNMT), DNMT3a and DNMT3b, and one maintenance methyltransferase, DNMT1 are involved in hepatocarcinogenesis. DNMT3a and DNMT3b are responsible for the creation of methylation patterns, while DNMT1 maintains clonal transmission of methylation patterns during replication. Increased levels of mRNA encoding for DNMTs might be associated with cell proliferation in cancer. Expression of DNMT1 in poorly differentiated HCC is significantly higher than in non-tumorous liver tissue [104]. Increased protein expression of DNMT1 is significantly correlated with the malignant potential and poor prognosis of human HCC, and an increase in the DNMT3a and DNMT3b mRNA levels in HCCs relative to their non-tumor tissues may be a predictor of poor survival [105]. The relationship between DNMTs mRNA expression levels and patient prognosis may justify a role for DNMTs inhibitor therapy in HCC patients with increased DNMTs mRNA levels.

In conclusion, the extent of global DNA hypomethylation and CpG hypermethylation correlates with biologic features and clinical outcomes of HCC. The aberrant methylation is a major event in both early and late stages of liver malignant transformation and might constitute a critical target for cancer risk assessment, diagnosis, classification, treatment and chemoprevention. In addition, a number of the putative tumor suppressor genes epigenetically silenced in HCC are already inactivated in the surrounding non-tumorous part of the liver [103].

2.3.3 Histone Modifications

Histone modifications, recognized as a “histone code”, are crucial in maintaining chromatin stability and play an important role in gene regulation and carcinogenesis [106]. Histone modifications, being involved in transcriptional regulation, are potentially oncogenic if deregulated deposition leads, for example, to loss of expression of a tumor suppressor gene [107, 108].

Several types of post-translational modifications can affect histones, including methylation, acetylation, phosphorylation and ubiquitination. These modifications can lead to alterations in gene transcription either directly or through chromatin remodeling, and influence DNA repair, DNA replication, cell cycle checkpoints and even the organization of chromosomes [109]. Modifications can occur in different histone proteins, residues and variants. In general, histone acetylation is associated with transcriptional activation, and deacetylation is linked with transcriptional repression. Thus, deacetylation is implicated in the silencing of tumor suppressor genes in carcinogenesis. The effect of histone methylation depends on the amino acid affected, and the amino acid location in the histone tail [109, 110]. Further complexity is observed when promoter hypermethylation and histone modifications work in concert to alter gene transcription.

There are few studies describing the specific modifications to histones that are involved in the development of liver cancer. HCC has been reported to display altered histone modification machinery and, as a result, altered cellular epigenetic state. Most of the known HCC-associated aberrant histone modification events affect expression of critical cellular genes and thus impair normal cellular activities. It was demonstrated that histone modifications at H3K4, H3K9 and H3K27 regulate the expression of tumor suppressor genes in HCC [111]. In a rodent model of dietary methyl deficiency that results in HCC, a progressive decrease in histone H4 lysine 20 tri-methylation and a gradual decrease in Suv4–20 h2 histone methyltransferase was detected in liver tumors. Moreover, a prominent increase in histone H3 lysine 9 trimethylation and in the expression of Suv39h1 histone methyltransferase was observed in preneoplastic lesions [112]. These findings indicate that some histone modifications occur early and are crucial to the development of HCC.

Genes encoding proteins involved in histone modifications, such as prothymosin alpha (PTMA) and SET nuclear oncogene, inhibitors of histone acetyltransferases complex, are highly expressed in HCC with shorter survival [9]. Metastatic tumor antigens (MTAs) 1, 2 and 3 represent another important group of proteins involved in histone modifications that play an important role in the pathogenesis and progression of a wide variety of cancers, including HCC [113]. These proteins are contained in the nucleosome remodeling and histone deacetylase complex which regulates transcription via histone deacetylation and chromatin remodeling.

In addition to chromatin histones, MTA proteins also deacetylate non-histone proteins. For example, the tumor suppressor p53 protein is deacetylated and inactivated by MTA2, resulting in inhibition of growth arrest and apoptosis. MTA2 over-expression is associated with HCC size and differentiation [114] and MTA2 might be a predictor of aggressive phenotypes and a possible target molecule for anticancer drug design in human HCC. Hypoxia-inducible factor-1α (HIF-1α) is also deacetylated and stabilized by MTA1, leading to enhanced angiogenesis. This suggests that MTA1 expression can be related to tumor progression and metastasis [115–118].

Hamatsu et al. [115] reported the association between high MTA1 expression levels and lower disease-free survival rate after curative HCC hepatectomy. Using immunohistochemistry, Moon et al. [116] reported that, in resected human HCC specimens, the overexpression of MTA1 protein was associated with HCC growth and vascular invasion. MTA1 is also referred as an important factor for invasion and metastasis in HBV-associated HCCs [117], since the HBX protein induces the expression of MTA1. In addition histone deacetylase 1 (HDCA1), the HDAC1 complex containing MTA1 protein, may be important in stabilizing HIF-1α, thus playing a role in angiogenesis and metastasis of HBV-associated HCC [117]. Again in HBV-related HCCs, it was recently reported that MTA1 is tightly associated with larger tumor size, worse histological differentiation, microvascular invasion, frequent postoperative recurrence, and poor prognosis [118]. Taken together these data indicate that the expression levels of MTA1 in HCC tissue might be an important prognostic marker after curative hepatectomy.

3 Conclusions and Perspectives

The integration of genetic, epigenetic, genomic, and proteomic data will be required for the effective personalization of HCC care through a better knowledge of the biological mechanisms involved in liver cell transformation. In particular the integration of genomic and proteomic data would be indispensable for a thorough understanding of the molecular features of HCC, since little correlation exists between mRNA and protein abundance in human tissues [119]. However proteomic analysis, that in principle should be the most useful method for the understanding of biological phenomena, is still impaired by major technical limitations for unbiased biomarker identification. Although HCC-specific protein profiles have been reported in liver tissue and serum of patients with HCC [120–122], results are very heterogeneous and difficult to exploit for the identification potential biomarkers or therapeutic targets.

An integrative approach for the analysis of different data sets seems to be therefore indispensable for the identification of common carcinogenic mechanisms, driven by the different alterations that lead to the dysregulation of the main signal transduction pathways involved in liver transformation and tumor progression. Several studies have exploited a combination of genetic, genomic and protein analyses, together with clinical data, to obtain a comprehensive prognostic classification of patients [7, 12, 29, 35, 123]. However, before this approach can be translated into current clinical practice, there is still a long way: it has to be kept in mind that there are several limitations before molecular profiling can be used in routine clinical care. First of all, the difficulty of obtaining sound molecular data that are really able to stratify the cohort of patients to be treated in subgroups at different risk of HCC progression; second, the cost of these diagnostic procedures is relevant and conflicts with a widespread use. Last but not least, these technologies should be used in selected centers as their performance and interpretation still require very experienced personnel. Another concern about the widespread use of these technologies is that it opens up to a very individualized way of curing patients: medicine would be shifting from a randomized approach to a ‘single patient’ approach. If this is not too risky for disease with a monogenic alteration (of which, GIST is a good example), it can be very risky in HCC, whose molecular heterogeneity may be too complex to allow a personalized approach.

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Chapter 7
Kidney Cancer Genomics: Paving the Road to a New Paradigm of Personalized Medicine

George M. Yousef, Nicole M.A. White, and Andrew H. Girgis

Abstract Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney. Unlike other cancers, its incidence has risen in the past 20 years. The most common subtype of RCC is clear cell RCC (ccRCC) which accounts for approximately 70–80% of cases. A number of genetic aberrations have been reported to be associated with RCC. These include mutations of the von-Hippel Lindau tumor suppressor (*VHL*) gene which can be associated with a hereditary form of RCC. Inactivation of VHL leads to the stabilization of hypoxia-inducible factors (HIFs) which activates a number of downstream target proteins and contributes to cell proliferation and migration. Currently, there are no established tumor markers for RCC in clinical practice. Recently, a number of molecular markers have been examined as potential diagnostic and prognostic markers for RCC but none have gained clinical application. The new era of molecular profiling has broadened the potential discovery of biomarkers for RCC. This approach allows simultaneous comparison of thousands of molecules in one experiment which will lead to a better understanding of the pathways that are involved in RCC pathogenesis. Molecular profiling can benefit RCC patients at multiple levels including the improvement of early diagnosis, accurate tumor subclassification, prognosis, and prediction of treatment response. In this chapter, we provide a comprehensive review of the genomics of renal cell carcinoma and describe known genetic alterations that are associated with each RCC subtype. We present the current status of tumor markers

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in RCC and discuss the use of molecular profiling in RCC through different approaches. We also describe the clinical applications of molecular profiling in RCC and how this approach may improve personalized medicine for RCC patients. Finally, we discuss the concept of "integrated genomics" and how this can be applied to further the understanding of the pathogenesis of RCC.

Abbreviations

AMPK	AMP-activated protein kinase
BHD	Birt-Hogg-Dubé
CAIX	Carbonic anhydrase IX
CAV1	Caveolin-1
ccRCC	Clear cell renal cell carcinoma
CGP	Cancer Genome Project
chRCC	Chromophobe renal cell carcinoma
CNA	Copy-number alteration
EPAS1	Endothelial PAS domain protein 1
FH	Fumarate hydratase
FLCN	Folliculin
GWAS	Genome-wide association study
HIF	Hypoxia-inducible factors
HIF2α	Hypoxia-inducible factor-2 alpha
HLRCC	Hereditary leiomyomatosis renal cell carcinoma
HRPCC	Hereditary papillary renal cell carcinoma
LOH	Loss of heterozygosity
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
NF2	Neurofibromin 2
PDGF	Platelet-derived growth factor
Phos-S6	Phosphorylated ribosomal protein S6 kinase
pRCC	Papillary renal cell carcinoma
pVHL	VHL protein
RCC	Renal cell carcinoma
TFE3	Transcription factor E3
TGFα	Transforming growth factor-alpha
TGFβ	Transforming growth factor-beta
UMPP	Ubiquitin-mediated proteolysis pathway
VEGF	Vascular endothelial growth factor
VHL	Von-Hippel Lindau tumor suppressor

1 Genomics of Kidney Cancer

1.1 Overview of Renal Cell Carcinoma Genetics

Renal cell carcinoma (RCC) is the most common neoplasm of the adult kidney. It represents a spectrum of subtypes with distinct genetic and morphological identities. The most common is the clear cell subtype (ccRCC) that constitutes approximately 70–80% of cases, followed by the papillary subtype (pRCC; 10–15%) and less common subtypes including chromophobe renal cell carcinoma (chRCC; 5%), medullary RCC, collecting duct carcinoma, and translocation carcinomas, among others [1, 2]. A number of genetic alterations are reported in renal cell carcinoma. Germ-line mutations of the von-Hippel Lindau tumor suppressor (*VHL*) gene have been associated with hereditary ccRCC. Recent study showed that family history of kidney cancer conferred ~2.8-fold increased risk of RCC [3, 4]. Sporadic ccRCC is associated with *VHL* inactivation through a number of different mechanisms including chromosome 3p deletion, somatic mutations, hypermethylation and more recently miRNAs [5–8]. VHL inactivation leads to the stabilization of the hypoxia-inducible factors (HIFs) with subsequent activation of a number of downstream target proteins such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor-alpha (TGFα), and transforming growth factor-beta (TGFβ).

Papillary RCC, on the other hand, is not commonly associated with 3p deletions but rather with trisomies of chromosomes 7, 16, 17, and loss of Y. Other RCC subtypes show multiple chromosomal aberrations that are not well defined [2, 9]. In translocation carcinomas, a breakpoint at Xp11, which harbours the transcription factor E3 (*TFE3*) gene, can result in subsequent fusion of *TFE3* with several partners depending on the exact translocation [1, 2]. Four distinct recipients have been identified; *PRCC* (1q21), *PSF* (1p34); *ASPL* (17q25) and *NonO* (Xq12) [10–13]. Furthermore, a number of new entities of RCC have been recently recognized. These include mucinous tubular and spindle cell carcinoma, clear cell papillary RCC, and tubulocystic carcinoma [14]. Most of these subtypes are recognized by their distinct morphology, and the underlying molecular changes remain to be elucidated.

1.2 Familial Kidney Cancer Syndromes

Familial RCC constitutes around 3–4% of all renal cancers. It tends to present at a considerably younger age, and is often bilateral and multifocal. Several (mainly autosomal dominant) hereditary renal cancer syndromes have been described, including VHL disease, Birt-Hogg-Dubé (BHD) syndrome, hereditary leiomyomatosis/RCC syndrome, and hereditary papillary renal cell carcinoma (HRPCC)

[15]. Many hereditary RCC syndromes are defined by known causal gene mutations and morphological manifestations [16]. Strong genotype-phenotype associations have been observed that predict relative risk of developing VHL disease-associated RCC, whereas the evidence is not as compelling for the remainder of the familial RCC syndromes.

1.2.1 von Hippel-Lindau (VHL) Disease

von Hippel-Lindau (VHL) disease is characterized by autosomal dominant germline mutations of the *VHL* tumor suppressor gene located on 3p25. It is most frequently associated with retinal and central nervous system haemangioblastomas, ccRCC, pheochromocytoma, and pancreatic islet tumours [17]. Approximately 75% of patients with VHL disease develop ccRCC by age 60, which is a leading cause of death among these patients [16].

Distinct genotype-phenotype correlations have allowed for the classification of two clinical types of VHL disease based on the absence (Type 1) or presence (Type 2) of pheochromocytoma. Type 1 patients, more commonly diagnosed (30–40%), are associated with *VHL* germline exon deletions or truncating mutations and the risk of developing RCC. Type 2 patients, on the other hand, harbour missense mutations of *VHL* which range from having no effect to complete functional loss of VHL protein (pVHL) [18]. There is also evidence to suggest that a specific subgroup of Type 1 patients who have a contiguous deletion of all or part of *VHL* and the adjacent *C3orf10* (*HSPC300*) gene have lower risk of RCC (proposed Type 1B phenotype) [19–21]. Type 2 disease is further subdivided into three subtypes: Type 2A (low risk of RCC), Type 2B (high risk of RCC), and Type 2C (pheochromocytoma only) [18]. Type 2A patients are at low risk of developing RCC and are associated with missense mutations that do not lead to pVHL destabilization, but rather impact pVHL target interactions with hypoxia inducible factor (HIF), elongin B, and elongin C. Type 2B patients are at high risk of developing RCC and are associated with missense mutations that lead to severe destabilization of pVHL. Type 2C patients are specifically susceptible to developing pheochromocytoma only and are associated with *VHL* missense mutations that retain comparable wild type pVHL function [18, 22, 23]. There was no significant association between *VHL* mutation type and prognosis [22, 24].

1.2.2 Birt-Hogg-Dubé (BHD) Syndrome

Birt-Hogg-Dubé (BHD) syndrome is characterized by autosomal dominant germline mutations of the *BHD* tumor suppressor gene, also known as folliculin (*FLCN*), located on 17p11.2. It is associated with high risk of developing cutaneous fibrofolliculomas, pulmonary cysts, spontaneous pneumothorax and bilateral, multifocal RCC [25].

Chromophobe and hybrid oncocytic RCCs (chromophobe/oncocytoma hybrids) are typically associated with BHD syndrome patients, however, a subset of familial ccRCC without a link to hereditary renal cancer syndromes were observed to carry pathogenic *BHD* mutations [26]. RCC is multifocal or bilateral in more than half of patients with BHD syndrome. The protein product of the *BHD* gene, tumor suppressor FLCN, plays a role in the 5′ AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signaling pathways. There are currently no clear genotype-phenotype correlations between *BHD* mutation type and relation to risk of developing RCC.

1.2.3 Hereditary Papillary RCC

Hereditary Papillary RCC is characterized by autosomal dominant germ-line activating mutations of the *MET* proto-oncogene, located on 7q31. Individuals with this syndrome are at risk of developing bilateral, multifocal, Type 1 pRCC. Approximately 30% of *MET*-carriers develop renal cancer by age 50 [27]. Activating *MET* gene mutations have also been detected in a subset of sporadic, Type 1 pRCCs [28].

1.2.4 Hereditary Leiomyomatosis/RCC (HLRCC) Syndrome

Hereditary Leiomyomatosis/RCC (HLRCC) Syndrome is characterized by autosomal dominant germ-line mutations of the fumarate hydratase (*FH*) tumor suppressor gene, located on 1q42.1. It is characterized by cutaneous leiomyoma, uterine fibroid and/or kidney cancer manifestations [29]. Renal tumours have been observed in approximately one third of HLRCC families and tend to manifest as solitary renal lesions, however bilateral and multifocal RCC cases have been identified [30].

2 Current Status of Tumor Markers in RCC

Unfortunately, there are currently no established tumor markers for RCC in clinical practice. Diagnosis of kidney cancer relies mostly on imaging studies that are not always reliable [31]. Prognosis of RCC is quite variable. Tumor size and stage offer the only viable tools to predict prognosis and these are not always accurate [32]. The most commonly used prognostic model for patients with metastatic disease is based on a multivariate analysis of clinical parameters that was developed at Memorial Sloan Kettering [33] and later validated and enhanced based on data from The Cleveland Clinic [34].

More recently, a number of molecular markers have been investigated, and although many show clinical potential, none have gained approved clinical application (Table 7.1; [35]). There are currently very few biomarkers that can guide clinicians

Table 7.1 Some of the available biomarkers for renal cell carcinoma and their clinical utility

Biomarker	Assay	Prognostic value
C-reactive protein	Plasma level	Independent prognostic factor for survival
IL-6	Serum level	Independent prognostic factor for survival
p53	Tissue analysis	Associated with risk for metastasis and poor survival in RCC
B7H1	Immunohistochemistry	Increased risk of CCRCC death
VEGF/VEGFR	Serum/plasma levels by ELISA	Response to sorafenib and sunitinib
HIF-1[alpha]	Tumor immunohistochemistry	Marker of progression of ccRCC
VHL	Gene sequencing	Mutation could mean higher response rate to antiangiogenic therapy
CA IX	Immunohistochemistry/western blot	Chance for early metastasis
GLUT-1	Immunohistochemistry	Sarcomatoid/higher grade disease
Circulating tumor cells	Magnet-assisted cell sorting	Predicts progressive phase of the disease
TIL	Stain the TIL for CD4, CD25 and Fox P3 and analyze using confocal microscopy. This can also be done using flow cytometry	Poor survival outcome
Tissue inhibitors of metalloproteinase-2	Immunohistochemistry	Increase in the odds of cancer death
Nuclear grade	Tissue under microscope	Grade 3; increase in the odds of cancer death

CA IX: Carbonic anhydrase IX; CCRCC: Clear cell RCC; GLUT: Glucose transporter; HIF: Hypoxia-inducible factor; RCC: Renal cell carcinoma; TIL: Tumor-infiltrating lymphocytes; VEGFR: VEGF receptor; VHL: von Hippel-Lindau.

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in the choice of therapy. Response to interleukin-2 (IL-2) is associated with the presence of alveolar features and an absence of papillary or granular features [36]. High levels of carbonic anhydrase (CA) IX are associated with a more favourable prognosis and a greater likelihood of a response to IL-2 [37]. There are, however, no biomarkers available to predict response to targeted therapy**.** Therefore, there is an urgent need for the identification of novel tumor markers for RCC that will improve patient diagnosis, prognosis and to guide clinicians to choose the optimal treatment for each patients.

3 Molecular Profiling: New Insights into RCC Susceptibility and Pathogenesis

More recently, a revolution happened in molecular biomarker discovery through the introduction of "molecular profiling" approaches that allow simultaneous comparison of hundreds or even thousands of molecules in one experiment [38, 39]. In addition to significant acceleration in biomarker discovery, this led to a better understanding of the "cross talk" that occurs between molecules to produce the disease phenotype. These high throughput technologies allowed a more in-depth insight into the complex mechanisms controlling carcinogenesis of RCC by interrogating information from classes of molecules, including genetic variants, genetic aberrations, epigenetics, transcriptomics, and proteomics analyses [31]. As a result, our focus started to switch from the single molecule approach to understanding the "mechanisms or pathways" altered in RCC [40].

The different approaches and applications of molecular profiling in RCC are summarized in Fig. 7.1. Stepping into the era of personalized medicine, there are a number of fields in patient management that require improvement, as outlined in Box 1. Molecular profiling has a potential significant impact towards developing better diagnostic, prognostic, and predictive markers of treatment efficiency [41]. Profiling approaches can also be useful in patient follow-up after surgery for early

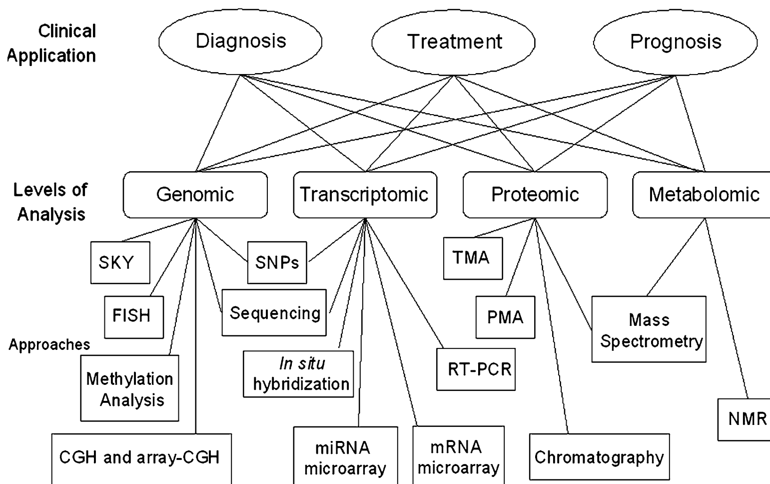

Fig. 7.1 Levels of molecular profiling analysis in renal cell carcinoma. Clinical applications through molecular profiling for RCC patients can be performed at different levels of analysis including genomic, transcriptomic, proteomic, and metabolomic profiling. These can be achieved through a number of different approaches. *SKY* special karyotyping, *FISH* fluorescence *in situ* hybridization, *CGH* comparative genomic hybridization, *SNP* single nucleotide polymorphism, *RT-PCR* reverse transcriptase polymerase chain reaction, *PMA* protein microarray, *NMR* nuclear magnetic resonance

detection of recurrence, and the sub-grouping of patients into smaller categories based on their tumor biology, thus allowing for individualization of treatment options. ***In Sects. 3.1, 3.2, 3.3, 3.4 and 3.5, we will discuss the value of molecular profiling in achieving a better understanding of the pathogenesis of RCC, in Sects. 4.1, 4.2, 4.3 and 4.4, we will outline the spectrum of clinical applications of molecular markers, and in Sect. 5, we will summarize their potential utility for the discovery of new therapeutic targets.***

Box 7.1: Fields that Require Research Efforts to Achieve Personalized Medicine in Kidney Cancer

- Early accurate non-invasive tools for diagnosis of kidney cancer
- Accurate classification of RCC subtypes, and characterization of the newly discovered entities.
- Accurate assessment of prognosis
- Prediction of response to treatment
- Discovery of new targeted therapies
- Accurate prediction of the risk of kidney cancer
- Discovery of markers to enroll patients into clinical trials

3.1 Genome-Wide Association Studies (GWAS) and Susceptibility of RCC

A genome-wide association study (GWAS) is defined as a case-control study comparing the genetic variation between people affected with a condition of interest to those who are unaffected, in order to identify genes involved in the disease, and may help prevent, diagnose, and treat the disease. GWAS have emerged as an important tool, commonly using hundreds of thousands of single nucleotide polymorphism (SNP) markers to discover regions in the genome that are associated with risk of cancer [42].

In kidney cancer, three genetic susceptibility genes/loci have been confirmed to be associated with the risk of RCC [43–45]. In individuals of European descent, genetic loci on 2p21, 11q13.3 and 12p11.23 were found to be associated with RCC risk. Three associated variants map to the endothelial PAS domain protein 1 (*EPAS1)* gene on 2p21, which encodes hypoxia-inducible factor-2a (*HIF2α)* and is strongly implicated as a renal cancer oncogene central to the *VHL–HIF* pathway. Two of these variants were also associated with former and current smokers, but not in never-smokers, suggesting the effect of *EPAS1* is dependent on tobacco smoking [43, 44]. The third variant was strongly associated with *VHL* alterations [43].

The locus on 11q13.3 does not map to a characterized gene. However it is flanked by two cancer-related genes, *MYEOV* and cyclin D1 [44]. Recent preliminary evidence suggests this variant is significantly associated with reduced risk of RCC, especially among normal-weight individuals, never-smokers and non-drinkers in the Chinese population [46].

Two associated variants on 12p11.23 map to the *ITPR2* gene [45]. One of them is also associated with waist-hip ratio phenotype, suggesting a genetic link between obesity and RCC risk, given that obesity is a well-established risk factor for RCC [47–49]. A recent study by Moore et al. [50] observed *VHL* germline variants were associated with a higher risk of *VHL* inactivation via promoter hypermethylation compared to *VHL* mutation in sporadic ccRCC, suggesting the utility of genetic polymorphisms as indicators of increased risk of epigenetic alterations and cancer susceptibility. They also observed a subset of tumors from current smokers lacking *VHL* alterations, suggesting an etiologically distinct subgroup of ccRCC [50]. Further GWAS are required to identify the genetic underpinnings behind RCC risk in distinct populations, such as African Americans, who are at higher risk of developing RCC [48, 51]. Also, evaluating the association of these variants with prognosis and treatment response is warranted.

3.2 Exome Sequencing Identifies Novel Mutations in RCC

Exome sequencing has emerged as a cost-effective alternative to whole genome sequencing where only protein-coding regions (~5% of genome) are sequenced for pathogenic gene mutations [52]. The Cancer Genome Project (CGP) recently conducted exome sequencing of ccRCC that revealed novel recurrent mutations of the SWI/SNF chromatin remodelling complex gene, PBRM1 (41%), and of genes encoding enzymes that methylate (SETD2, 3%) or demethylate (JARID1C and UTX, 3%) key lysine residues of histone H3 [53, 54]. The CGP also identified mutations of the tumor suppressor neurofibromin 2 (*NF2*) in non-*VHL* mutated ccRCCs [53, 54]. These results illustrate the complex genetic architecture of ccRCC, and emphasizing the contribution of chromatin aberrations in ccRCC tumorigenesis.

In addition to *VHL*, the *PBRM1* and *SETD2* genes map to the frequently deleted 3p21 region, suggesting a link between frequent overlapping biallelic inactivation of these genes and ccRCC tumorigenesis [55]. Recently, Niu et al. [56] demonstrated knockdown of *JARID1C* in *VHL*-deficient ccRCC cells to significantly enhance tumor growth, suggesting *JARID1C* is a tumor suppressor.

An independent exome sequencing study by Guo et al. [57] confirmed several mutations catalogued by the CGP and also identified 12 additional mutations previously unreported in ccRCC. Furthermore, this study observed a significant enrichment of mutations targeting the ubiquitin-mediated proteolysis pathway (UMPP) in ccRCC and that alterations in the UMPP were significantly associated with overexpression of HIF1α and HIF2α.

3.3 Genome-Wide Copy-Number Alteration (CNA) and Loss of Heterozygosity (LOH) Analyses in ccRCC

High-throughput arrays have allowed for the interrogation of tumour-specific chromosomal and allelic changes in RCC at a much higher resolution than traditional cytogenetic methods. At least three regions on chromosome 3p have been implicated in sporadic ccRCC; one locus is on 3p25-26 harboring the *VHL* gene, the second is on 3p21-22, and the third is on 3p13-14 [1]. In addition to VHL, recent data suggest the presence of other putative tumor suppressor genes at the 3p region, such as *RASSF1A* and *SETD2* located on 3p21 and *NRC-1* on 3p12 [54, 58]. Recent analysis has revealed peak deletions, specifically targeting *VHL* (on 3p25) and CDKN2A and CDKN2B (on 9p21), and peak amplifications of MYC (on 8q24) in subsets of ccRCC [59].

A number of studies suggested the accumulation of additional genetic alterations during the process of tumor progression and metastasis [60–64]. Metastasis was found to be associated with losses of 3p, 8p and 9p, 13q and gains of 17q and Xq. Also, a correlation was observed between metastasis and increase in the copy number of genes located at 1q [61].

3.4 miRNAs: A New Dimension in the Pathogenesis of RCC

miRNAs are small non-coding RNAs that negatively control gene expression. They have been shown to be involved in the regulatory functions of diverse biological processes including cell mobility, differentiation, development, proliferation and apoptosis [65]. Aberrant miRNA expression has been reported in many cancers and accumulating reports show that they play novel roles as oncogenes or tumor suppressors [66–68]. Recent evidence showed the diverse clinical uses of miRNAs in cancer as diagnostic, prognostic and predictive markers [69].

Recently, a number of studies documented the differential expression of miRNAs in kidney cancer [70–77]. White et al. [71] identified 166 miRNAs that were significantly dysregulated in ccRCC when compared to normal kidney tissue. miR-122, miR-155 and miR-210 had the highest overexpression while miR-200c, miR-335, and miR-218 were the most downregulated.

Evidence is accumulating regarding the involvement of miRNAs in RCC pathogenesis. A recent study showed an effect of the oncogenic miRNA cluster miR-17-92 on tumor cell proliferation [78], and preliminary evidence showed that miRNAs can affect key molecules in the VHL-HIF-hypoxia pathway [5, 7]. miRNAs have also been shown to be epigenetically regulated in ccRCC. Voqt et al. [79] studied 12 RCCs found that miR-34 was methlylated in 58% cases while miR-34b/c was methlylated in 100% cases. The inactivation of miR-34a and miR-34b/c was concomitant in most cases. The proposed mechanisms of miRNA involvement in RCC pathogenesis have been recently reviewed [80].

3.5 New Molecular Classification of RCC

Recent reports suggest the presence of great heterogeneity in RCC and that even tumors with the same morphology (e.g. ccRCC) can be further sub-classified based on their molecular signature. This can have a great impact on patient management, since these "biological" subtypes can have different prognosis and may be subject to different types of targeted therapy.

VHL is inactivated in almost 90% of sporadic ccRCC tumors [8]. It is involved in a number of different pathways but is best characterized for its role in the regulation of the hypoxia induced factors, HIF1α and HIF2α, which are key regulators of the hypoxia response [81]. Under normal oxygen conditions, VHL is the recognition component of a complex that is responsible for the degradation of HIF1α and HIF2α [82]. When VHL is inactivated, the HIFs become constitutively activated and can induce a number of genes that promote tumor growth by enhancing cell proliferation and angiogenesis [83]. Although HIF1α and HIF2α have been both shown to play significant roles in ccRCC pathogenesis, recently, it has been shown that they can have different effects [84].

Gordon et al. [85], classified VHL-deficient tumors into two groups based on their HIF expression; one subtype expressed both HIF1α and HIF2α (H1H2), while the other expressed HIF2α only (H2) (Fig. 7.2). Interestingly, distinct pathways were shown to be significantly dysregulated in each of these groups. The H1H2 tumors showed increased MAPK and mTOR signaling, while the H2 group showed increased c-Myc activity. This suggests that these subtypes may have different clinical outcomes and patients would benefit from targeted therapies that are

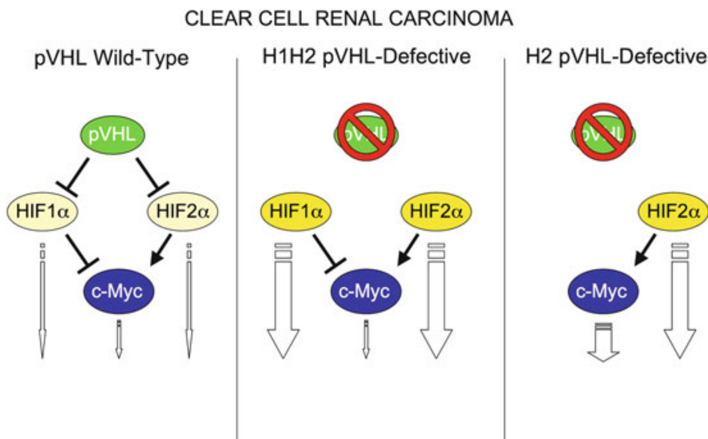

Fig. 7.2 Clear cell renal cell carcinoma subtypes. pVHL targets HIFα for proteasomal degradation. Accordingly, tumors with wild-type pVHL have low levels of HIFα. pVHL-defective tumors can be subdivided based on whether they accumulate both HIF1α and HIF2α (H1H2) or HIF2α alone (H2). In the former, HIF1α antagonizes c-Myc. In the latter, this antagonism is lost and c-Myc activity is therefore increased (Reprinted from Kaelin [86], with permission from Elsevier)

designed specifically for the ccRCC subgroup in which the belong [86]. More recently, distinct chromosomal aberrations were identified in each of these subtypes, adding to the growing evidence that these two subgroups are distinct [87]. Another study identified two distinct biological subtypes of ccRCC based on gene expression signatures [88]. These two subtypes also showed significant differences in disease-free survival

Klatte et al. [89] showed that there are distinct cytogenetic aberrations associated with Type 1 and Type 2 pRCC. Type 1 tumors frequently had trisomy 17, while Type 2 tumors were associated with loss of chromosome 1p and 3p, and gain of 5q. Type 2 was also associated with worse overall survival than Type 1 but was not retained as an independent prognostic factor.

4 The Clinical Application of Molecular Signatures

A wide spectrum of clinical applications is gradually evolving from molecular profiling of RCC at multiple levels of analysis [38, 90]. This includes diagnosis, accurate subclassification, prognosis, and prediction of treatment response, as outlined below.

4.1 Diagnosis of RCC

Molecular profiling has been used to determine the presence of a "signature expression profile" in RCC that can accurately distinguish between cancerous and normal kidney tissue. A number of studies have analyzed differential gene expressions in RCC at the mRNA level [91–94]. At the protein level, there are few reports on proteomic profiling [95–101] that identified a limited number of potential biomarkers [102, 103].

The differentially expressed genes and proteins are candidate diagnostic markers that await validation as tissue markers or as non-invasive serum and/or urine markers for early detection of RCC. miRNAs have also been recently shown to have great diagnostic potential in RCC. Recent reports suggest that miRNAs are present in stable form in body fluids and as such, they can be useful as non-invasive diagnostic tests. In addition to distinguishing normal from cancerous tissues, potential usefulness of molecular profiling in RCC include the potential ability to determine the tissue of origin in tumors of unknown primary [69]. Moreover, urinary markers may prove to be useful non-invasive biomarkers. For example, 14-3-3-beta/alpha was higher in urine samples from patients with RCC than in samples from healthy volunteers [104].

4.2 Accurate Classification of RCC Subtypes Using Molecular Signatures

RCC is a group of heterogeneous subtypes, each with distinct morphology, prognosis and response to therapy [105]. Distinguishing between subtypes cannot be done by imaging analysis and relies on histomorphology. There are, however, a significant number of cases where histopathology is not conclusive. For example, oncocytoma can be easily mistaken for chRCC, and the eosinophilic variant of ccRCC can be mistaken for oncocytoma or chRCC. Also, papillary configuration might be encountered in different histological subtypes and is not restricted to pRCC. A recent study showed a significant inter-observer variability in diagnosing different subtypes among pathologists [106, 107]. Moreover, some subtypes have overlapping morphological features. Added to this is the recognition of collision tumors (two separate tumors developing close to each other) and hybrid tumors (two types within the same tumor). Furthermore, some of the newly recognized entities, like translocation carcinomas, have histological patterns that are overlapping with other subtypes. Since immunohistochemistry lacks both sensitivity and specificity for accurate subtyping of kidney tumors, the creation of an "unclassified" category of RCC (which is characterized by morphological features that fits more than one category) was a necessity.

A useful application for molecular profiling is the use of distinct molecular profiles to accurately distinguish between RCC subtypes. Using a number of different platforms, several groups have shown that gene expression profiling can be used for renal tumor classification, complimentary to morphological criteria [108–112]. Yang et al. [113], demonstrated the viability of using molecular signatures for the accurate classification of renal tumors. Gene expression analyses showed that oncocytoma and chRCC are also closely related at the molecular level. A distinct pattern of gene expression can, however, distinguish these two closely related tumors. Another study used mRNA expression profiles to properly distinguish between ccRCC and chRCC [92].

Also, specific miRNA signatures have shown to be able accurately distinguish between kidney cancer subtypes. Youssef et al. [114] developed a unique classification system that can accurately distinguish between the RCC subtypes with high precision (Fig. 7.3). A unique feature of this classifier is that it is based on differential expression between pairs of miRNAs within the sample in question. Similar findings were reported by other groups [115]. Petillo et al. [116] showed that miR-203 and miR-424 were overexpressed in ccRCC when compared to pRCC and that miR-203 was downregulated in oncocytomas. Another group showed that 18 miRNAs were significantly different between the RCC subtypes [117]. miR-21, a known oncogenic miRNA, was shown to be upregulated in papillary and clear cell carcinomas, but not in the other subtypes [118]. Recently, a genome-wide DNA methylation study was able to accurately distinguish between Type 1 and Type 2 pRCCs and discriminate chRCCs from oncocytomas [119].

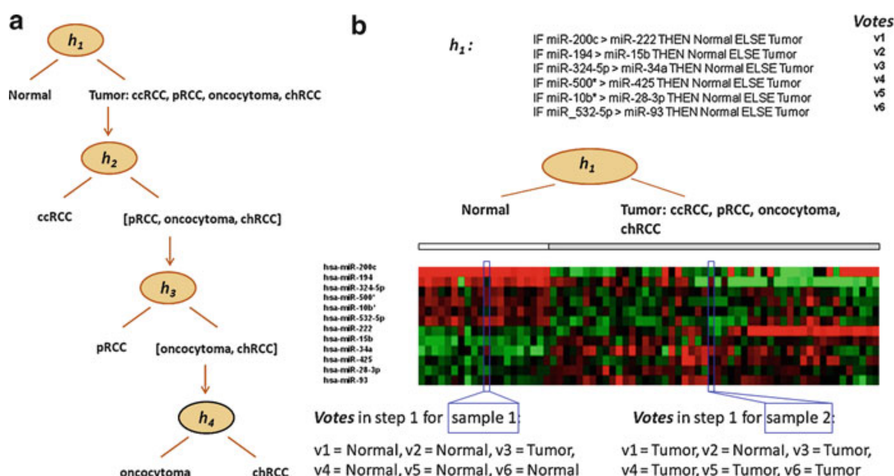

Fig. 7.3 RCC subtypes can be accurately diagnosed using miRNAs. An overview of the decision tree hierarchic microRNA (miRNA) classifier system. The decision tree has a total of four steps. Each step consists of a group of miRNA pairs, which based on their differential expression can classify a sample as belonging to one of the two possible outcomes in a single step (Reprinted from Youssef et al. [193], with permission from Elsevier)

4.3 Prognosis of RCC

Recent evidence shows that the integration of molecular markers can lead to significant improvement in the accuracy of the available clinical parameters that are currently used to assess prognosis in RCC [120].

4.3.1 Chromosomal Aberrations as Prognostic Markers

A growing body of preliminary evidence have shown copy number aberrations in ccRCC to be dynamically related to patient prognosis and suggest the utility of chromosomal aberrations as prognostic markers in RCC. Apart from gain of chromosome 5q which was associated with a better overall survival [121], most chromosomal aberrations are related to worse prognosis, such as associations of the loss of 4p, 9p and 14q and gains of 7q, 8q, 20q, with higher TNM stages, higher grade and/or worse prognosis [122–127]. 9p loss has been observed in association with poor outcome in several studies [54, 128]. In locally advanced ccRCC, the loss of heterozygosity (LOH) of 8p and 9p was found to be a strong predictor of recurrence post nephrectomy, in an analysis by Presti et al. [129]. It was also observed that LOH of 8p was a better predictor of recurrence compared to tumor grade [54, 129]. Moreover, copy-number alterations in chromosomes 1q, 12q and 20q have been associated with metastatic ccRCC [126]. These associations, however, need to be further validated on larger cohorts.

In pRCC, 1q gain was shown to be a poor prognostic marker [130]. In addition, loss of 1p, 3p, or 9p and the absence of trisomy 17 were all associated with poorer prognosis [89]. Also, amplifications of 8q were associated with MYC oncogene activation and overexpression in high-grade and aggressive Type 2 pRCC [131]. There is much less evidence that assesses chromosomal aberrations as prognostic markers in other subtypes of RCC.

4.3.2 mRNA Prognostic Markers

Earlier reports identified a number of potential prognostic markers for RCC. Lower *PTEN*, *EPCAM*, and higher carbonic anhydrase IX (*CAIX*), *VEGF-R2* and *VEGF-R3* were all associated with poorer prognosis in papillary RCC [89]. CAIX is gaining attention as a potential prognostic biomarker for RCC. A number of studies showed that high *CAIX* expression is associated with favourable prognosis [132–134]. These findings were, however, not reproducible in other studies [135]. CAIX was also proposed as diagnostic marker (when incorporated into imaging studies) and a predictor of treatment efficiency [136]. In addition, *B7H1* overexpression was found to be associated with poor survival [137]. Also, VHL alterations were found to have useful prognostic information [138]. *IMP3 is a*nother potential marker [139, 140]. Earlier reports have shown that p53 overexpression is associated with sarcomatoid differentiation and poor prognosis [141–144]. Other identified molecular markers of prognostic significance include *bFGF*, *VEGF*, Interleukin-8, *MMP-2*, *MMP-9*, vimentin, major histocompatibility complex (*MHC*) class 2, and e-cadherin [145–149]. Epidermal growth factor receptor (*EGFR*) is another proposed prognostic factor [150].

The use of microarray analysis led to the identification of batches of genes, or gene signatures, which can be of prognostic significance. Takahashi et al. [109] showed that a group of 40 genes could accurately distinguish between patients who died of cancer and those who did not developed metastasis. In another study, Vasselli et al. [151] examined 58 primary tumors of patients with metastatic RCC and identified a 45-gene signature that was associated with poor prognosis. Another study identified *ADFP* as a potential prognostic biomarker for ccRCC and showed that high *ADFP* expression was associated with a better cancer-specific survival and cancer-free survival [110, 152].

A recent microarray analysis identified two subgroups within ccRCC, based on gene expression profiling, that differ in biological behaviour despite similarity in histology [153]. Another microarray-based analysis showed that approximately 40 genes can accurately make the distinction between patients with a relatively non-aggressive form of the disease compared to patients with aggressive disease [109]. Molecular signatures were shown to supersede conventional staging in predicting outcome. Another study identified 89 differentially expressed genes in RCC [154]. One of these, vimentin, has been shown to be a marker of poor prognosis. Other studies showed that the integration of expression profiling data with standard clinical parameters will enhance the assessment of prognosis in RCC [109].

Kosari et al. [155] identified a 35 gene signature that is associated with RCC tumor aggressiveness. The majority of these genes were downregulated in aggressive ccRCC and metastasis. They identified the gene survivin as being inversely correlated with survival in an independent data set that was confirmed using immunohistochemistry.

Interestingly, Jones et al. [156] identified a metastatic 155-gene signature in primary tumors that can be used to differentiate ccRCC patient with distant metastasis at the time of surgery from patients with localized disease, suggesting that patients presenting with distant metastasis represent a biologically distinct subgroup. Sultmann et al. [157] independently validated this gene set on a different platform. This concept was further supported by a study that examined a metastatic signature across a number of tumor types and found that solid tumors carrying the gene expression signature were more likely to be associated with metastasis and poor clinical outcome [158].

4.3.3 miRNAs as Prognostic Markers

miRNA signatures associated with tumor progression and metastasis have been recently reported. Heinzelmann et al. [159] defined a miRNA signature of 33 differentially expressed miRNAs distinguishing between metastatic and non-metastatic ccRCC. These include miR-451, miR-221, miR-30a, miR-10b and miR-29a. They also showed a number of miRNAs associated with progression-free and overall survival. White et al. [160] identified 65 miRNAs that were significantly altered in metastatic compared to primary ccRCC. Another study reported miR-155 expression to correlate with tumour size [71]. Slaby et al. [161] suggested the utility of miR-106b underexpression as an indicator of early metastasis in ccRCC patients post-nephrectomy.

Lin et al. [162] conducted a preliminary study assessing potential functional SNPs in miRNA and miRNA-related genes in association with survival and recurrence in RCC patients. They identified seven SNPs associated with survival and five with recurrence. Five of the seven variants linked to *GEMIN4*, a gene involved in pre-mRNA splicing and ribonucleoprotein assembly, whereas the remaining two map to pre-mir-608 and *DICER* (involved in pre-miRNA maturation). The five variants associated with tumor recurrence map to pre-mir-146a, pre-mir-196a-2, pre-mir-631, pre-mir-608, and pre-mir-423 [162]. They also observed associated haplotypes of *DICER* and *DROSHA* (also involved in pre-miRNA maturation) with survival and recurrence, as well as a cumulative effect of multiple unfavourable variants with increased risk of death.

4.3.4 Prognostic Proteomic Markers

A study found that high caveolin-1 (CAV1) protein expression level in the tumor cell cytoplasm may be an independent poor prognostic marker of both overall and

tumor specific survival in ccRCC patients [163]. In addition, when examined by immunohistochemistry, increased levels of HIF-1α and phosphorylated ribosomal protein S6 kinase (Phos-S6) were associated with disease-specific survival and tumor progression [164].

4.3.5 Prognostic Epigenetic Markers

Epigenetic alterations can occur at the DNA level as methylation, the RNA level as RNA interference and miRNAs, and at the protein level as post-translation histone modifications and polycomb group protein complexes [165, 166].

Arai et al. [167] conducted genome-wide methylation profiling of ccRCC and matched non-cancerous renal cortex and observed two methylation subclasses for both tumor and non-malignant tissue that were associated with significantly different survival. Another study demonstrated global hypermethylation as an independent indicator of aggressiveness in early stage confined ccRCC [168]. Methylation status of *DLEC1* tumor suppressor was associated with more advanced stages and grades [169]. *GREM1* methylation was associated with increased Fuhrman grade and decreased overall survival in ccRCC [170]. Several hypermethylated genes and miRNAs show promise as independent poor prognostic markers for RCC management such as gamma-catenin, *RASSF1A*, *BNC1*, collagen, type XIV, *COL14A1*, *UCHL1*, *APAF-1*, *DAPK1*, *hsa-miR-9-1* and *hsa-miR-9-3* [171–176].

There is evidence to support global histone modification levels as prognostic markers in RCC. Rogenhofer et al. [177] demonstrated lower levels of H3K27me1, H3K27me2 and H3K27me3 in RCC with tumor relapse compared to benign renal tissue. Lower levels of H3K27me1 and H3K27me3 were also associated with shorter progression-free survival. Ellinger et al. [178] observed lower levels of H3K4 in correlation with Furhman grading, staging, lymph node and distant metastasis. Lower levels of H3K4 were also associated with shorter progression-free and cancer-specific survival. Mosashvilli et al. [179] observed an inverse correlation between histone H3 acetylation levels and stage, distant metastasis, Fuhrman grade and RCC progression. They also observed a correlation between histone H4 deacetylation levels in correlation with stage and grade.

4.4 Predictive Markers of Treatment Outcome

Managing advanced metastatic RCC is a clinical challenge. New targeted therapies have led to improvements in response and survival over traditional treatments, however most patients ultimately develop resistance. Response rates vary among patients and the optimal combination and sequence of therapy is yet to be defined. There are currently no validated biomarkers that can predict treatment outcome in RCC management. However, there is a growing body of preliminary evidence demonstrating the potential utility of molecular markers in that regard.

Genetic polymorphisms in key genes associated with sunitinib response and/or toxicity have been recently reviewed [180]. A recent exploratory study found that genetic polymorphisms in three genes involved in sunitinib pharmacokinetics are associated with progression free survival (PFS) in mRCC patients treated with this drug [181]. Likewise, in a phase-III clinical trial of pazopanib in RCC, three polymorphisms in *IL8* and *HIF1α*, and five polymorphisms in *HIF1α*, *NR1I2*, and *VEGFA*, showed a significant association with PFS and response rate, respectively [182].

Serum/plasma levels of VEGF, soluble VEGFR-2, CAIX, TIMP-1, and Ras p21 have shown prognostic value in sorafenib treated RCC patients [183]. Also, TIMP-1 was demonstrated as an independent poor prognostic marker in sorafenib treated patients [183]. miRNAs represent another class of predictive markers for treatment outcome with successful potential use in other cancers [184–186]. They remain unexamined in RCC.

5 The Role of Genomics in RCC Therapy

Understanding the pathogenesis of RCC is a cornerstone towards the development of new molecular targeted therapies. Currently, anti-angiogenic therapies and mTOR inhibitors are the first line treatment for metastatic cancer but their response rates are in the moderate range. More in-depth understanding of the pathways affected in RCC will allow for the introduction of new targeted therapies [40]. Interestingly, targeted therapies that are used for other cancers might be also applicable to RCC if the same pathway is affected. Molecular profiling analysis can have a potential promise in enrollment of patients for clinical trials, based on their biological behaviour rather than the anatomical site of their tumors. Recently, initial data showed the feasibility of using genomic and transcriptomic data from integrative sequencing of tumors as a means to identify the most suitable clinical trial for each individual patient [187]. If this is validated on large scale studies, it will represent a revolutionary improvement in personalized medicine. Finally, miRNAs represent new potential therapies with the unique advantage of controlling the expression of multiple targets by altering the level of a single miRNA [188].

6 Conclusion and Future Perspective

Accumulating evidence demonstrate the potential ability of genomic analysis to pave the road to a new era of personalized medicine in kidney cancer. There are, however, a number of challenges that need to be addressed during the transition from bench to bedside, as recently reviewed [38]. Among these challenges is the ability to analyze and extract meaningful and clinically useful information from these large data sets. The concept of "integrated genomics" represents a promising solution

for this. By simultaneously analyzing different molecular levels of changes in the kidney cancer genomes, including DNA copy number aberrations, methylation, mutation, mRNA and miRNA expression, you can get a better understanding of the overall changes that affect certain biological processes in RCC. Employing this approach overcomes to a large extent the limitation of overlooking critical genes that are disrupted at low frequencies when assessed by a single mechanism [189, 190]. Moreover, this approach facilitates the discovery of tumor suppressor genes exhibiting multiple concerted disruptions (MCD), where each allele may be disrupted by a different mechanism. Also, an oncogene could be activated by two separate mechanisms such as DNA amplification with a simultaneous activating mutation or DNA hypomethylation. In addition to enhancing the ability to detect candidate driver genes, this integrative approach is also useful for detecting deregulated pathways [189, 191]. Integrative software for genome wide integration of genomic and epigenetic data to decipher their effect on gene expression and disease phenotype are emerging [192].

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Chapter 8
Pancreatic Cancer Genomics

Vincenzo Corbo, Andrea Mafficini, Eliana Amato, and Aldo Scarpa

Abstract Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with the worst prognosis among all solid tumors [1]. Although surgical resection offers the only hope for cure, it is possible in only 20% of patients that present with local disease [2]. Indeed, most patients are diagnosed at an advanced stage, when the disease is inoperable. Whether dismal prognosis is a result of late diagnosis or early dissemination to distant organ is still a debate. Systemic chemotherapy provides temporary benefits in controlling advanced disease and prolonging survival in the adjuvant setting but this happens in a small proportion of patients. Several factors are supposed to contribute variably to the intrinsic chemotherapic resistance of pancreatic cancer and include: (i) the presence of a dense stromal component (termed desmoplastic reaction) that significantly reduces drug delivery [3]; (ii) the transformation of epithelial cells into a mesenchymal phenotype (referred to as epithelial to mesenchymal transition, EMT) [4]; and (iii) the presence of pancreatic cancer stem cells [5]. To complicate our understanding of chemoresistance, there is the marked molecular heterogeneity among primary tumors and metastatic deposits (discussed in details below) [6].

Keywords Pancreatic cancer • Cancer heterogeneity • Metastasis • Clonal evolution • Next generation sequencing

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1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with the worst prognosis among all solid tumors [1]. Although surgical resection offers the only hope for cure, it is possible in only 20% of patients that present with local disease [2]. Indeed, most patients are diagnosed at an advanced stage, when the disease is inoperable. Whether dismal prognosis is a result of late diagnosis or early dissemination to distant organ is still a debate. Systemic chemotherapy provides temporary benefits in controlling advanced disease and prolonging survival in the adjuvant setting but this happens in a small proportion of patients. Several factors are supposed to contribute variably to the intrinsic chemotherapic resistance of pancreatic cancer and include: (i) the presence of a dense stromal component (termed desmoplastic reaction) that significantly reduces drug delivery [3]; (ii) the transformation of epithelial cells into a mesenchymal phenotype (referred to as epithelial to mesenchymal transition, EMT) [4]; and (iii) the presence of pancreatic cancer stem cells [5]. To complicate our understanding of chemoresistance, there is the marked molecular heterogeneity among primary tumors and metastatic deposits (discussed in details below) [6].

Accounting for more than 85% of all pancreatic tumors, PDAC is generally referred to as the pancreatic cancer and it is definitely a genetic disease [7]. A distinct set of genetic alterations can be identified in pancreatic cancer and in its precursor lesions [7]. Furthermore, genetically engineered mouse models (GEMMs) that display several of those specific alterations fully recapitulate human disease [8, 9]. Finally, a small proportion of patients diagnosed with pancreatic cancer (about 5%) have a familial form of the disease [10].

Several studies have described three distinct precursor lesions of pancreatic cancer: pancreatic intraepithelial neoplasia (PanINs), mucinous cystic neoplasms (MCNs), and intraductal papillary mucinous neoplasms (IPMNs) [11]. Of these, PanINs represent the most frequent and well-characterized premalignant lesions. Histologically, PanINs are classified into PanIN-1, PanIN-2, and PanIN-3 lesions, depending upon the degree of cytologic and architectural atypia. A PanIN to pancreatic cancer progression model has been proposed based on the results of different molecular studies showing an increasing number of genetic alterations in higher grade PanINs (Fig. 8.1).

Most of the genetic alterations that characterize pancreatic cancer have been identified through candidate gene approaches and relying on conventional 'Sanger' sequencing that are unsuited to identify low-frequency gene alterations. Recent technological advances enable comprehensive genome-wide studies of individual tumors that promise to uncover the pancreatic tumor heterogeneity [12]. However, big efforts should be put in place in order to adequately manage the multitude of data that will be generated through massive-scale studies. Indeed, these new technologies are to be considered only instrumental in identifying putative causal mutations whose role and implications in cancer needs to be deeply characterized before introducing them as clinical biomarkers or therapeutic targets. In the next

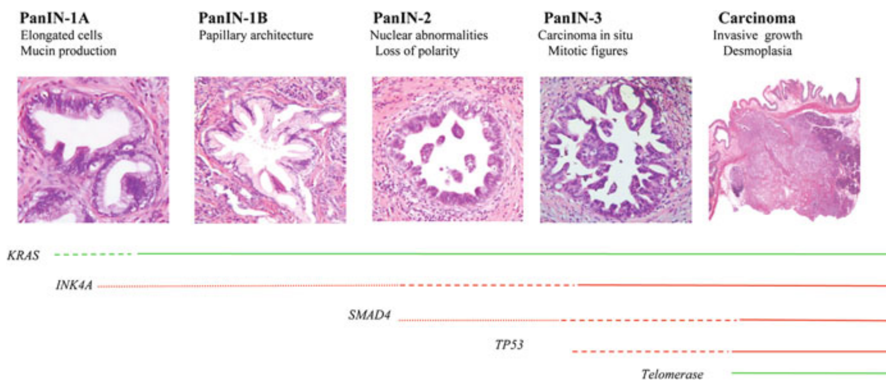

Fig. 8.1 Precursor lesions of pancreatic cancer: **pancreatic intraepithelial neoplasia (PanINs)**. PanINs represent progressive stages of neoplastic growth that precede the onset of the invasive carcinoma. The progression from low-grade lesions to carcinoma (from the *left to the right*) is associated with an increasing number of genetic alterations. *Lines* represent the stage of onset of these alterations; the *thickness* of the line indicates the frequency of the alteration; whereas the *color* corresponds to the type of alteration (*green*, activation; *red*, loss of function)

subsections we report the most relevant molecular signatures of pancreatic cancer to date. Different methodological approaches have been used in recent years to explore pancreatic cancer at different molecular levels (genome, transcriptome, and methylome) leading to a plethora of molecular features, whose clinical implications are also discussed.

The new clinical "calls" arising from genome-wide comprehensive studies are also debated.

2 Molecular Genetics of Pancreatic Cancer

2.1 The Molecular Backbone of Pancreas Cancer Involves Anomalies in Four Genes

Four genes alterations are found in the majority of sporadic PDACs: *KRAS* oncogene activating mutation and inactivation of three tumor suppressors including *INK4A*, *TP53* and *SMAD4* [13, 14].

Activating mutations in the *KRAS* oncogene occur in virtually all PDACs and therefore represent the most common anomaly encountered in this tumor type [15]. Mutations of this gene are detectable in the low-grade precursor lesions of PDAC and therefore represent one of the earliest genetic abnormalities observed in pancreatic cancer [16]. Somatic mutations of *KRAS* are restricted to the GTP-binding pocket (mainly a single-base substitution in codon 12) and result in a constitutively active protein that drives pancreatic tumorigenesis [17]. This is also

confirmed by genetically engineered mouse models that conditionally express oncogenic KRAS (KrasG12D) that recapitulate develop pancreatic cancer tumorigenesis in humans [18]. However, a small proportion of PDAC, possibly accounting for less than 10% of cases, lacks *KRAS* mutations suggesting that other genes belonging to Ras/Raf/MAPK pathway (through which KRAS acts) might be involved in pancreatic cancer. In line with this, *BRAF* mutations have been found in *KRAS* wild type cancers [19] thus confirming the importance of the Ras/Raf/MAPK pathway as well as supporting the hypothesis of mutually exclusive mutations within the same signaling pathway. From a therapeutical point of view, these observation point out the possible use Ras/Braf inhibitors in pancreatic cancer [20].

Moreover, *KRAS* point mutations stand out as an ideal marker for early diagnosis of pancreatic cancer since they are found in pre-invasive lesions and their detection is technically feasible at high sensitivity in different biological samples [21, 22]. Despite this, *KRAS* mutations are not exclusive of pancreatic cancer and can be also found in non-neoplastic patients with a relatively high risk of over-diagnosis.

The *INK4A* gene encodes for a protein belonging to the family of cyclin-dependent kinase (CDK) inhibitors and as such is involved in the regulation of cell cycle progression through G1-S phase [23]. Inactivation of *INK4A* is observed in more than 90% of pancreatic cancer cases by diverse mechanisms [13, 24]: (i) homozygous deletions in approximately 40% of cases; (ii) intragenic mutations associated with loss of heterozygosity (LOH) in another 40%; (iii) and epigenetic silencing by promoter methylation in the remaining 10% of cases. As for *KRAS,* genetic alterations of *INK4A* are also observed in precursor lesions of pancreatic cancer strongly suggesting its causative role in pancreatic carcinogenesis [16]. Nevertheless, conditional loss of the *Ink4a* locus in the KrasG12D mouse model of pancreatic cancer led to acceleration of precursor lesions development, a greatly reduced tumor latency with an increase in undifferentiated and anaplastic PDAC, which showed micrometastasis to lung and liver [25].

Inactivation of *TP53* occurs in at least 50% of pancreatic cancer patients mainly by intragenic mutations associated with LOH [26]. Mutations of *TP53* are observed in high-grade precursor lesions of PDAC suggesting that these alterations are likely to be a late event in pancreatic carcinogenesis [27]. This feature renders *TP53* alterations potentially useful to differentiate low-grade lesions from those that are more likely to progress towards invasive cancer. Differently from *KRAS* mutations, *TP53* alterations span throughout the entire gene and therefore their detection is more technically demanding. A major limitation to the introduction of *TP53* mutations detection as a diagnostic tool is the presence of gene mutations in heavy-smokers and in individuals with a history of exposure to environmental toxins.

Mutations of *TP53* could be also exploited for treatment of pancreatic cancer through synthetic lethality (SL) approaches. SL arises when the combined loss of function of two or more genes results in cell death, whereas a mutation in one gene does not affect cell survival [28]. Pancreatic cancer that harbors mutations of *TP53* has a deregulation of G1-S checkpoint of the cell cycle that significantly reduces the efficacy of standard DNA-damaging agents. These tumors still retain a physiologic

G2-M checkpoint; therefore the concomitant abrogation of G2 checkpoint control will kill p53-deficient cells through induction of mitotic catastrophe [28]. Attempts have been done in this direction by evaluating the efficacy of drugs targeting protein involved in G2 checkpoint in a panel of p53-wild type and p53-mutated pancreatic cancer xenografts [29]. In this study, the combination of DNA-damaging drug with abrogation of G2 checkpoint leads to an enhanced antitumor effect compared to standard chemotherapy alone [29].

SMAD4 (also known as DPC4) is a tumor suppressor gene that is found inactivated in approximately 55% of pancreatic cancers by either intragenic mutations associated with LOH or homozygous deletions [13, 30]. Mutations of *SMAD4* are a relatively late event in pancreatic tumorigenesis and are associated with the loss of SMAD4-dependent TGF-ß signaling [23, 31]. Interestingly, it has been suggested that genetic alterations of *SMAD4* are associated with poor prognosis of pancreatic cancer patients after surgical resection [32]. This feature together with the fact that immunolabeling for SMAD4 correlates with gene status [33] render inactivation of *SMAD4* a valuable prognostic test for pancreatic cancer.

2.2 Low Frequency Gene Mutations

Activating mutations in oncogenes other than *KRAS* have been reported at low frequency in pancreatic cancer and include alteration of *AKT2* and *MYB* [34–38]. A subset of low-frequency somatic mutations found in sporadic cases are those germline variants that are associated with familial forms of pancreatic cancers [7] and include: (i) mutations of *STK11/LKB1*; (ii) mutations in *BRCA2*; (iii) and alterations in *FANCC* and *FANCG* genes. Germline variants of *STK11/LKB1* are associated with Peutz-Jeghers syndrome, with patients having more than 100-fold increased risk of developing pancreatic cancer [39]. Mutations in the DNA cross-linking repair gene *BRCA2* are associated with a 3.5-10-fold increased risk of developing pancreatic cancer [39]. Other DNA repair genes belonging to the Fanconi anaemia family of genes (*FANCC* and *FANCG*) have been implicated in familial and young age of onset pancreatic cancer [39, 40].

2.3 Massive-Scale Genome Analysis is Shedding Light in Our Comprehension of Cancer Biology

Massive-scale analysis of cancer genomes mainly rely on technical advances that enable to cover the entire spectrum of somatic alterations at different macromolecular level [12, 41].

In 2008 Jones et al. [42] performed the first comprehensive analysis of the genetic complexity of pancreatic cancer studying 24 cases by: (i) mutational

screening of all protein-coding genes (exome), (ii) SNP arrays to evaluate copy number variations and (iii) next-generation sequencing analysis of transcriptomes to assess gene expression of each sample. About 99.6% of the cancer exome was explored identifying a total of 1,562 somatic mutations. The majority were single base substitutions among which missense mutations were mostly represented. Comparing mutational data from this study with those from mutational surveys of other solid tumors, it became evident that the average number of 63 somatic alterations in pancreatic cancer is considerably less than that in breast or colorectal cancer [43] pointing out an intrinsic biological characteristic of normal pancreatic epithelial cells that divide infrequently [44]. Gene deletions or amplifications were less common than base substitutions. Homozygous deletions involved both known tumor suppressor genes (*TP53, SMAD4, and INK4A*) and genes not previously identified. Gene amplifications were the less common form of genetic alterations and when present involved known oncogenes. Gene expression analysis was used to inform mutational and copy number analyses in order to help prioritize altered gene as most likely to be causal genes. A set of 91 candidate genes was identified. This obviously included all the known causal genes such as *KRAS, SMAD4, INK4A,* and *TP53* and genes not previously associated with pancreatic cancer (for example *ARID1A*). Noteworthy, the vast majority of alterations occurring in pancreatic cancer are low-frequency alterations whose causative role in pancreatic carcinogenesis needs downstream functional studies.

In addition to a high genetic complexity of pancreatic cancer this study pointed out a high degree of heterogeneity among different cancer samples that was partially solved categorizing altered genes into cellular pathways and processes through which their protein products act. In this way, it was possible to identify 31 gene sets grouped in 12 partially overlapping core signaling pathway that were each altered in at least 67% of cancers (Fig. 8.2). Within each of these pathways the genes that were altered in any given cancer varied widely, and importantly only one gene of a specific pathway was altered in an individual cancer.

Taken together the results of this study have changed definitely the perspective through which look at this very complex disease. Indeed, the fact that most genes are mutated in only a small fraction of tumors implies the necessity of an in depth analysis of functional gene groups to assess the causative role of altered genes in pancreatic cancer. From a therapeutic point of view, this study paves the way to the concept of personalized cancer medicine due to the varying genetic alterations detected in individual tumors. Finally, another important concept emerges relative to the development of drugs that target functional pathways rather than their individual component as a preferable therapeutic strategy. However, other important issues in the design of efficient treatments should be considered such as the need of predictive markers that help identifying which tumors are strongly dependent on a specific pathway as well as the possible activation of alternative mechanisms of resistance that in turn implies targeting of multiple pathways.

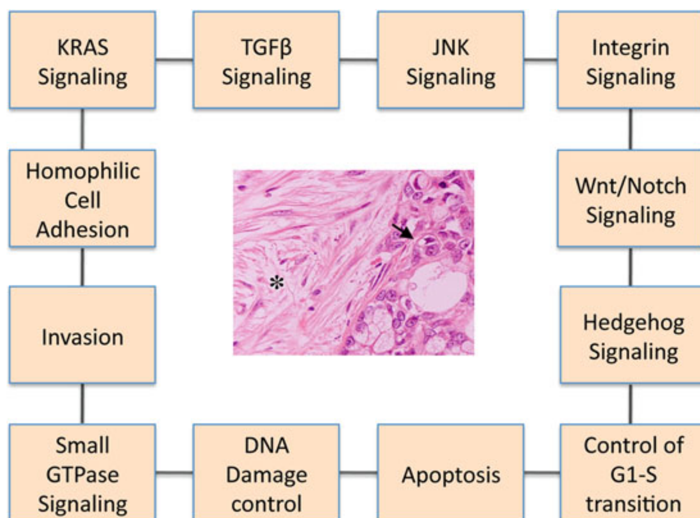

Fig. 8.2 Core signaling pathway and processes in pancreatic cancer. Depicted are the 12 pathways that grouped the genes altered in most pancreatic cancers. Interestingly, pathways are partially overlapping and not all pathways and processes are altered in any given pancreatic cancer. In the *middle*, H&E section of pancreatic cancer tissues: *asterisk* refers to the fibrous stroma in which neoplastic cells (*arrow*) are embedded

2.4 Metastasis Reflect Genetic Heterogeneity of the Primary Cancer

Metastasis represents the major cause of morbidity and mortality in pancreatic cancer, the events that lead to the spread of cancer to vital organ are yet poorly understood. The inherent complexity of genomic alterations in late-stage cancers, coupled with numerous heterotypic interactions that occur between tumor and stromal cells, represent the main challenges in our quest to understand and control metastasis.

Pancreatic tumor heterogeneity and the clonal relationship between metastasis and their primaries has been explored in two different studies by massive-scale sequencing comparison of different cancer samples within the same individual [6, 45].

In their study, Campbell et al. [6] annotated somatically acquired genomic rearrangements of 13 patients with pancreatic cancer showing a high degree of inter-patients heterogeneity concerning the number and type of rearrangements. Intrachromosomal rearrangements were the major structural variations (SVs) identified and a specific pattern of SV, they "called fold-back inversion", was identified consisting in a duplicated genomic region with the two copies head away in opposite orientations from the breakpoint. The authors argued that the rearrangements observed in pancreatic cancer are indicative of telomere dysfunction and abnormal cell-cycle control, specifically a dysregulated G1-S transition and an intact G2-M

checkpoint. Indeed, fold-back inversion could be the consequence of breakage-fusion-bridge cycles that are often initiated by telomere loss [46–48]. Somatic rearrangements were also genotyped across multiple lesions from pancreatic cancer patients to unravel clonal relationship among metastases. Interestingly fold-back inversions occur frequently in all metastases from any given patient strongly suggesting that are an early event during cancer development.

Although the majority of SVs occur before metastatic dissemination (rearrangements present in all metastases), ongoing clonal evolution in primary tumors was hypothesized based on the presence of metastatic samples lacking specific SVs shared by primary tumor and other metastases from same individuals. The authors suggest as possible explanation the existence of different subclones of the primary that seeded metastases independently, thus pointing out at the clonal nature of metastasis itself. Also evidence of clonal evolution within metastases was found, as SVs private to a specific metastatic sample were present. The analysis of rearrangements in different metastatic deposits (for example abdominal vs lung) also indicated that metastasis from a given organ system are more closely related to each other than metastases from different organ. Although suggesting a considerable heterogeneity among metastatic-initiating cells, findings from these study also showed that the majority of somatic structural variations occur at early stage and persist throughout tumor development. These features render structural variations ideal target for the development of personalized tumor biomarkers. Efforts in this direction have been done recently by using somatic translocations annotated by massively sequencing of primary samples to monitor residual and recurrent disease in blood sample from the same individuals [49]. This approach, termed PARE (Personalized Analysis of Rearranged Ends), was shown to be a highly sensitive clinical tool for colorectal and breast cancer, but it is obviously applicable in other tumor types including pancreatic cancer.

Another group evaluated the clonal relationship among neoplastic lesions from same individuals at genetic level using comparative lesion sequencing approaches [45]. This approach consists in analyzing the genetic alterations identified in one cancer samples in additional geographically or temporally distinct samples from that same patient [50]. The genetic landscape of single metastatic lesion from seven patients reported in Jones et al. [42] was used as a reference and compared with the mutational status of primary pancreatic cancers and matched metastases. The comparison of mutational spectra among cancer samples from same individuals allowed to dichotomize mutations into two classes; (i) founder mutations, which correspond to the largest category (mean of 64% per patient) of mutations present in all samples from a given patient; and (ii) progressor mutations (mean of 36% per patient), which correspond to mutations present in one or more of the metastatic samples, but not the primary sample analyzed. According to this two class of mutations, lesions can be also classified into parental clones and subclones (containing progressor mutations beyond founder mutations). Interestingly, parental clones contained the majority of deleterious genetic alterations and chromosomal instability including alterations affecting known driver genes of pancreatic cancer (*KRAS, INK4A, TP53, SMAD4/DPC4*). Evolutionary maps constructed for each

patient showed that, despite the large number of founder mutations in the parental clones, numerous progressor mutations accumulate in association with clonal evolution and metastasis. Analysis of both founder and progressor mutations in multiple geographically distinct regions of primary cancer from each patient was used to address if subclonal evolution (indicated by progressor mutations) occurred within primaries. Such approach led to the identification within primary tumor of many subclones that contained both founder mutations and one or more progressor mutations. Subclones arising within primaries are able to seed distant metastasis since their genetic signature was highly similar to that of specific metastases in the same patient. Although the possibility that metastasis itself seed metastases cannot be excluded, the findings of these studies strongly support the hypothesis that metastatic subclones are pre-existent within primary cancers.

2.5 The Time Frame for the Evolution of Preinvasive Lesions into Metastatic Disease is Long

The comparative lesion sequencing data was also used to perform a temporal analysis of clonal evolution of pancreatic cancer. A mathematical model was generated to integrate the categorization of founder and progressor mutations of each sample with other relevant parameters including: published cellular proliferation rates of normal and neoplastic pancreatic cells [44]; rates of passenger mutation per cell division [51]; data from pancreatic genome analysis [42]. Based upon these data, the timeframe from tumor initiation to the development of a parental clone of cancer cells was estimated to occur in an average of 11.7 years. Interestingly, almost 7 additional years would be required for a single clone to develop metastatic ability and only 2 years for metastatic subclones to further progress, leading patients to death. This proposed evolutionary timeline implies extraordinary clinical relevance, as it leaves a huge window of opportunity, estimated in approximately 21 years, to diagnose pancreatic cancer in a time frame suitable for curative treatment. Despite this, we have to take into account that patients with very small or clinically undetectable primary tumors still have a high risk for developing metastases. This implies that additional efforts are needed to definitively unravel the mechanisms and temporal pattern of metastatic process.

2.6 Metastatic Clones May Appear Early During Cancer Development

The prevailing dogma in pancreatic cancer biology is that the preneoplastic lesion does not possess invading capability though sharing molecular features of the invasive carcinoma. Very recently, Rhim et al. demonstrated in GEMM that cells from low-grade PanINs are able to breach basement membrane and reach the

circulatory system [52]. These cells had the appearance of mesenchymal cells and express markers commonly associated with EMT. The presence of such cells was also independently confirmed in some samples of human PanIN. Independently, another group predicted through a mathematical modeling approach of radiological and pathological data obtained from autoptic sampling of pancreatic cancer patients that even small primary tumors may produce microscopic metastases [53]. Overall, the findings from these two studies strongly suggest that clinical trials comparing systemic chemotherapy to upfront surgery should be considered to significantly improve patients' outcome.

Part 2. Key Points

- Alterations of *KRAS, INK4A, TP53 and SMAD4* are the most common genetic anomalies of pancreatic cancer
- Driver genes (e.g., *KRAS*) might be useful for clinical management of pancreatic cancer patients with several limitations
- Genome-wide comprehensive studies indicate a high degree of genetic heterogeneity of pancreatic cancers
- Metastasis, the most deadly feature of pancreatic cancer, shows a complex genetic signature
- The majority of deleterious alterations occur early during pancreatic tumorigenesis and before metastatic spread
- Epithelial to mesenchymal transition might have a decisive role in metastatic dissemination
- Novel insights into temporal pattern of metastatic spread are expected to deeply impact the clinical management of this lethal disease

3 Chromosomal Instability and Epigenetic Drivers in Pancreatic Cancer

The complex genomic landscape of PDAC is characterized by copy number changes (deletions and amplifications), structural variations (e.g., inversions and translocations), and point mutations [42, 46, 54–56] resulting from chromosomal instability. Complex methylation pattern of the genome are also at work in this disease.

3.1 Chromosomal Alterations in Pancreatic Cancer

Various techniques for whole-genome analysis have led to the identification of many regions of genomic gain and loss, harboring genes involved in PDAC pathogenesis and progression. An overview of the most common alterations found

Table 8.1 Regions of genomic gains and losses identified in PDAC using chromosomal CGH

Study	Sample type(s)[a]	Regions of high frequency gain	Regions of high frequency loss[b]
Solinas-Toldo et al. (1996)	27 primary tumors	16p, 20q, 22q, 17q	9p
Mahlamaki et al. (1997)	24 primary tumors	20q, 8q, 11q, 12p, 17q	18q, 9p, 15q
Fukushige et al. (1997)	12 cell lines, 6 primary tumors	20q, 8q, 20p, 7p, 7q, 11q, 5p, 14q, 18p	18q, 9p, Y, 6q, 3p, 21q, 4q, 8p
Curtis et al. (1998)	12 cell lines	19q13.1, 20q, 5p, 7p, 11q, 3q25-qter, 8q24, 10q	9p, 12, 18q, 8p, 4, 10p
Ghadimi et al. (1999)	9 cell lines	3q, 5p, 7p, 8q, 12p, 20q	8p, 9p, 17p, 18q, 19p
Schleger et al. (2000)	33 primary tumors	8q, 2q (high copy number amplifications at 5p, 8q22-ter, 12p12-cen, 19q12-13.2, 20q)	18q, 10q, 8p, 1q
Shiraishi et al. (2001)	27 primary tumors	1q, 8q, 5q14-q23, 7p, 7q, 12p, 20q	8p, 17p, 19p, 3p, 6q, 9p, 18q
Harada et al. (2002)	32 primary tumors	13q, 15q, 20q	NA
Mahlamaki et al. (2002)	31 cell lines	8q, 11q, 17q, 20q	9p, 17p, 4q, 6p
Lin et al. (2003)	27 primary tumors	8q, 7q, 3q, 1q	9p, 17p, 4q, 6p
Kitoh et al. [57]	15 primary tumors (17 total, 15 PDAC and 2 acinar cell)	17p, 9p, 18q, 19p, 8p	5p, 8q, 20q, 1q, 7p, 12p

Adapted from Samuel et al. [58]
[a]*PDAC* Pancreatic ductal adenocarcinoma
[b]*NA* Not applicable

in PDAC during the last two decades are provided in Tables 8.1 and 8.2. Recurrent amplifications and deletions were implicated as players in tumor development. Indeed, these alterations may contribute to the dysregulation of expression levels of oncogenes and tumor suppressor genes in cancer cells, the accumulation of which is correlated with tumor progression [2, 63].

Several studies combined different strategies for the genome-wide copy number analysis of PDAC including array-based comparative genomic hybridization (CGH) and various biological validation approach with knockdown genes and immunohistochemical assays [55, 57, 59, 61, 62, 64]. These works have demonstrated that focusing on targets of structural changes may allow the identification of genes involved in carcinogenesis and might be candidate biomarkers or therapeutic targets. In particular, *SMURF1* has been successfully identified as having the greatest potential as a 7q21.3-22.1 amplification target. It has been demonstrated to work as a growth-promoting gene in PDAC through overexpression and might be a good candidate as a therapeutic target [64]. Moreover, the frequent copy number gain and overexpression of the development-related transcription factor GATA-6 was recently associated to pancreatic carcinogenesis as it plays a predominant role in the initial specification of the pancreas and in pancreatic cell type differentiation [55].

Table 8.2 Regions of genomic gains and losses identified in PDAC using CGH (BAC/PAC, cDNA) or array CGH (SNP arrays)

Study	Sample type(s)[a]	Regions of high frequency gain	Regions of high frequency loss[b]	Additional information
CGH (BAC/PAC, cDNA)				
Heidenblad et al. [59]	16 cell lines, 15 low passage cell lines	8q, 12p, 7q, 18q, 19q, 6p, 8p	9: p24, p21, q32 10: p12, q22 12q 24, 18q23	BAC, 3,200 clones; cDNA, 25,648 clones
Aguirre et al. [60]	13 primary tumors, 24 cell lines	1: p13.1, p12 5: p15.33-15.31, q31.1 6: p22.1-21.32, p21.1 7: p22.2-22.1, p15.1-14.3, q11.21-21.11, q21.11-32.2 8: p12-11.21, q12.1-12.3, q21.3-24.3 9: p21.3-13.2 11: q14.1-14.2 12: p12.3-13.3, q15 13: q12.11-14.13, q34 14: q11.2, q24.3, 17: q12-23.2, q23.2-25.3 18: p11.21-q12.1 19: p13.11-q13.32, q13.32-13.43 20: p13-q13.33 22: q11.1-12.1, q12.2	1: p36.21-36.11, 35.3-34.3, p21.2-21 2: p25.3-24.3, p12-11.2 3: p24.3 4: q31.22-32.1, q34.1-35.2 5: q23.2-23.3 6: q21-22.31, q23.3-q24.3, q27 8: p23.3-12 9: p24.3-21.2 11: q14.2-14.3 12: q12-13.12, q13.12-33.3, q14.1-15, q21.2-24.33 16:p13.3-12.2 17: p13.3-q11.1 18: q11.2-21.1, q22.1-23 19: q13.2-13.4313.43 21: p11.2-q11.2, 21q22.2-22q22.3 22: q11.1-13.2	cDNA, 14,160 clones
Holzmann et al. [61]	6 primary tumors, 13 cell lines	7p12.3; 8q24, 11q13; 20q13 (most frequent) 1: p22 2: q22-23, q35-36	NA	cDNA, 498 selected clones; 812 selected clones

		3:q27		
		5: p15.2		
		7: p12.3, q21.3		
		8: q24.12-13		
		10: q22.2, q 25.1-26.1		
		11: q12-13, q23-24		
		12: p1, q13-15		
		13: q14		
		14: q32.32		
		18:p11.22, q11.2		
		20: q13.1		
		X: q22.2b-3a		
Mahlamaki et al. (2004)	13 cell lines	3p, 5p, 7q, 8q24, 11q13, 15q, 17q, 19q, 20q	NA	cDNA, 12,232 clones
		19: p1.2, q13.1, q13.3		
Bashyam et al. (2005)	22 cell lines	6p21, 7q21	6q25	cDNA, 39,632 clones
		11: q13, q22	8:p23, p22	
		12: p12, p11	9: p21, q33	
		14q24, 17q12, 19q13[a]	10: q22, a24	
			11: p15	
			16q23	
			18: q21, q23	
			21q22, Xp11[b]	
Gysin et al. (2005)	25 cell lines	5: p14.3-15.1, q33-34	3:p21-14	BAC, 2,464 clones
		6: p21.1	4: q32-35	
		8: p23.1-22, q24.1-24.2	6: p24, p26	
		10: p14	8: pp23.2-22	
		11: q13, q22, q23.1-24	9:p21, q21, q32	
		12: p12.1-11.2	10: q22.1	
		17: q21.3	11: p15.1-14	

(continued)

Table 8.2 (continued)

Study	Sample type(s)[a]	Regions of high frequency gain	Regions of high frequency loss[b]	Additional information
		19: q31.1, q13.2, q13.1-13.4 20q11.1-qtel	13: q21, q32 16: q23 18: q21.1 21: q22.1-22.2 X: p22.3, p27	
Nowak et al. (2005)	9 cell lines, 7 low-passage cell lines, 17 xenografts	7: p21.1-11.2, q31.32, q33 8: q11.1-24 11: p13 14: q22.2 20: p12.2, q11.23-13.3	1: p36.33-34.3, p13-13.2 3:p26, p25.2-22.3, p22.1-14.1 4: q28.3, q31, q35.1 5: q14.3 6: p, q 8: p23.2-12 9: p, q22.32-31.1 13: q33.2 15: q11.2 16: p13.3 17: p 18: q11.21-23 19: p13.3-13.12, q13.2 21: p, q 22: p, q	BAC, 5,200 clones
Loukopoulos et al. [62]	44 primary tumors	1: q25 2: p16, q21-37 3: q25 5: p14, q11-13 7: q21, p22 8: p22, q21-23 10: q21	1p36, 4p16, 7q36, 9q34 11: p15, q13 14: q32-33 16: p13 17: p11-13, q11-25 18: q21-tel 19: p13	BAC, 800 duplicated target clones

		12: p13 13: q22 15: q12-22 18: q11 20: q11	21: q22 22: q11-12	
Harada et al. (2009)	6 cell lines, 23 primary tumors	1: p21.1 5: p14.1 8: q22.1-22.2, q22.3, q22.3-24.11, q24.12-24.13, q24.13-24.21, q24.21, q24.21-24.22, q24.22 19: q12-13.11, q13.12[a]	8: p23.3-23.2, p22 9: p24.3-24.2, p23, p21.3 10: q23.1 12: p13.31 13: q21.1-21.2 18: q21.2 X: p22.33[b]	BAC/PAC, 3,125 clones
CGH (SNP arrays)				
Calhoun et al. (2006)	26 cell lines	NA	2: (141.7), (213.2) 3: (60.3), (160.2) 4: (29.2), (92.2) 5: (92.9) 6: (102.1), (157.0) 8: (15.5), (114.4) 9: (9.0), (22.0), (114.8) 10: (18.4), (73.3) 12: (76.0) 17: (11.9) 18: (46.8), (49.6), (60.5), (74.0)	Affymetrix CentXba and CentHind Oligonucleotide array (115,353 SNP)

(continued)

Table 8.2 (continued)

Study	Sample type(s)[a]	Regions of high frequency gain	Regions of high frequency loss[b]	Additional information
			20: (15.0) 21: (35.5), (41.9) X: (43.0), (141.4)[c]	
Harada et al. (2009)	27 primary tumors	1q, 2, 3, 4, 5, 7p, 8q, 11, 14q, 17q	1p, 3p, 6, 9p, 13q, 14q, 17p, 18q	Affymetrix GeneChip Human Mapping 100 K Set
Jones et al. (2008)[d]	14 low-passage cell lines, 7 xenografs	NA	NA	Illumina Infinium II Whole Genome Genotyping Assay(BeadChip platform) at 1,069,688 (1 M) SNP loci

Adapted from Samuel et al. [58]

Abbreviations: *BAC* Bacterial artificial chromosome, *CGH* Comparative genomic hybridization, *NA* Not applicable, *SNP* Single nucleotide polymorphism

[a]Only high-level amplifications reported

[b]Only homozygous deletions reported

[c]Number in parentheses indicates position (Mb)

[d]This study only cited homozygous deletions and high-level amplifications: frequency not calculated

Conventional techniques such as chromosomal CGH have the intrinsic limit of detecting somatic copy number alterations only at chromosomal level. Array based CGH provides further localization of alterations at the cytoband level and the new sequencing technologies have enabled detection of somatic copy number changes at the base-pair level. Resulting evidence indicates that PDAC bears specific pattern of genomic alterations (deletion, fold-back inversion, tandem duplication), as compared to other types of cancer [6].

Other recent studies based on high throughput sequencing approaches described numerous original regions prone to frequent large genetic alterations during PDAC carcinogenesis. One hundred and forty-four minimal regions identified in 119 independent loci are subjected to such changes and play potential role in tumor progression, with encoding for *p16INK4A*, *TP53*, *MYC*, *KRAS*, and *AKT2* previously described as duplicated or deleted in PDAC [60]. Lucito et al. by using Representational Oligonucleotide Microarray (ROMA) identified 31 amplifications and 25 deletions involving more than 500 genes in familial pancreatic cancer [65]. Birnbaum and colleagues used high-resolution aCGH and candidate gene sequencing to study the genome of 39 pancreatic adenocarcinomas [66]. Frequent genetic gains were detected on chromosome arms 1q, 3q, 5p, 6p, 7q, 8q, 12q, 15q, 18q, 19q, and 20q. Losses were more frequent than gains and were observed on 1p, 3p, 4p, 6, 8p, 9, 10, 11q, 15q, 17, 18, 19p, 20p, 21, and 22 as already reported [54, 67]. The target genes included known or suspected tumor suppressor genes such as *CDKN2A/B*, *FHIT*, *PTEN*, *RB1*, *RUNX1-3*, *SMAD4*, *STK11/LKB1*, and *TP53* and also new genes like *NRG3* or *MACROD2* recently related to pancreatic cancer but not yet well characterized [6].

3.2 Epigenetic Alterations in Pancreatic Cancer Genome

Cancer is a complex disease characterized by multiple genetic and epigenetic genomic alterations [68–70]. DNA methylation is one of the most important epigenetic alterations and plays a critical functional role in development, differentiation and diseases [70]. Through the activity of DNA methyltransferases (DNMTs), DNA methylation occurs at the cytosine residue in the context of 5′-CG-3′ (CpG dinucleotide) across human genome [71]. During the developmental process, DNA methylation plays an essential role in X chromosome inactivation in female somatic cells and in the mono-allelic silencing of parentally imprinted genes [72]. Once these DNA methylation patterns are acquired in the early embryo stage, these patterns are inherited and maintained in successive cell generations. Promoter regions are usually enriched with CpG dinucleotides, known as CpG islands; and hypermethylation of these islands correlates with transcriptional silencing of tumor suppressor genes (Fig. 8.3) [72]. Conversely, increased expressions of oncogenes were associated with hypomethylation [73]. This hypomethylation is known to contribute to cancer cell phenotypes through loss of imprinting (LOI) and genomic instability that characterizes tumors [73]. Furthermore, tumorigenesis of several

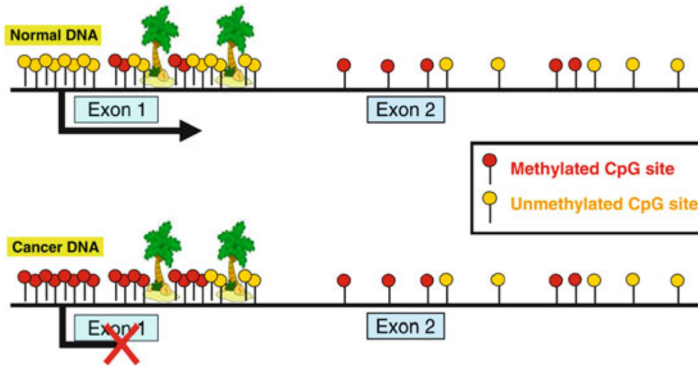

Fig. 8.3 Schematic representation of DNA methylation occurring in the regulatory region of a gene in normal and cancer cells. *Palm trees* refers to CpG island spanning gene promoter and first exon. In a normal cell CpG islands are usually unmethylated and the transcription process can start. In the cancer cell methylation at specific sites along CpG Island leads to gene inactivation or downregulation, despite a general status of hypomethylation throughout the genome

cancers was also marked by specific methylation changes in their genomes [74]. Therefore, it is useful to construct a global methylation profile to discover candidate genes and to predict therapeutic outcomes [75] and patient survival in cancer [76].

Aberrant DNA methylation contributes to pancreatic cancer development and progression [77–79] and the detection of aberrant DNA methylation is being evaluated as a strategy to improve the diagnosis of pancreatic cancer [80]. While the causes of aberrant CpG island methylation during the cancer development are not well understood, the identification of differentially methylated CpG islands in cancer relative to normal tissues may lead to the development of cancer-specific markers of cancer and may also identify important pathways that merit therapeutic targeting [81]. As the extent of DNA methylation alterations become more apparent, robust high-throughput technologies are being evaluated that can efficiently interrogate genome-wide DNA methylation profiles. The introduction of genome-wide screening techniques has accelerated the discovery of a growing list of genes with abnormal methylation patterns in pancreatic cancer, and some of these epigenetic events play a role in the neoplastic process [82].

Several genome-wide strategies have been developed to interrogate methylated CpG islands [83–85] and to identify genes whose expression is under epigenetic control [78, 86]. Newer array platforms provide an opportunity to more efficiently explore promoters for evidence of differential methylation. In an effort to identify biomarkers for PDAC, many groups have used high-throughput molecular profiling technologies, including oligonucleotide and cDNA arrays and Serial Analysis of Gene Expression (SAGE), to analyzed gene expression data [87–92]. Many candidate genes have been identified through these studies; however, the mechanism for the regulation of these genes is not fully understood. Among the substantial number of hypermethylated genes identified in pancreatic cancer, several may be

functionally involved in tumor growth, invasion, metastasis and chemoresistance. Using the candidate gene approach, Sato et al. identified seven overexpressed genes (*CLDN4*, *LCN2*, *MSLN*, *PSCA*, *S100A4*, *SFN* and *TFF2*) in pancreatic cancer when compared to normal pancreatic duct were due to hypomethylation [77]. In particular, the authors describe that Mesothelin and Claudin 4 genes are frequently hypomethylated in PDAC (92 and 89%, respectively) and their methylation status correlates with their expression pattern. On the other hand, hypermethylation occurs during cancer, and may lead to gene silencing. Hypermethylation is responsible for the inactivation of numerous tumor suppressor genes and can occur independently or additionally to intragenic mutation [93, 94].

INK4A is equally subjected to mutation or hypermethylation, whereas *RASSF1A*, *Cyclin D2*, *SOCS-1*, *APC* are tumor suppressor genes silenced only following hypermethylation of their promoting sequence in PDAC [95–97]. By comparing the whole methylome of PDAC with healthy tissue using high throughput technologies, Omura et al. determined that *MDF1*, *miR-9-1*, *ZNF415*, *CNTNAP2* and *EVOLV-4* were the most frequently methylated loci among 88,000 probes tested. Interestingly, only miR-9-1 and CNTNAP2 have previously been linked to cancer progression or initiation [94].

Tan and colleagues [98] have recently employed a global methylation profiling platform to comprehensively survey a large panel of CpG sites across 800 genes in pancreatic cancer genome. They compared the DNA methylation profiles of the pancreatic tumors and normal tissues in order to unravel methylation markers for diagnostic purposes. Methylation markers were correlated with global gene expression profiles to identify candidate genes that were transcriptional regulated by methylation. By correlating methylation profiles with drug responses, they have identified candidate methylation markers (*GSTM1* and *ONECUT2*) that alter the expression of genes critical to gemcitabine susceptibility in pancreatic cancer. These results are in line with previous studies suggesting that aberrant DNA methylation can affect the sensitivity of cancer to chemotherapeutics by altering expression of genes critical to drug response [75, 99–101]. More recently, other authors attempted to identify CpG island methylation alterations between pancreatic cancers and normal pancreata and their associated gene expression changes [102]. They analyzed the methylation profile of 27,800 CpG islands covering 21 Mb of the human genome in nine pairs of pancreatic cancer versus normal pancreatic epithelial tissues and in three matched pairs of pancreatic cancer versus lymphoid tissues from the same individual using an Agilent 244 K CpG island microarrays platform. More than 1,500 known loci commonly differentially methylated in pancreatic cancer compared with normal pancreas were identified and the DNA methylation status was then integrated with gene expression profiles of the same samples before and after treatment with the DNA methyltransferase inhibitor 5-aza-2’-deoxyxytidine and the histone deacetylase inhibitor, trichostatin A. This work recognized a large group of aberrantly methylated and differentially expressed genes in pancreatic cancers and provided a more comprehensive list of hypermethylated and silenced genes that have not been previously described as targets for aberrant methylation in cancer. Noteworthy, the enrichment of aberrant methylation of the WNT pathway gene

members supports a significant role for alterations of this pathway during pancreatic tumorigenesis. Indeed, the pancreatic genome project supported the evidence for aberrant WNT pathway signaling during pancreatic tumorigenesis [42].

The development of early detection strategies, using molecular markers, should lead to an improved clinical outcome for PDAC. In this regard, epigenetic changes (aberrant DNA hypermethylation) hold promise as novel screening/diagnostic markers of PDAC, especially for high-risk individuals such as those with a strong family history of PDAC [10, 103]. The detection and quantification of DNA methylation alterations in pancreatic juice is likely a promising tool for the diagnosis of PDAC. This diagnostic potential has been evaluated in clinical samples from patients with different pancreatic diseases. Fukushima et al. first by a methylation-specific PCR (MSP) assay detected aberrant methylation of *ppENK* and *INK4A* in (30/45) 67% and (11/45) 5% of pancreatic juice samples, respectively, collected during surgery from patients with pancreatic cancer; differently, in 20 pancreatic juice samples from patients with benign pancreatic disorders methylation was absent [104]. Other authors using a panel of three genes (*NPTX2*, *SARP2* and *CLDN5*) identified by microarray approach as very frequently methylated in pancreatic cancer, found aberrantly methylated DNA in 75% of pancreatic juice samples from patients with pancreatic cancer, but not in samples from benign counterparts [93]. However, quantifying pancreatic juice methylation using real-time quantitative methylation-specific PCR (qMSP) could better predict pancreatic cancer than detecting methylation using conventional MSP [80, 105].

Another recent work proved that measuring methylation patterns specific for PDAC in blood samples is possible with a sensitivity of 81.7%, and can be the first step towards a sensitive, specific and non-invasive diagnosis tool [106].

Altogether these increasing evidences indicate that aberrant methylation in PDAC could represent novel diagnostic and therapeutic targets for this disease. However, many fundamental questions about the biological and clinical significance of DNA methylation have yet to be addressed, such as how and when such epigenetic defects occur during pancreatic ductal carcinogenesis, and how our knowledge of epigenetic features in PDAC should be translated into the clinical setting. Additional studies are needed to identify the best set of methylation markers for early detection and/or risk assessment, to determine the detection technologies best suited for each application (as well as their cost performance) in the clinical setup, and to establish the sensitivity and specificity of these selected markers in larger studies.

Part 3. Key Points

- PDAC bears specific pattern of genomic alterations (deletion, fold-back inversion, tandem duplication)
- The majority of structural variations occur early and persist throughout pancreatic cancer development
- Somatic genomic aberrations represent ideal marker for disease monitoring
- DNA methylation is the most relevant epigenetic process in pancreatic cancer

- Specific DNA methylation pattern can affect the sensitivity of pancreatic cancer to chemotherapeutics
- Detection of DNA methylation in pancreatic juice and blood from cancer patients potentially represents a sensitive, specific and non-invasive diagnostic tool

4 Transcriptomics

4.1 Expression Profiling of Pancreatic Cancer

Gene expression (transcriptome) and miRNA profiling are challenging techniques because, rather than assessing the presence/absence of a genetic lesion, they focus on quantifying the relative abundance of a given nucleic acid (messenger or microRNA). This feature may be reversible and subjected to modulation by factors other than the neoplastic process. This implies a different approach during the data analysis, which is aimed at identifying differentially expressed RNAs between groups of samples displaying different biological features. Dealing with relative abundance, two further constraints must be considered:

1. changes at the RNA level may be masked by successive events (i.e. modulation of the translation or sequestering of the RNA);
2. the choice of the reference sample (cell type or tissue) is crucial for the analysis and often is not straightforward.

The first constraint is generally addressed by validating significant findings at the protein level, usually by means of antibody-based methods for protein detection (i.e. immunoblot, immunohistochemistry, flow cytometry).

The second constraint is particularly tricky to solve for pancreatic cancer, due to the fact that neoplastic cells of this cancer type display a ductal phenotype, while normal pancreas is mostly composed of acinar cells. Despite this, recent works have shown that pancreatic cancer could arise also from acinar cells [107–110]; therefore, it is not trivial to consider the differences between normal acinar cells and pancreatic cancer cells.

Moreover, cancer cells in pancreatic adenocarcinomas are surrounded by a thick layer of desmoplastic reaction that is missing in healthy pancreatic tissue. To solve this last issue, several approaches have been used, including the use of chronic pancreatitis samples (having a comparable desmoplastic reaction) as a reference [90], laser capture microdissection of normal and neoplastic pancreas [111–114], fine needle aspiration of tumor cells [115, 116], or the use of normal and pancreatic cancer cell lines [89].

The desmoplastic reaction itself has recently attracted interest, since data from animal and cellular models have shown that it is an essential component in pancreatic cancer biology [117] and that neoplastic and stromal cells modulate each other's gene expression by molecular crosstalk [118, 119]. In this scenario, it is

easy to understand that we have just begun the trip toward making sense of gene expression modulation in pancreatic cancer. In the next subsections, we describe milestones achieved to date with implications on pancreatic cancer biology, diagnosis, prognosis, and possible therapeutic approaches. The future integration of –omics data by bioinformatic approaches and systems biology tools is also briefly discussed.

4.2 mRNA Profiling Studies

4.2.1 Heterogeneity of Samples and Methods

The list of gene expression profiling studies focused to pancreatic cancer to date is quite long; most of the published works relied on cDNA microarrays hybridization, followed by hierarchical clustering and differential expression analysis. Due to the different starting material used for the comparison (whole or microdissected tissue, cell lines) and to the analysis platform (array type, number of probes, statistical approaches and softwares), the overlap between the lists of differentially regulated transcripts (usually amounting to hundreds of genes from each work) is fairly small. Table 8.3 lists genes that have been found differentially regulated in eight comparable profiling studies [87–90, 113, 121–123]; only genes reported in more than two studies have been included. Some of these genes were already known as involved in cancer, have been detected by traditional methods in independent studies and even proposed as markers in pancreatic cancer (e.g. CSPG2 [124], PLAUR [125, 126], CEACAM6 [127]).

Another reason for the reduced overlap between gene profiling studies is the low statistical power deriving from the small number of cases analyzed compared to the large amount (thousands) of genes that can be screened with microarrays. This is one of the main reasons that render the validation of results from cDNA microarrays mandatory. Usually, a reduced number of highly and consistently disregulated genes are validated in each work to support the consistence of cDNA microarray data analysis. Table 8.4 shows genes that have been described/validated in more than two studies (adapted from [128]). Gene annotation and molecular pathway analysis are used to seek a deeper biological insight out of these long lists of candidate markers, together with literature search aimed at identifying genes that have already been reported as disregulated in other tumor types.

4.2.2 Searching for Significant Pathways for Subclassification with Therapeutic

The use of gene annotation and molecular pathway analysis has been useful to detect concordant changes in the expression of genes involved and influencing one another in definite molecular pathways (Fig. 8.4).

Indeed, gene expression profiles have shown modulation of the S100 calcium-binding protein family, of players involved in EGFR/KRAS/MAPK [129, 130] or

Table 8.3 Genes shown to be differentially expressed in PDAC

Gene ID	Expression (Cancer vs normal)	Concordant studies (total = 8)
S100P	Overexpressed	5
TSSC3	Overexpressed	4
LAMC2	Overexpressed	4
ANXA1	Overexpressed	4
ITGB4	Overexpressed	4
OSF-2	Overexpressed	3
DAF	Overexpressed	3
SLC2A1	Overexpressed	3
COL1A2	Overexpressed	3
PLAUR	Overexpressed	3
KRT19	Overexpressed	3
SFN	Overexpressed	3
LCN2	Overexpressed	3
CEACAM5	Overexpressed	3
FER1L3	Overexpressed	3
KRT17	Overexpressed	3
IFI27	Overexpressed	3
FN1	Overexpressed	3
FXYD3	Overexpressed	3
S100A11	Overexpressed	3
CEACAM6	Overexpressed	3
LUM	Overexpressed	3
CSPG2	Overexpressed	3
TRIM29	Overexpressed	3
NAP1L1	Overexpressed	3
KLK1	Down-modulated	3

NOTE: data derived from eight comparable profiling studies. Only genes reported in more than two studies have been included
Adapted from Grutzmann et al. [120])

Table 8.4 Overexpressed genes in PDAC validated in more than one publication

Gene identified by SAGE and/or microarray study	Confirmatory test for gene overexpression		Number of independent studies
	Nucleic acid based	Protein based	
14-3-3 sigma, stratifin (SFN)	Northern Blot, RT-PCR	IHC	4
Claudin 4 (CLDN4)	RT-PCR	–	2
Lipocalin 2 (LCN2)	RT-PCR	IHC	2
Mesothelin (MSLN)	In situ hybridization, RT-PCR	IHC	2
Prostate stem cell antigen (PSCA)	RT-PCR	–	2
S100A4	RT-PCR	–	3
S100A6	–	–	2
S100P	RT-PCR	–	5

Adapted from Rodriguez et al. [128]

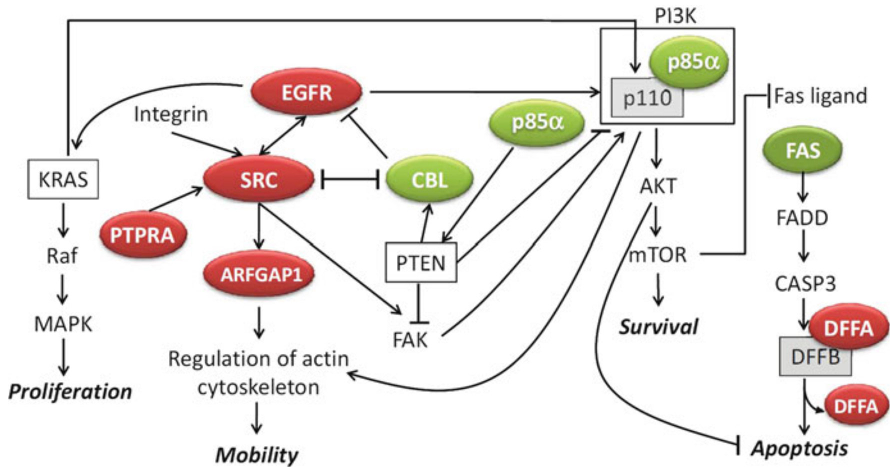

Fig. 8.4 Example of pathway analysis from a gene expression profiling of pancreatic cancer. Up- (*red*) and down-modulated (*green*) genes belonging to the EGFR/KRAS and PI3K/mTOR pathway are *highlighted* (Adapted from Donahue et al. [129])

PI3K/mTOR pathways [129, 131]. In particular, the role of EGFR and KRAS in response to gemcitabine and erlotinib respectively has been recently re-evaluated in light of the gene expression profiles by Collisson et al. [130]. More specifically, starting with data from 66 microdissected pancreatic cancer specimens, they could classify tumors into three groups by a gene signature of 62 differentially regulated genes. The same signature classified 11 cell lines into two groups: "classical" or "quasi-mesenchymal". Interestingly, classical cell lines were more sensitive to erlotinib while quasi-mesenchymal cells were more sensitive to gemcitabine, suggesting that, despite the almost ubiquitous KRAS mutation, EGFR may be more or less influent depending on the tumor molecular subtype and the whole pathway modulation should be considered, especially at its relevant "checkpoints". This idea was also supported by a previous work by Jimeno et al.: by profiling xenografted pancreatic cancers treated with the EGFR inhibitor erlotinib, they proposed a molecular signature for sensitivity to EGFR inhibitor involving core components of the EGFR pathway.

As for the integrin signaling, PI3K/mTOR pathway has been deeply investigated in experimental models of several tumors and several inhibitors are available or under testing at present [132]. A recent survival-based gene expression profiling of pancreatic cancers has indicated expression of the PI3K regulatory subunit p85a and of CBL (indirectly activated by p85a and inhibitor of EGFR and SRC) as associated to better prognosis.

An approach to gene expression profiling data that has been rarely used but could give added value to this research is the meta analysis of already published and publicly available datasets. Using this approach, Grutzmann et al. [131] showed the possibility to combine multiple datasets to obtain an improved list of

disregulated genes; they also showed the possibility to validate gene signatures produced by a given work (and claimed to be diagnostic valuable) on cDNA data from another work in the meta analysis set. More specifically, they showed that the gene signature proposed by Friess et al. (110 genes) [123] could correctly discern chronic pancreatitis from pancreatic cancer in the dataset from Logsdon et al. [90]; moreover, the gene signature of Logsdon et al. (74 genes) performed equally well on Friess et al. samples, despite the two signatures only shared 20 genes.

A different approach to cDNA microarrays validation for diagnostics was proposed by Buchholz et al. [116] starting from a gene signature of 558 transcripts derived from previous studies, a "diagnostic" array was designed and used to assay samples from cytological examination or from resected pancreatic cancer. This array separated pancreatic cancer from benign samples with 95% accuracy, again showing the possibility to use gene expression signatures for diagnostics [116].

Finally, the diagnostic performance of a transcriptomic gene signature has been investigated in ductal cells obtained from pancreatic juice of patients with pancreatic cancer and non neoplastic disease undergoing ERCP (endoscopic retrograde cholangiopancreatography). Starting from a signature of 21 genes, Ishikawa et al., restricted the set to a minimum of five genes (H2BFB, RASAL2, PLOD2, adlican, epiplakin1) that allowed 82% accuracy in differentiating pancreatic cancer from non-neoplastic pancreas [133]. This study was partly confirmed by a previous study applying RT-qPCR on pancreatic juice ductal cells from patients with pancreatic cancer [134]. Indeed, genes highly expressed in this study (SUMO1, AC133, CEACAM7) were consistent with the above cited microarray data, though not being part of the final signature.

4.3 miRNA Profiling Studies

Being part of the trascriptome, micro RNAs (miRNAs) have recently emerged as a group of small non-coding RNA oligonucleotides (17-25mer) involved in gene expression control: synthesized as long precursors, they mature in the cytoplasm following a series of cleavages by dicer/TRBP and AGO2. Finally, as mature single-strand RNAs, they are incorporated in RISC (RNA-induced silencing complex) and direct its post-transcriptional gene silencing based on partial complementarity between the miRNA sequence and the target genes sequences; this implies that, usually, increased expression of one miRNA can down-modulate the expression of several gene products. This has been shown in various models of developmental processes, but their expression has also been reported to be altered in various diseases and in cancer [135]. Real-time PCR and Microarray platforms have allowed the simultaneous screening of a high number of miRNA as it was for cDNA microarrays and several studies have focused on miRNA expression also in pancreatic cancer.

A first study profiling 222 miRNAs in 32 cell lines of pancreatic, lung, breast, colorectal, prostate, head and neck and haematopoietical cancers showed

Table 8.5 miRNA expression patterns in pancreatic cancer cell lines and tissues

MicroRNA	Expression	Independent studies
miR-15b	Up-regulation	2
miR-21	Up-regulation	7
miR-100	Up-regulation	3
miR-107	Up-regulation	4
miR-125b-1	Up-regulation	2
miR-146	Up-regulation	2
miR-155	Up-regulation	4
miR-181a	Up-regulation	2
miR-196a	Up-regulation	2
miR-221	Up-regulation	5
miR-222	Up-regulation	4
miR-223	Up-regulation	4
miR-375	Down-regulation	3

Adapted from Rachagani et al. [138]

consistence between the miRNA pattern of most malignant cells and their respective tissues of origin, suggesting the possibility to use the miRNA profile to assess disease primitivity when it is uncertain [136].

A second, larger study involved profiling of 540 samples from solid tumors (pancreas, breast, colon, lung, prostate and stomach) and comparison with normal tissues; the study showed a large variability in miRNA expression between different tumors and between tumors and normal tissues. However, some miRNAs (miR-21, miR-191, miR-17-5-p) were overexpressed in all tumor types and miR-218-2 was consistently down-regulated in pancreas, colon, prostate and stomach [137]. A number of studies on pancreatic cancer cell lines and tissues have further dissected the contribution of miRNAs to pancreatic cancer and consistent findings have been summarized in Table 8.5. In particular, miR-155, miR-21, miR221 and miR-222 have been often reported as overexpressed in pancreatic cancer and, notably, also in pancreatic intraductal papillary mucinous neoplasms. Interestingly, miR-155 appear to down-regulate p53-induced nuclear protein 1, while miR-21 targets PTEN, PDC4, propomyosin 1 and TIMP3, inhibiting apoptosis.

Given its tight relation with gene expression, miRNA and transcriptome profiling are expected to be somewhat co-ordinately disregulated in neoplastic disease. An early trial to integrate data from these two molecular profiling platforms is reported in the next subsection.

4.4 Upcoming: Integrative Studies

Many of the gene expression and miRNA profiling studies to date have tried to propose a panel of markers with diagnostic or prognostic predictivity; despite having encouraging performance on their own data sets, or even on other data

sets when cross-validated by meta analysis, these panels display few overlaps across studies when it comes to the identity of the single genes/miRNA. One of the possibilities to increase the confidence in the identification of valuable candidate markers is to integrate different molecular profiling technologies by using them to analyze the same samples simultaneously. This approach has been recently used to stratify 25 pancreatic cancer patients in two survival groups [129]. Nucleic acids of these patients were profiled by DNA copy number, gene expression and miRNA expression analysis; each of the three platforms' results were used to define molecular signatures predictive of patients survival (68 DNA copy number aberrations, 500 cDNA and 31 miRNA respectively). Finally, the analysis was repeated upon integrating data in a composite score that considered concordance (on a gene-by-gene) between expression data from the three platform. The integrated approach reduced the minimum dimension of the survival-related gene signature to 171 transcribed genes with concordantly disregulated DNA copy number and/or miRNA expression. The vast majority of these transcribed genes (134) had concordant miRNA expression (i.e. increased miRNA expression for down-modulated transcripts and *vice versa*), 20 showed concordant DNA copy number variation alone and 17 had concordance of data from all the three platforms. Molecular pathway analysis showed modulation of many members of PI3K/mTOR and EGFR/KRAS pathway; in particular the concomitant expression of p85α and absence of p-SRC and the expression of CBL where validated as predictors of better prognosis. It should be noted that SRC was included among survival-linked genes only after the integrated analysis, indicating that pooling molecular profiles from different platforms may provide a better snapshot of gene expression. This is a prototype of studies that we shall see in the next years.

4.5 Gene Expression, Data Bases and Systems Biology

As above mentioned, most gene expression studies on pancreatic cancer rely on a small number of cases, given the difficulties in obtaining enough good starting material from microdissected primary adenocarcinomas. As a consequence, one of the efforts that have been done is the collection of gene expression data into publicly available databases. Probably the most comprehensive collection is hosted by the Pancreatic expression database (www.pancreasexpression.org): born under the 6th frame European project Mol-Diag-Paca, it has been recently updated. It contains over 60,000 measurements on pancreatic cancer specimens, not only from gene expression profiling but also from proteomics and genomics assays. Its interface allows combining expression data with resources from NCBI, Ensembl, Uniprot, and Reactome. Other well known and used gene expression databases are GEO (http://www.ncbi.nlm.nih.gov/geo/), ArrayExpress (http://www.ebi.ac.uk/arrayexpress/query/entry), Oncomine (www.oncomine.org) and SMD (http://genome-www5.stanford.edu/MicroArray/SMD/). Besides the integration of multiple data sets from different annotation databases, new promises

also come from the field of systems biology. By combining multiple data from each gene's expression with information regarding the interaction of gene products into different pathways, systems biology tools (e.g. cytoscape, www.cytoscape.org) allow to build a network of interactions between molecular pathways, where important checkpoints can be visualized and analyzed. This could further empower the approach of molecular pathway analysis, allowing to group pancreatic cancer samples with apparently different profiles into subsets of tumors with a similar resulting pathway unbalance.

Part 4. Key Points

Intrinsic characteristics of pancreatic cancer render expression profiling studies particularly challenging

Gene expression signatures are promising tools for diagnosis of pancreatic cancer

Gene signatures might help in discriminating patients that are likely to respond to specific therapies

miRNA profiles can be useful to assess disease primitivity when uncertain

Transcriptomic profiling is technically feasible in diverse biological samples, thus representing a useful non-invasive diagnostic tool

Integrative analyses of expression profiles identified candidate therapeutic pathway in pancreatic cancer such as EGFR and PI3K/mTOR

5 Conclusions and Perspectives

The genomic landscape of pancreatic cancer revealed that beyond known and well characterized alterations, a plethora of different molecular features defined a very complex and heterogeneous disease. When ignored, molecular heterogeneity can lead to failures in therapeutic treatments, as drugs that may have efficacy in subgroups of patients with specific molecular phenotypes may show marginal response when tested in a large groups of unselected patients.

Massive scale sequencing provides now the unprecedented opportunity to dramatically improve diagnosis and treatment of pancreatic cancer patients by covering the marked genetic heterogeneity of this disease.

Examples of tumor genomic profiles used to guide clinical decisions are already available. Although far from being introduced into a routine clinical setting, findings from comprehensive genome-wide studies indicate a new perspective through which look at the clinical management of PDAC towards a personalized cancer medicine.

The most relevant clinical issue in pancreatic cancer is the metastatic spread, as metastases represent the principal cause of pancreatic cancer-related deaths. Recent works have highlighted a high degree of genetic complexity and heterogeneity of metastatic deposits. At the same time, intriguing insights into temporal pattern of tumor clonal evolution have significantly improved our understanding of the metastatic process. Despite far from identifying all the pre-requisite events that trig-

ger metastatic dissemination, these findings have important clinical implication both from a diagnostic and therapeutic point of view. Indeed, they defined a suitable time-window to diagnose pancreatic cancer before metastatic spread occurs. Moreover, there is the suggestion of an inherent disseminating capability of pancreatic cancer cells that could re-prioritize therapeutic interventions that are commonly scheduled for pancreatic cancer patients.

Beyond important clinical implications, the genetic complexity of pancreatic cancer have also pointed out the need to re-design experimental approaches to the study of cancer biology. Indeed, the biological behavior of such a complex disease cannot be predicted by its individual components without taking into account the interactions occurring between the different molecular levels. In line with this, the International cancer genome consortium (www.icgc.org) has recently launched an initiative that foresees the creation of a catalogue of molecular alterations of individual cancers by the systematic analysis of all the molecular compartment of cancer cells (genome, transcriptome, and methylome) in the larger set of well-characterized cancer tissues [139].

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Chapter 9
Breast Cancer Genomics: From Portraits to Landscapes

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Abstract Breast cancer is the most frequent female cancer and still one of the major causes of death although early diagnosis and improved therapies have had a great impact on survival after breast cancer diagnosis. However, there are still many unresolved problems in breast cancer such as the fraction of breast cancers that do not respond to current therapies and considerable overtreatment due to imperfect prognostication. The application of genomics to breast cancer has led to the identification of clinically relevant molecular subtypes, especially the distinction of luminal A and luminal B subtypes within the class of hormone receptor positive cancers. Many prognostic signatures have been developed and two of them are being applied in oncologic decision making yet their utility most likely does not go beyond the distinction of luminal A and B subtypes that show a highly different proliferative potential. Integration of copy number variation has identified even more subclasses with distinct clinical characteristics. Genome wide association studies have identified many single nucleotide polymorphisms that are associated with breast cancer risk and several of them resist in validation studies. Their application for the design of risk based preventive strategies has been proposed. Next generation sequencing shows a wide variation of driver mutations in breast cancer, most of them within interrelated signaling pathways. Several genes such as TP53 or PIK3CA show frequent mutations but many mutations are almost private. Sequencing also identified several actionable mutations, among which those that occur in genes more frequently involved in other cancers that could indicate specific treatments. Better prognostication and response prediction by means of genomic analyses and mutation screening will almost certainly contribute to the improvement of therapy and to the reduction of unnecessary toxicity. Breast cancer genomics has

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also led to a conceptual shift in our understanding of the process of metastasis that seems to be determined from very early stages of the disease although additional mutations occur at later stages.

1 Introduction

Breast cancer is the most frequent cancer in women and a frequent cause of death. Yet breast cancer can be detected relatively early through now widely used preventive screening in women above the age of 50. Early detection translates into the diagnosis of smaller, lower stage and grade cancers that have a better prognosis. Breast cancer therapy has impressively improved mainly through the development of more active chemotherapy, through the introduction of anti-estrogen therapies, tamoxifen or aromatase inhibitors, for hormone receptor positive cancers and through the development of humanized monoclonal antibodies and kinase inhibitors for the treatment of cancer with amplifications of the HER2 gene, a member of the epidermal growth factor receptor family also called ERBB2. Breast cancers that do neither express hormone (estrogen and progesterone) receptors nor HER2 are treated with chemotherapy only and many of these triple negative cancers have a poor prognosis with limited response rates to chemotherapy. Many new drugs that are being tested in the clinics will hopefully further increase survival after diagnosis of breast cancer but the treatment of triple negative cancers, early relapse under anti-estrogen therapy, resistance to anti-HER2 therapies, brain metastasis and latent micro-metastasis that eventually grow for still unknown reasons remain clinically relevant problems. In addition, it is assumed that many early stage breast cancers are cured by surgery alone or by surgery plus hormone therapy. Chemotherapy could be avoided for these patients but the difficulty to reliably identify low risk cancers still leads to considerable overtreatment with drugs that can also provoke irreversible cardiotoxicity.

High incidence, good response to targeted drugs, the presence of pathological subtypes (HR+, HER2+, TN), relatively long yet heterogeneous survival and considerable overtreatment determine a special attention that genomics research has dedicated to breast cancer from the very beginning. Unsupervised molecular classifiers, prognostic and predictive signatures have been developed and attempts to integrate molecular data of various nature such as gene expression, structural genome alterations including translocation, copy number alterations, insertions, deletions, and inversions, DNA methylation, microRNA expression and gene mutations have been undertaken applying microarray and, more recently, next generation sequencing technologies. Breast cancer genomics has therefore become the avant-garde of cancer genomics and nearly all conceptual and technical aspects of cancer genomics have been addressed first in breast cancer.

Given the huge number of papers in breast cancer genomics (21,305 entries in Pubmed) it is impossible to list up all these studies and we apologize for not being able to review each study. Many excellent reviews are available for the field [1–6]

and we limit our effort here to providing an overview over the most important contributions that influence the conceptual framework of breast cancer biology in our present understanding.

2 Molecular Subtypes

The assessment of the tumor subtype is crucial for the determination of treatment since anti-estrogen treatments are effective only in HR+ cancers and anti-HER2 therapies rely on the presence of the amplification of the ERBB2 gene. In routine analyses this is today obtained by highly standardized and reliable immunohistochemistry procedures for ER, PGR and HER2. Ambiguous results of HER2 immunohistochemistry (2+ staining in a scale of 0 to 3+) are further analyzed using fluorescence in situ hybridization (FISH) to directly detect gene amplifications. The treatment decision is mainly based on this classification inasmuch as HR+ cancers receive anti-hormone treatments, HER2+ cancers receive anti-HER2 treatments while no targeted treatment exist as yet for triple negative cancer. The decision of whether to add chemotherapy or not, especially for HR+ cancers, is taken on the base of the clinical and pathological parameters. All HER2+ cancers and most TN cancers are treated with chemotherapy.

The first application of genomics to breast cancer was an approach to molecular classification using unsupervised hierarchical clustering. Hierarchical clustering is a mathematical approach to group samples together based on the similarity of the pattern of gene expression. Perou and co-workers applied this approach in two early studies [7, 8] showing that gene expression reproducibly identifies molecular subtypes that are coincident with the pathological types that are, however, further subdivided. HR+ cancers can be divided in luminal A, B and C subtypes and TN cancers show clusters corresponding to a "normal like" and to "basal like" expression phenotypes. HER2+ cancer show no further subdivision (see Fig. 9.1). This analysis showed a clear distinction of HR+ and HR− cancers reviving the discussion of whether these cancers derive from different cells and are to be considered different cancers. In fact, Gruvberger and colleagues showed that HR+ and HR− cancers yield remarkably different gene expression patterns and can easily be distinguished by hierarchical clustering or neural networks even when the top discriminators are removed. Moreover, only very few of the genes that discriminate the two types are actually estrogen responsive in cellular models [9]. This would indicate that the two types constitute different diseases rather than steps in the evolution of the cancer.

Molecular subtypes, also referred to as intrinsic subtypes, are clinically relevant inasmuch as subtypes within the pathological subtypes, such as luminal A and B cancers, show different disease free survival [10]. This has further been corroborated by several studies [11–14] and a 50 gene classifier (PAM50) has been developed on the base of the genes that can identify molecular subtypes [15]. Later on, a classifier that identifies the most frequent subtypes (ER+/HER2−/Low Proliferative, ER+/HER2−/High Proliferative, HER2+ and ER−/HER2−) using only three

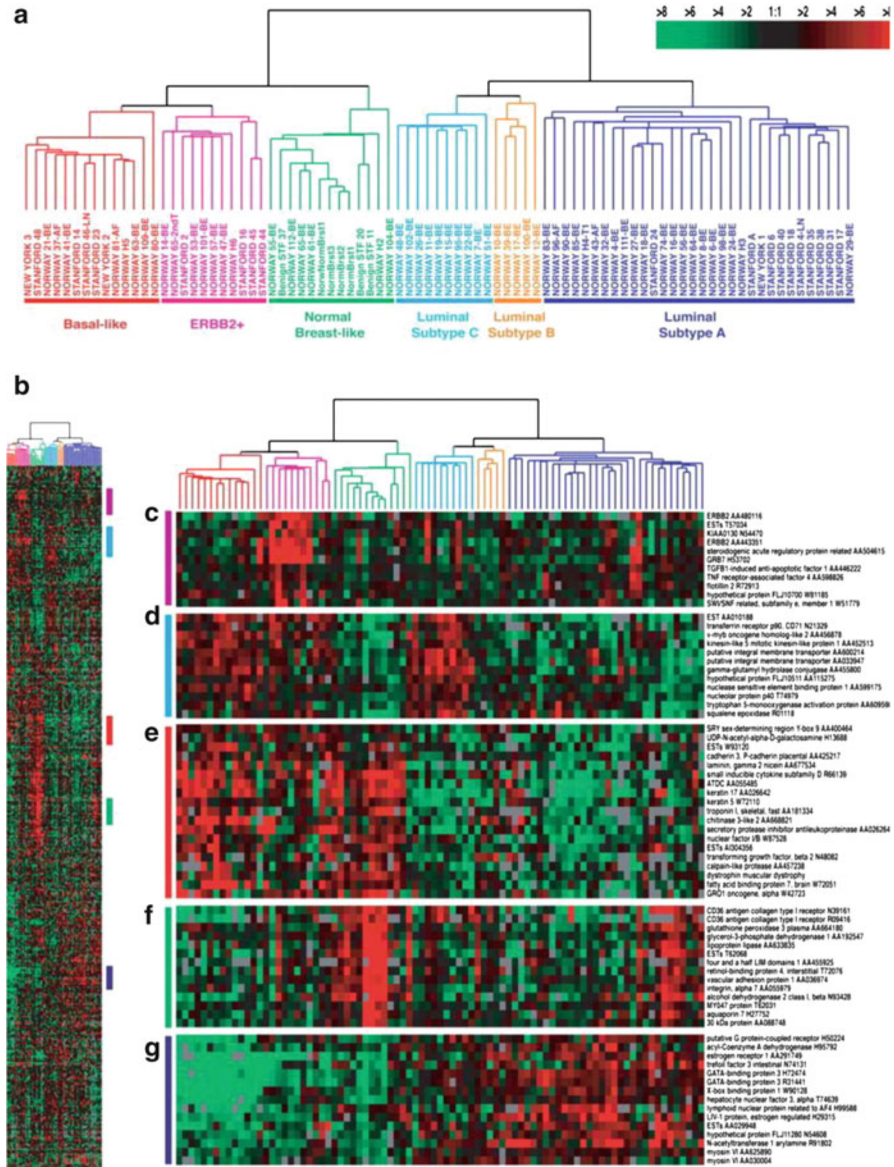

Fig. 9.1 Gene expression patterns of 85 experimental samples representing 78 carcinomas, three benign tumors, and four normal tissues, analyzed by hierarchical clustering using the 476 cDNA intrinsic clone set. (**a**) The tumor specimens were divided into five (or six) subtypes based on differences in gene expression. The cluster dendrogram showing the five (six) subtypes of tumors are colored as: luminal subtype A, *dark blue*; luminal subtype B, *yellow*; luminal subtype C, *light blue*; normal breast-like, *green*; basal-like, *red*; and ERBB2+, *pink*. (**b**) The full cluster diagram scaled down. The *colored bars* on the *right* represent the inserts presented in **c**–**g**. (**c**) ERBB2 amplicon cluster. (**d**) Novel unknown cluster. (**e**) Basal epithelial cell-enriched cluster. (**f**) Normal breast-like cluster. (**g**) Luminal epithelial gene cluster containing ER (From Sorlie et al. [8] with permission)

genes (estrogen receptor, ERBB2 and aurora kinase A) has been developed [16]. The three gene assay is, however, inferior to the PAM50 assay in predicting the response to chemotherapy [17]. With the integration of the proliferation marker KI-67 into the clinical decision making [18] it is unclear whether the three gene assay can add any information, since AURKA and KI-67 both identify highly proliferative cancers. The distinction of molecular subtypes within HR+ cancers, i.e. luminal A and B subtypes, has been questioned since two meta-analyses show that luminal cancers constitute a continuum rather than two distinct classes [19]. Medullary breast cancers have a distinct gene expression profile and constitute a subgroup of basal-like (TN) breast cancers [20]. Claudin-low tumors have been proposed as an additional subgroup of TN cancers [21].

3 Breast Cancer Progression

Breast cancer initiates as the premalignant stage of atypical ductal hyperplasia (ADH), progresses through the preinvasive stage of ductal carcinoma *in situ* (DCIS) to the potentially lethal stage of invasive ductal carcinoma (IDC) (for review, see [22]). It was expected that tumor progression is associated with distinct gene expression profiles that distinguish the various progression steps. Ma and colleagues reported on gene expression analyses of breast cancer specimen derived from patients with foci of different progression stages using laser capture microdissection coupled to microarray gene expression analysis [23]. Surprisingly they found that the expression profiles of the distinct pathological stages were more similar to each other than tumors of the same stages in different patients. The strong individual variability dominated over weaker variations in gene expression during tumor progression. Significant global alterations in gene expression mainly occur at ADH, the earliest phenotypically recognized stage of progression, while the later stages of DCIS and IDC show a stable gene expression phenotype [23]. Even if more detailed analyses of the gene expression changes during breast cancer progression have allowed for the identification of differentially expressed genes [24] the variation of gene expression is relatively limited once transformation has occurred. Authors who tried to identify mediators of metastasis by comparing primary breast cancers with metastases from the same patients came to opposing conclusions. Weigelt et al. showed a striking similarity of primary tumors and their metastases [25, 26]. Other groups, though confirming the general similarity of primary tumors and matched metastases, identified many genes that are differentially expressed [27–29] several of which concordantly in at least two of the studies [27]. However, these studies, despite some mechanistic evidence from in vitro analyses, cannot rule out whether the gene expression events observed are the cause or the consequence of the acquisition of a metastatic phenotype. It therefore remains unclear whether the metastatic potential of the tumor is predetermined by the transforming event or acquired during tumor progression. MDA-MB-231 breast cancer cells that show lung [30, 31], bone [32, 33] or brain [34] specific metastases could be enriched

through selection in mouse xenografts indicating that metastable gene expression changes can confer a site specific metastatic potential. Yet this only shows that certain gene expression events are required or at least facilitate the selection of a metastasis target tissue but cannot be taken as a proof of acquisition of the metastatic phenotype during tumor progression. It is beyond any doubt that acquired genetic determinants influence metastasis [35] yet it remains unclear how independent they are from the molecular events leading to malignant transformation. The possibility to predict the outcome of breast cancers by analyzing the bulk of the primary tumor [36], where molecular events present in a tiny subclone that eventually metastasizes would go undetected, makes a point in favor of predetermination of the metastasis risk through the initial transformation event and/or type of tumor originating cell.

Structural genomic alterations during breast cancer progression have been analyzed on single disseminated cancer cells obtained from bone marrow of breast cancer patients. Single-cell comparative genomic hybridization showed acquisition of additional genetic changes in micrometastases as compared to the microdissected cells of the primary tumor. Micrometastases from patients who were cured by surgery showed less genomic alterations than primary tumors of patients with manifest metastases and micrometastases present at the time of surgery in the latter patients showed less alterations than the matching manifest metastases [37] compatible with the acquisition of the true metastatic phenotype after dissemination from the primary site or with predetermination by the driving mutation. In any case, invasive tumors are likely to disseminate cells from very early stages of carcinogenesis on. Most of the steps necessary for metastasis can therefore occur long before the tumor is detected [38]. For a further discussion and models of tumor progression to metastasis see [39].

4 Tumor Heterogeneity

Tumors, and breast cancers make no exception, show evident morphological heterogeneity and it is widely assumed, that single, morphologically distinct subclones of tumors show different growth patterns and invasion potential. This heterogeneity corresponds to molecular differences as for instance gene expression profiles. Barry and co-workers have analyzed a series of breast cancers from which they took several fine needle biopsies followed by microarray gene expression analysis. Samples from different areas of the single tumor clearly revealed heterogeneous gene expression patterns. yet when the expression profiles were used for classification according to the hormone receptor status or for the application of prognostic or predictive signatures, the different samples of the same tumor yielded highly concordant classifications despite the detectable molecular heterogeneity [40]. This is consistent with the relative stability of the expression phenotype through tumor progression and with an early determination of the metastatic potential discussed above. However, when single mutations are addressed rather than gene expression

profiles, tumor heterogeneity can influence the classification as it has been shown for the HER2 status [41]. A similar study on pretreatment biopsies confirmed the reproducibility of molecular classification despite variable composition of the tumor sample yet also showed that response predictors work better if more homogenous samples are used [42].

5 Prognostic Signatures

5.1 Whole Genome Based Signatures

Early genomic studies on breast cancer showed the presence of subtypes with different risk of metastasis. This prompted the research into the development of molecular signatures to predict outcome. Signatures are sets of genes whose expression values are associated with the parameter under study, in general disease free or overall survival or, for predictive signatures, response to therapy. Hence, classification is supervised using datasets of samples with known outcome and selecting genes that discriminate between cases of different risk. In most signatures, the contribution of the single genes to classification is weighted. The actual expression value multiplied by the weight yields a score for each gene and the addition of the scores of all genes yields a cumulative score for each sample analyzed. New cases can be classified by simply considering the distance of the score of the sample from the centroid of the cumulative score of the two classes, low and high risk, in the reference set. In 2007 Dupuy and Simon published a critical analysis of many molecular signatures showing that most of these studies had considerable flaws in their design [43]. In order to obtain validated signatures that can be developed for the application in the clinics, the authors recommend to use separate datasets for the development of the signature (training set) and for the validation of the signature (validation set). The scores for each gene are calculated for the training set and must be applied to the validation set as they are.

A great number of molecular signatures for breast cancer have been designed given the high (also commercial) interest in the issue. An additional aspect may be that it is relatively easy to obtain gene expression signatures discriminating low and high risk cases that are validated on independent datasets. This is mainly due to the strongly different risks for HR+ versus HR− and grade 1 versus grade 3 cancers. Other cancers such as prostate cancer or lung cancer do not show easily distinguishable risk classes. The discussion is therefore not so much whether the signatures work or do not work as whether they work better than classical, histopathological prognostication or not. Among the many breast cancer signatures reported we will discuss here only very few in order to highlight the potential and the pitfalls of prognostic signatures. For more exhaustive reviews we refer to [3, 5, 44, 45].

In 2002 van't Veer and colleagues reported a 70-gene signature obtained through the analysis of the gene expression profiles of 78 lymph node negative

breast cancer patients with at least 5 years of follow-up using inkjet-synthesized oligonucleotide microarrays [46]. The same group reported on a validation study on 295 samples of consecutive cancers of lymphnode negative and –positive patients from the Netherland's Cancer Institute with a median follow-up of 6.7 years, 61 of which were part of the first study [47]. This study claimed the superiority of the prognostic classification obtained over the prognostication based on clinical and histo-pathological criteria following the guidelines of St. Gallen and NIH. The prognostic power of this test was confirmed in several retrospective studies [48–50]. Interestingly, it is able to identify patients with a favorable long-term outcome even among HER2 amplified cancers [49]. The application of the signature has been reported to yield only small differences in survival but to outcompete St. Gallen guidelines in terms of quality of life due to reduced use of chemotherapy and cost-effectiveness [51]. A prospective validation of the 70-gene signature is ongoing within the MINDACT trial (Microarray In Node-negative and 1–3 node positive Disease may Avoid ChemoTherapy) [52]. Patients with a discordant prognosis, high by classical (Adjuvant! Online) and low by genomic (70-gene) analysis or vice versa are randomized to be treated according to one of the two risk assessments. A preliminary analysis showed the feasibility of the trial where 27% of the patients had discordant risk [53]. The 70-gene classifier has been approved by the FDA. The application of this signature was found to potentially reduce the use of chemotherapy [54]. Where the signature has been applied it has, however, only a reduced impact on the choice of adjuvant treatment [54, 55]. This might be due to the unwillingness of the oncologist to leave patients untreated who are classified as high risk by classical risk assessment, and to the already very restrictive Dutch CBO guidelines [54]. Routine application would certainly require an improved technical success rate since at present for almost a quarter of the samples no risk assessment is possible [54, 55]. The 70-gene signature is commercialized under the name "MammaPrint" by Agendia, Amsterdam, Netherlands.

Wang and co-workers reported on a similar signature in 2005. They developed two separate signatures for HR+ and HR− tumors of 60 and 16 genes, respectively, using Affymetrix microarrays and analyzing 115 (training set) and 171 (validation set) patients [56]. The intended use of this signatures is to avoid unnecessary adjuvant chemotherapy for low risk patients and in the cohort analyzed, the signature was superior to classical prognostication. The ability of the signature to predict benefit from tamoxifen therapy in HR+ cancers was also reported [57].

Sotiriou and colleagues developed the genomic grade index, a procedure based on Affymetrix microarray data to discriminate grade 2 breast cancers into a group similar to grade 1 cancers and another similar to grade 3 cancers based on the assumption that grade 2 does not represent an independent biological entity [58]. The gene expression grade index (GGI) is based on 97 genes that were found to discriminate grade 1 from grade 3 cancers in a cohort of 64 HR+ cancers. When applied to more than 500 samples the GGI was able to correctly predict grade 1 and 3 cancers and grade 2 cancers were divided into two groups similar to grade 1 and 3 cancers. The risk of recurrence followed this assessment. Grade 2 therefore appears to contain tumors of very different risk of relapse that can be distinguished

applying GGI [58]. GGI identifies molecular classes of HR+ cancers that strongly resemble the intrinsic subtypes of luminal A and B [59]. High GGI is associated with increased response to chemotherapy in both HR+ and HR− breast cancer [60]. The test has also been developed for application on formalin fixed paraffin embedded (FFPE) material [61]. Independent retrospective validation confirmed the prognostic power of the method [62, 63] including the prediction of relapse of HR+ cancers under letrozole treatment [64]. A study that compared GGI to the proliferation marker KI-67 and to the mitotic index showed that GGI essentially reflects the proliferation status of the tumors but performed best in assessing it [65]. GGI is commercialized as MapQuant Dx Genomic Grade by Ipsogen SA, Marseille, France. A similar approach has led to the definition of a five gene molecular grade index [66] that shows its prognostic potential if combined with the prognostic markers HOXB13 and IL17BR [67].

Paik and colleagues developed a real time polymerase chain reaction based prognostic classifier based on the analysis of 16 prognostic and 5 houskeeping genes [68]. The test, called “recurrence score” (RS) is intended for quantifying the likelihood of distant recurrence in tamoxifen-treated patients with node-negative, HR+ breast cancers. By analyzing published microarray gene expression studies the authors identified 250 candidate genes that they tested on the samples of three independent clinical trials to identify the 16 prognostic genes. Most of the genes of the classifier test the estrogen responsiveness, proliferation status, HER2+ expression and invasion potential. The recurrence score delivers a prognostic classification into “low”, “intermediate” and “high” risk of distant recurrence with 6.8, 14.3 and 30.5% actual recurrence rates in the National Surgical Adjuvant Breast and Bowel Project clinical trial B-14 that was used for validation [68]. RS also predicts locoregional recurrence [69]. Although RS has been designed for the prognostication of lymph node negative cancers it also revealed that LN+ patients with low RS score might not benefit from chemotherapy [70]. Its prognostic potential has been confirmed in the TransATAC study for node-negative and node-positive (LN ≤ 3) postmenopausal patients treated with anastrozole or tamoxifen [71]. Tested on tumors of the National Surgical Adjuvant Breast and Bowel Project (NSABP) B20 trial RS revealed its potential to predict benefit from chemotherapy [72]. RS and Adjuvant! Online have been shown to be independently associated with the risk of recurrence yet the combination of RS with Adjuvant! Online is inferior than its combination with standard clinical and histo-pathological parameters [73]. The application of RS has been reported to determine a change in the treatment decision in 25–44% of the patients [74–78] and greatly increased the oncologist’s confidence in their treatment decision [79]. The application of RS has led to a decrease in use of chemotherapy in HR+ cancers [80]. RS is being validated in the “Trial Assigning IndividuaLized Options for Treatment” (TAILORx; NCT00310180) where also the management of patients with an intermediate RS will be addressed [81]. RS is commercialized by Genomic Health, Redwood City, CA under the name of Oncotype DX and has been incorporated into the guidelines of the National Comprehensive Cancer Network and the American Society of Clinical Oncology.

5.2 Predictive Signatures

Prognostic signatures contain elements of response prediction since the main problem they address is the possibility to treat patients with HR+ cancers with endocrine therapy alone. Hence, the prediction of relapse under tamoxifen or aromatase inhibitors is the main clinically relevant information that prognostic signatures can provide. This aspect has been addressed systematically in two predictive signatures [82, 83]. The comparison of these signatures with more general ones showed comparable predictive potentials with some degree of independence that derives from the fact that various signatures identify different subsets of luminal A cancers. The combination of several signatures worked therefore best [84]. Still, most low risk cancers were node negative cancers and low risk node positive cancers had a worse survival. The application of the signatures to the treatment decision for node positive cancers is therefore highly unlikely and the added value is limited.

Prognostic signatures identify a significant effect of adjuvant chemotherapy in high risk but not in low risk patients [60, 85, 86] (with exceptions [87]) and could build the base for analyses weighting risks and benefits of chemotherapy in low risk patients. Concordantly, the response to chemotherapy in locally advanced breast cancer is associated with low expression of estrogen receptor associated genes and high expression of proliferation and immune genes. The higher the risk of recurrence the higher the benefit from chemotherapy [88]. ABC transporter genes that are generally linked to drug resistance, on the contrary, are not associated with risk yet predict response to chemotherapy [89]. For response prediction in Paclitaxel and Fluorouracil, Doxorubicin, and Cyclophosphamide based therapies a 30 gene classifier has been developed and validated that apparently relies less strongly on known prognostic factors [90]. Predictors of the response to anthracyclin based regimens are challenged by evidence that only HER2+ cancer respond, probably due to the co-amplification of the target, topoisomerase 2a (TOP2A) [91] but HER2-patients with elevated TOP2A also respond [92]. Response to the aromatase inhibitor Letrozole in the neoadjuvant setting has been shown to be associated with the expression of 205 probesets yet this has not been independently validated [93]. Resistance to targeted drugs is most likely linked to compensatory mutations in the targeted gene or in other genes of the same pathway as it has been shown for the resistance to Trastuzumab [94]. Mutational analysis rather than expression profiling appears therefore best suited for the search of resistance genes. Yet a next generation sequencing based analysis of resistance to aromatase inhibitors has not led to the identification of highly prevalent resistance associated mutations [95].

5.3 Functionally Defined Signatures

Signatures developed using all the genes of the human genome or large part of them invariably lead to enrichment for proliferation associated genes. In fact, the speed of

tumor growth is proportional to the frequency of metastases since fast growing and larger tumors disseminate more cells into the bloodstream thereby increasing the probability that a cell will form a colony in another tissue. However, the proliferative status of the tumor is already well assessed by KI67 immunohistochemistry and the preponderance of proliferation associated genes will eventually hinder other important metastasis associated biological processes from emerging. Several groups have therefore set out to test the possibility to develop prognostic signatures starting from lists of genes involved in specific biological processes.

Chang and colleagues analyzed gene expression in response to serum stimulation in fibroblasts derived from various sites of the human body and showed that the expression of these genes is associated with the development of metastases for various cancers [96]. This signature is referred to as "core serum response" or as "wound response signature" (WRS). WRS genes were chosen to minimize overlap with cell cycle genes, but instead appeared to represent other important processes in wound healing, such as matrix remodeling, cell motility, and angiogenesis, processes that are likely to contribute to cancer invasion and metastasis. WRS has been validated using the same dataset on which the 70-gene signature had been validated where it showed a similar, yet independent, discrimination power as both, the 70-gene signature and molecular subtypes [97].

Several authors have developed gene signatures based on inflammation related genes based on the known link between cancer and inflammation [98]. These studies identified inflammation related genes in inflammatory breast cancers that have an unfavorable prognosis and applied these genes to non-inflammatory breast cancers where they were able to discriminate cancers with high and low risk of metastasis [99–101].

The general metastasis signature developed by Ramaswamy et al. contains many genes that are expressed in part or exclusively by stromal components of the tumor, fibroblasts, endothelium and tumor infiltrate [36] and these genes alone can predict outcome [102]. Finak et al. developed a signature using only the stromal part of breast cancers for analysis and showed its prognostic potential [103]. Several groups have shown that genes involved in the immune response can be used to develop prognostic signatures that can improve prognostic assessment for TN cancers where other signatures are less discriminatory [104–106]. The proportion of tumor initiating or stem cells are widely held to determine the aggressiveness of a tumor. The combination of genes specifically expressed in human embryonic stem cells with the invasive gene set, a signature developed analyzing breast cancer cells with stem-like features (CD44+ CD24−) [107], allowed for the development of the consensus stemness ranking with prognostic and predictive potential [108]. CD44(+)/CD24(−)/CD45(−) breast cancer stem cells isolated from HR+ cancers showed a hyperactive phosphoinositol-3-kinade (PI3K) pathway [109].

Epithelial to mesenchymal transition also leaves its trace on expression profiles and negatively correlates with survival [110]. Insulin and the insulin-like growth factor (IGF1) are involved in breast carcinogenesis and the treatment of the breast cancer cell line MCF7 with IGF1 induces gene expression changes that are related to outcome in human breast cancers [111] and the expression status of insulin related

genes in the absence of treatment allows for the definition of a prognostic signature with similar but independent prognostic potential as the recurrence score [112]. Bild and colleagues developed cellular models for oncogenic mutations and showed that the related expression profiles can correctly identify tumors in which specific pathways are deranged and pathway signatures show prognostic and predictive power [113]. The analysis of chemokine gene expression in breast cancer has led to the identification of CXCL12/SDF1 whose expression positively correlates with disease free and overall survival despite the prominent role of the CXCL12/CXCR4 axis in metastases [114].

Despite considerable interest in these functional aspects of breast cancer, none of these signatures has been developed for clinical application. Signatures that are designed starting from subgroups of genes, invariably lack some information relevant for the definition of a correct prognosis. It is therefore expected that such signatures must be combined with each other or with general, proliferation dominated signatures in order to increase the prognostic power.

6 Limits of Prognostic Gene Expression Signatures

Since the publication of the first prognostic signature there is a debate whether molecular classifiers deliver clinically relevant information that justifies their introduction into the routine. Despite the fact that the term breast cancer encompasses at least five subtypes, most of the signatures discussed here could not address subtypes since they are based on relatively small patient cohorts. Pathological subtypes are considered different diseases yet none of the studies had a sufficient number of cases to address the possibility that cancers of a specific subtype might follow different routes to metastasis and therefore have different prognostic signatures. Most breast cancer signatures are dominated by the strong effect of proliferation (see Fig. 9.2). Interestingly, this is not true for all cancers. Proliferation signatures do not reliably discriminate high and low risk lung cancers (our unpublished observation) indicating that breast cancer but not lung cancer might contain subtypes with highly different proliferation potentials, such as luminal A and luminal B breast cancers. The many signatures developed show only a marginal overlap and this is true even for signatures developed on the same microarray platform. This is at least in part due to cohort effects given the relatively low numbers of cases analyzed. Ein-Dor and co-workers were able to generate several 70-gene signatures with a similar discrimination power as the original 70-gene signature using the data on which the latter had been developed just by leaving out single patients [115]. Their interpretation is that many, probably thousands of genes actually discriminate highly proliferative from less proliferative cancers and the subset that is selected during the development of a signature strongly depends on the actual cohort analyzed. In order to obtain a stable, universal signature thousands of patients must be analyzed [115].

Fan et al. showed that four different signatures, intrinsic subtypes, 70-gene profile, wound response, recurrence score, performed similarly on a validation set of

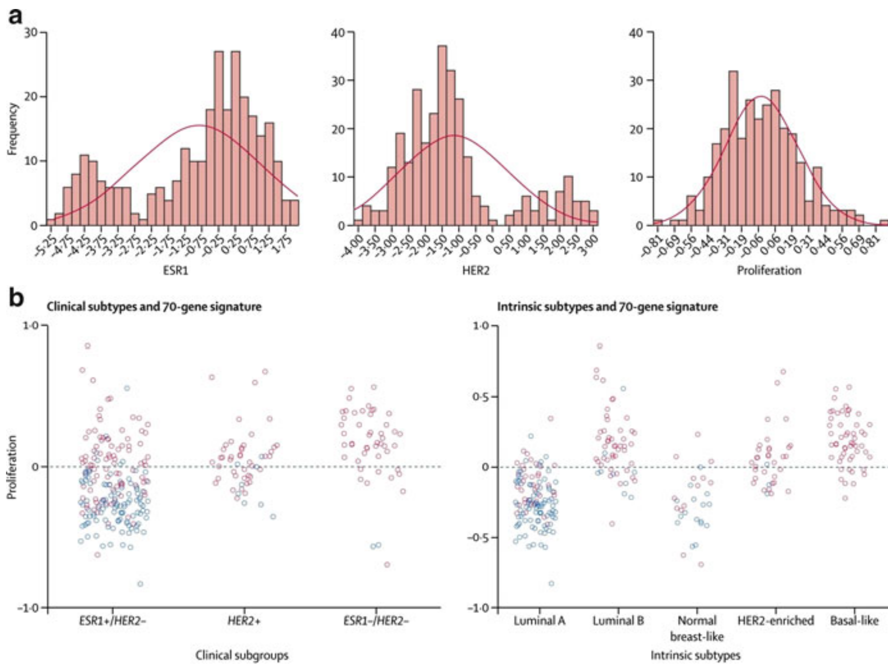

Fig. 9.2 Oestrogen receptor, HER2, and proliferation mRNA expression in breast cancers and the effect of proliferation on the prognostic power of first generation prognostic signatures (**a**) Distribution of ESR1, HER2 (also known as ERBB2), and AURKA mRNA expression. ESR1 and HER2 mRNA concentrations have a bimodal distribution, whereas the proliferation surrogate (AURKA) has a normal distribution; hence cutoff s to define high or low proliferation status cannot be solely defined based on the data distribution. (**b**) Relation between AURKA expression (a surrogate of proliferation), 70-gene prognostic class, and clinical or intrinsic breast cancer subtypes in the NKI-295 dataset. Information about the intrinsic subtypes and about the classification of tumors into good prognosis (*blue circles*) or poor prognosis (*red circles*) groups based on the 70-gene signature was retrieved from supplementary data from Fan and colleagues (From Reis-Filho et al. [3] with permission)

samples yielding 77–81% correct classifications [116]. Yet the signatures were not independent and their combination did not improve classification. Embarrassingly, Venet and coworkers showed that more than 90% of signatures composed of at least 100 randomly selected genes as well as signatures not related to breast cancer, including a signature for postprandial laughter, were significantly associated with breast cancer outcome and 28 of 47 published breast cancer signatures did not perform better than random signatures [117]. They point out that the simple measure whether a signature yields a significant discrimination is not sufficient. The discrimination must be significantly better than that obtained with random signatures. But why are random signatures able to discriminate high and low risk cancers? Breast cancer subtypes have profoundly different expression profiles and different risk of recurrence. Proliferation as the main risk discriminator affects many

genes, so many that the chance that some of them are contained in a random sample of 100 genes is relatively high. Fan et al. calculated a metagene composed of genes that show expression correlation with the proliferating cell nuclear antigen (PCNA), a proliferation marker. Over 50% of the breast cancer transcriptome correlated with this metagene [117].

The main limitation of gene expression signatures for breast cancer is, however, the limited accuracy in predicting outcome. The intended use of prognostic signatures is to withhold chemotherapy from women with low risk cancers and therefore, the cost of misclassification is to be confronted with the cost of overtreatment. This also explains why the application of molecular classifiers can increase the number of patients treated since oncologists change their treatment decision more easily for discordant cases with classical risk low, genomic risk high than for cases with classical risk high, genomic risk low. Yet if the combination of signatures does not improve classification [116] how can this be obtained? We propose that the development of second generation signatures should consider the following aspects:

- Use of large datasets
- Development of subtype specific signatures
- Incorporation of genes that are not related to proliferation
- Combination of functionally defined signatures.

Metastases derive from invasive primary tumors that disseminate tumor cells. Hence, any invasive tumor can give rise to metastases. The molecular profile of the primary tumors is associated with the risk of metastases but even low risk cancers can develop metastases, though with a lower probability. Absolute classification without occasional misclassified samples is therefore impossible. This is also demonstrated by the comparison of samples that are misclassified applying several different signatures. Different signatures built on functional categories including several not related to proliferation tend to misclassify the same tumors. The comparison of tumors that metastasized but are classified as low risk with those correctly classified as high risk yields a gene list that distinguishes low and high risk cases indicating that the misclassified cases have a truly low risk profile [118]. These cases cannot correctly be classified by gene expression profiling and constitute a true limit of prognostic signatures.

7 miRNA Signatures

MicroRNAs (miRNAs) are small non-coding RNA molecules ranging in size from 19 to 24 nucleotides that regulate gene expression in a sequence-specific fashion. Each miRNA can have multiple targets and each mRNA gene can be targeted by multiple miRNAs. MiRNAs can act as oncogenes or tumor suppressors according to their location in genomic regions that are amplified or deleted in cancer, abnormal expression in tumor versus normal tissue is frequently observed for miRNAs that

target tumor associated protein coding genes. MiRNA expression profiles are related to clinical and biological features of tumors [119]. Despite the involvement of many miRNAs in breast cancer carcinogenesis and progression and despite the fact that genomic techniques have contributed to provide the evidence for this involvement (for reviews see [120, 121]) no prognostic or predictive miRNA signatures have been developed for application in the clinics. MiRNA expression profiles can be used to distinguish breast cancer subtypes although luminal and basal cancers are not as clearly separated as they are by mRNA profiles [122]. A similar analysis of mammary tumors in the mouse has confirmed the potential of miRNA profiles to distinguish subtypes [123]. The correlation between miRNA and mRNA based profiles might not be straightforward since the enzymes involved in miRNA generation are also differentially expressed in breast cancer subtypes [122]. MiRNA expression data have also been extracted from RNA-sequencing studies confirming the stability of the expression phenotype during progression from ductal carcinoma in situ to invasive cancer observed by mRNA expression profiling (see above) although some significant miRNA expression changes could be observed [124]. The analysis of miRNA expression and mutations by deep sequencing revealed a rather limited role of miRNAs in breast carcinogenesis and progression although miRNA expression recalls mRNA based subtype classification [125]. Deep sequencing (see also below) has also allowed for the identification of many new miRNAs in breast cancer tissues [126] and miRNA sequencing from circulating DNA has been shown to be prognostic for locally advanced breast cancer [127]. A genetic screen has recently identified the miR-200, miR-15/16 and miR-103/107 families and miR-145, miR-335, and miR-128b that are involved in the growth regulation of cancer stem cells through the control of the expression of several transcription factors [128].

8 Genome Wide Association Studies

Two major breast cancer genes, BRCA1 and 2, that confer a high risk to develop breast cancer at some point in once life have been identified. Mutations in other genes encoding for proteins that like BRCA1 and 2 act in DNA repair and germline mutations in TP53 and PTEN also contribute to familiar breast cancer but account for only a minor part of it. A considerable part of cases with a clear genetic component cannot be explained by these mutations and other high penetrance mutations have not been identified. Several mutations or polymorphisms that only slightly increase the life time risk to develop breast cancer might act in concert in a polygenic model. Large genome wide association studies (GWAS) have the necessary statistical power for the detection of such low penetrance, high incidence variants and several of such variants have been identified [129–132]. Easton et al. reported on a GWAS involving 4,398 breast cancer cases and 4,316 controls followed by validation on existing studies with a total of 21,860 cases and 22,578 controls where they identified five novel independent loci that were associated

with breast cancer, four of which contained putatively causative genes (FGFR2, TNRC9, MAP3K1 and LSP1) [129]. Hunter et al. performed GWAS on 1,145 women with invasive breast cancer and 1,142 controls and identified four SNPs in intron 2 of FGFR2 that were confirmed in the validation phase [130]. Another smaller study where only breast cancer patients and controls with Ashkenazi ancestry were enrolled confirmed the FGFR2 polymorphism [131] as well as a much larger study involving 6,145 cases and 33,016 controls [132]. The latter two studies also identified additional markers. The seven most strongly associated SNPs (rs2981582 – FDFR2; rs3803662 – TNRC9, LOC643714; rs889312 – MAP3K1; rs1317198 – LSP1; rs13281615 – na; rs13387042 – na; rs1053485 – CASP8) confer a relative risk per allele ranging from 1.07 to 1.26 which is to limited to have any clinical relevance. However, the combination of these alleles could identify women with a slightly elevated risk and could be used to define risk based instead of age based screening programs [133]. In accordance to the results of gene expression studies there is now overwhelming evidence that the association of SNPs with breast cancer risk might vary with subtype [134–141]. Loci that modify the BRCA1 and −2 associated risk have also been identified [142–144] (see also references therein).

9 Copy Number Alteration, Structural Genomic Alterations and Integrated Genomics

Amplification and overexpression of several genes (HER2, EGFR, MYC, CCND1, and MDM2) has been shown to have prognostic value [145] and HER2 amplification [146] identifies a breast cancer subtype with particularly bad prognosis if untreated and is a response marker for anti-HER2 antibodies [147] and kinase inhibitors. Early copy number alteration studies using microarrays identified many amplifications and deletions with subtype specific patterns associated with outcome [148–153]. Genetic instability of tumors can result in the accumulation of amplifications, deletions and inversions of smaller or larger pieces of chromosomes as well as in translocations that often lead to expressed fusion genes. Not all structural genomic events lead to a change in gene expression. Deletions are often compensated by increased expression of the remaining allele and expression of amplified genes, especially at low copy numbers, can be down-regulated by the control of transcription levels. It is therefore generally necessary to show an alteration in gene expression in order to postulate a functional effect of structural alterations [120]. The integration of functional and structural genomic data delineates the mechanisms by which the transcriptional program is altered [154, 155] and helps to identify driver genes of breast cancer development and progression [156, 157]. Chin and coworkers obtained array comparative genome hybridization data and matched microarray gene expression profiles for 101 breast cancer and identified many genes with high-level amplification and/or overexpression at the loci 8p11, 11q13, 17q12, and 20q13. 66 of these genes are considered potential targets for drugs (“druggable”) and nine are matched by existing drugs (FGFR1, IKBKB, ERBB2, PROCC, ADAM9, FNTA,

ACACA, PNMT, and NR1D1) [158]. The study also confirmed the association of structural and functional genomic alterations inasmuch as amplification events are associated with expression subtypes, however, subtypes were identified even in the absence of highly amplified genes indicating that the latter are not sufficient to explain the former. Uniparental disomy caused by the loss of one allele and the duplication of the other one frequently occurs in breast cancer, in particular in TN cancers [159]. Russnes et al. performed CNA analysis of 595 breast cancers and developed an algorithm for the classification of cases into two classes with whole-arm gains and losses and complex rearrangements, respectively. The presence or absence of complex rearrangements was prognostic of high risk of recurrence independently from subtype [160].

A recent study integrated copy number alteration, SNP, TP53 mutation and gene expression data for almost 2,000 breast cancers subdivided into training and validation set [161]. 39% of the gene expression events observed were influenced by SNPs, germline (copy number variation, CNV) or somatic (copy number alteration, CNA), approximately half of these events were classified as events in *cis* (within 3,000 kb of the variant) and *trans* (beyond 3,000 kb). The effect on gene expression was in the order of CNA > SNP > CNV. The high resolution assessment of amplified and highly overexpressed genes led to the identification of 45 putative driver events that included known (ZNF703, PTEN, MYC, CCND1, MDM2, ERBB2, CCNE1) and unknown (among which MDM1, MDM4, CDK3, CDK4, CAMK1D, PI4KB, NCOR1) events. The analysis of deletions added new entries (PPP2R2A, MTAP and MAP2K4) to the frequent deletion of PTEN as candidates for driver deletions. The combination of these data allowed for the identification of ten robust molecular subtypes with characteristic patterns of somatic CNAs that were reproduced in the validation set. These subtypes were only partially overlapping with the intrinsic subtypes as analyzed using PAM50 and were associated with disease free survival [161]. This large study also fails to identify subtypes with truly low risk consistent with intrinsic limits of prognostic classification discussed above. Their clinically relevance is therefore limited.

10 Epigenomics of Breast Cancer

Gene expression is also regulated by epigenetic events such as histone modifications [162], polycomb/trithorax protein complexes [163], and DNA methylation [164]. Epigenetic regulation is metastable over many generations of somatic cell divisions and can contribute to the determination of the tumor phenotype. The different epigenetic mechanisms cooperate in the determination of the transcriptional activity of the genome.

Epigenetic analyses in breast cancer start conceptually from the debate whether methylation of the estrogen receptor α gene, ESR1, might determine the HR− negative phenotype, a debate that is linked to the question of whether HR+ and HR− tumors are different stages of tumor development or independent cancer

types [165]. At present, there is no conclusive evidence for an induction of the HR− state through ESR1 methylation [165]. Nonetheless, microarray methylation profiling has been reported to be predictive of the response to hormone therapy [166] and associations of DNA methylation of five genes with the HER2-status have been shown [167]. Kovalchuk et al. reported on DNA methylation, histone modification and miRNA expression in a rat model of estrogen induced breast carcinogenesis that preceded the formation of atypical duct hyperplasia [168]. FOXC1 hypomethylation has been reported for a CD44+ subpopulation of breast cancer cells with a stem-cell like phenotype [169] and this appears early during human breast cancer development [170]. Only four differentially methylated genes were identified through the comparison of inflammatory with non inflammatory breast cancers (TJP3, MOGAT2, NTSR2 and AGT) [171], other more frequent molecular subtypes of breast cancer appear to be distinguishable also at the level of DNA methylation that frequently affects developmental genes [172]. Two clusters with differential methylation profiles and distinct estrogen receptor-, TP53-, ErbB2-status and grade, characterized by the methylation status of HDAC1, TFF1, OGG1, BMP3, FZD9 and HOXA11, were identified [172]. Taken together, differential DNA methylation certainly occurs in breast cancer yet the present evidence does not indicate a prominent role in the determination of subtypes and prognosis.

To the best of our knowledge, no large scale breast cancer histone modification or chromatin immunoprecipitation microarray profiling studies have been published, probably due to the inherent technical features that make these approaches still difficult to apply to high throughput studies. These limits are likely to be overcome by next generation sequencing approaches [173].

11 Next Generation Sequencing

Next generation sequencing (NGS) [174] is a new technology that allows for high throughput analysis of the sequences of single DNA molecules. Instead of obtaining a sequence derived from many identical or almost identical DNA molecules, NGS delivers sequences derived from a single DNA molecule. It is therefore possible to observe mutations even if they occur in only a fraction of the sample analyzed (deep sequencing) as it often occurs for molecularly heterogeneous tumors. NGS also allows for parallel sequencing of many DNA molecules (massive parallel sequencing) to obtain sequence information of the whole genome (whole genome sequencing). Combined with specific capturing methods, NGS can be limited to only the protein coding fraction of the genome (exome sequencing). The termini of larger genomic fragments can be sequenced and aligned to the genome for the identification of inversions, deletions and translocations (paired-end sequencing). Sequencing of cDNA obtained from a biological sample yields the sequences of all transcribed mRNAs (whole transcriptome shotgun sequencing or RNAseq) where the abundance of the single transcript is proportional to the number of sequences obtained. RNAseq therefore delivers information on both, transcript abundance and

mutational status. In addition, NGS can be applied to any other technique that involves sequencing including analysis of chromatin immunoprecipitates (ChIp-seq) and bisulfite sequencing for methylation analyses. These techniques are being applied to breast cancer yielding the first complex insights into the mutational landscape of breast cancer. Paired-end sequencing breast cancers showed a higher number of structural alterations than expected and a high variability in the number of such mutations [175]. Deletions, tandem duplication, inversions, translocations, and rearrangements within amplified regions were observed [175]. Many translocations and duplications give rise to transcribed fusion genes [176–178]. Several fusion genes were observed in many breast cancers [178, 179], in particular fusions involving microtubule-associated serine-threonine kinase (MAST) and members of the Notch family [178]. Redundant fusion genes show subtype specificity [179]. Yet most events are rare or even private and it has been proposed to use these events as molecular markers for the detection of minimal residual disease [180]. Early detection of breast cancer through the detection of breast cancer specific DNA fragments in the blood by sequencing of circulating DNA has also been shown to be feasible [181]. A strategy for diagnostic deep sequencing of BRCA1 and −2 and other potential cancer genes has been developed [182] and might confirm a higher than expected prevalence of BRCA1 and 2 mutations in sporadic cancers [183]. NGS can be performed on formalin fixed paraffin embedded material [184].

Many of the problems already approached using classical and microarray based techniques are now being addressed using NGS. Mutational analyses show, as expected from earlier data, the presence of new mutations in metastatic samples as compared to the primary cancer they derived from Shah et al. [185]. Banerji et al. performed whole-exome sequencing on 103 breast cancers of any subtype and matched normal tissues revealing a total of 4,985 somatic mutations in protein coding portion of genes and their adjacent splice sites. Six genes were identified to be recurrently mutated (CBFB,TP53, PIK3CA, AKT1, GATA3 and MAP3K1) [186]. This study, while confirming the general rearrangement spectrum, identified a recurrent new fusion gene, MAGI3–AKT3 [186]. Shah et al. reported RNAseq of 80 cases and genome/exome sequencing of 65 cases, all TN breast cancers. PIK3CA (10.2%), USH2A (Usher syndrome gene, 9.2%), MYO3A (9.2%), PTEN (7.7%), RB1 (7.7%) were the most frequently mutated genes [187]. Ellis and co-workers sequenced 77 HR + samples from two aromatase inhibitor trials. Mutations in MAP3K1 and its substrate MAP2K4 were associated with luminal A cases. Identification of aromatase resistance markers was not straightforward, excluding a single resistance mutation [95]. Stephens et al. analyzed 100 breast cancer of various subtypes and identified several new driver mutations (AKT2, ARID1B, CASP8, CDKN1B, MAP3K1, MAP3K13, NCOR1, SMARCD1 and TBX3). The study also revealed a particular frequency of cytosine mutations at TpC dinucleotides. Age at diagnosis was associated with the number of mutations encountered [188]. Many mutations were identified in more than one study and general concepts of the mutational landscape of breast cancer emerged:

- The number of mutations in each single tumor is highly variable
- Higher number of mutations are associated with more aggressive disease

- Mutations cluster in genes belonging to specific pathways
- Several genes are mutated with high frequency
- Many mutations are almost private
- Several mutated genes show polymorphisms that were associated with BC risk in GWAS.

Several of the mutations identified such as BRAF, EGFR, ERBB2, ERBB3, and AKT3 could indicate a treatment given the availability of specific drugs ("actionable mutations"). It is therefore possible that NGS will find a rapid application in the clinics, at least for therapy resistant tumors that could be treated with targeted drugs not normally used for breast cancer if the targeted mutation is present. Concordantly these studies identified central pathways, the TP53/RB, MAPK, PI3K/AKT/mTOR pathways, that are affected by mutations concurring in the development of breast cancer (see Fig. 9.3).

12 Outlook

Breast cancer is frequent, often early detected and in many cases successfully treated. Yet there remain (i) a considerable overtreatment of cancers whose risk is inappropriately assessed by existing classifiers, (ii) a considerable number of patients, mainly with triple negative disease, who show low response rates to existing therapies and (iii) primary or acquired resistance to targeted drugs. Genomics can contribute to alleviate the effects of the shortcomings of present diagnostics:

- Improved prognostic procedures could be used to restrict the use of chemotherapy to patients who are likely to benefit from it,
- Patients with poor prognosis who, on the basis of improved predictive assessment, are unlikely to benefit from existing therapies could be included in clinical trials of new therapies without prior treatment [189],
- Many targeted therapies can be used for the treatment of breast cancer if the individual mutational status is known for each single patient,
- Resistant subclones can be detected with high sensitivity.

For this purpose, prognostic and predictive classifiers whether based on gene expression signatures, integrated genomics or next generation sequencing, must certainly be improved. NGS will play a major role in the future, perhaps especially RNAseq since it combines transcript counting with mutation analysis. Clinical parameters will remain important and must be combined with genomic features of the tumor and histopathological parameters will also continue to play a role especially in assessing tumor heterogeneity. Genomics will therefore not simply substitute pathology.

Genomics has delivered huge amounts of data on many aspects of tumor biology yet the mere possession of data does not automatically increase our understanding.

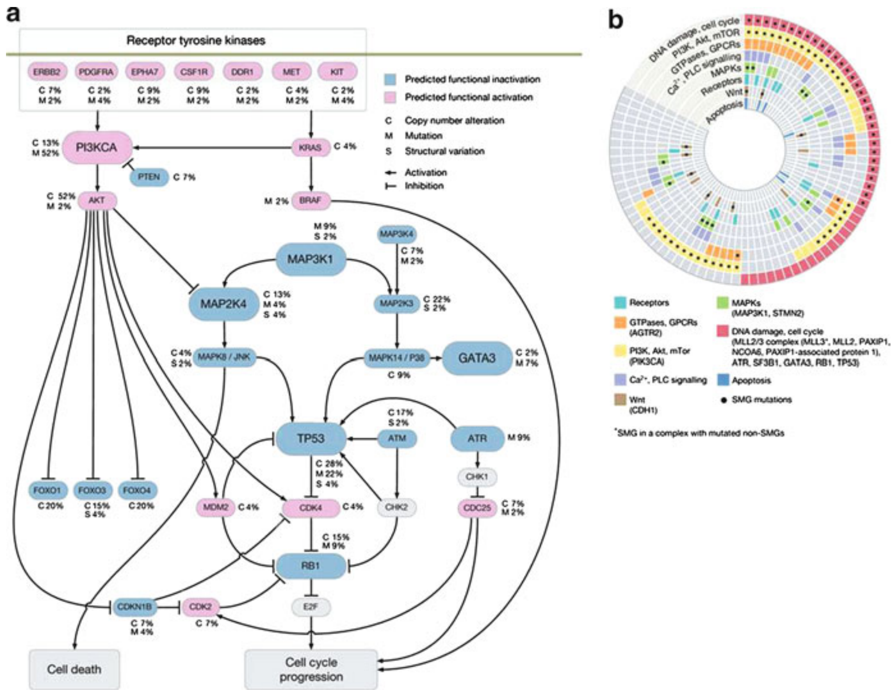

Fig. 9.3 Human breast cancer mutation pathway summary. (**a**) Only genetic alterations identified in 46 whole genome sequencing cases are shown. Alterations were discovered in key genes in the TP53/RB, MAPK, PI3K/AKT/mTOR pathways. Genes colored *blue* and *red* are predicted to be functionally inactivated and activated, respectively, through focused mutations including point mutations and small indels (M), copy number deletions (C), or other structural changes (S) that affect the gene. The inter-connectedness of this network (several pathways) shows that there are many different ways to perturb a pathway. (**b**) Eight interaction networks from canonical maps are significantly over-represented by mutations in 77 luminal breast tumors (46 whole genome sequencing and 31 exome cases). In the *concentric circle* diagram, tumors are arranged as *radial spokes* and categorized by their mutation status in each network (*concentric ring color*) and significantly mutated gene mutation status (*black dots*). Tumor classification by pathway analysis shows many tumors unaffected by a given significantly mutated gene often harbor other mutations in the same network (From Ellis et al. [95] with permission)

Much of the understanding will rely on functional analyses. It appears that after many big international projects on data collection, functional analyses should also be systematically approached by large coordinated projects creating a major advance in high throughput tools for functional studies. Gene function is normally analyzed by a limited number of functional assays and cellular models using overexpression or knockdown constructs and this can be organized to yield truly comparable data in many cell systems under standardized conditions. Data from single gene/single cell models must be integrated with complex data from human tumors and healthy tissues by systems biology approaches that can model gene interactions and polygenic determination of phenotypic traits.

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Chapter 10
Genomic Landscape of Ovarian Cancer

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Abstract Epithelial ovarian cancer (EOC) remains one of the most challenging areas of cancer research as it is a highly heterogeneous disease from both molecular and etiological points of view. Furthermore, EOC is the fifth leading cause of cancer-related deaths among women, and the leading cause of death from gynecological cancer. Early detection is paramount to increase survival, but only 25% of all EOC are found at an early stage; furthermore, tumors that appear similar based on traditional clinical and histopathologic features may respond very differently to therapy. At the biological level, the most relevant need is for a new molecular classification of EOC that would enable identification of targetable pathways and predict outcome of disease; at the clinical level, the open issues are early detection of disease and early identification of patients with drug-resistant cancers so that alternative therapeutic modalities can be offered.

Microarray-based technologies are powerful tools that may potentially help in understanding the relationship between clinical features of cancers and their underlying biological alterations by measuring the simultaneous structural alteration/expression of thousands of genes. The genomic landscape in EOC, herein

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described, refers to genomic, functional genomic, and epigenomic studies published in the last 10 years. On the basis of this genomic landscape, the following can be affirmed: (i) all approaches have contributed to the identification of tumor subtypes, but none of the proposed genetic signatures has been sufficiently confirmed or validated; (ii) the clinical question of early identification remains unanswered. In fact, even if there are promising data from epigenetic-based analysis of blood samples from EOC patients, their predictive power is still too low for population-based screening; (iii) genomic and methylation analyses have only recently been carried out on a genome-wide level, and accordingly only a limited number of promising prognostic signatures and predictors have emerged; (iv) gene and miRNA expression analyses, based on more mature technologies, have provided a larger number of promising prognostic signatures and predictors.

In the case of early detection, improvement in terms of accuracy and further confirmation of reliability as specific markers in adequately-sized prospective studies are needed; in the case of prognosis and prediction, it is imperative to confirm potential genetic signatures in large, well annotated independent sets of patient samples coming from multicenter randomized phase III clinical trials. The use of these type of sample sets, combined with the introduction of new high throughput technologies and the integration of data raised by different genome-wide approaches, will hopefully enable a global view of the DNA-RNA relationships and ultimately lead to identification of clinically useful biomarkers.

1 Introductory Remarks

In traditional pathology, tumors are classified according to the organ of origin and, subsequently, on the basis of the tissue of origin. Considering this histopathological classification, only epithelial ovarian cancer (EOC) and the main open questions for this pathology will be discussed in this chapter. The genomic landscape of a tumor considers genomic, functional genomic, and epigenomic aspects as described in Sect. 3; it refers only to studies performed on clinical materials, and is focused mainly on the results of multi-gene studies, and whenever applicable, by high-throughput technologies such as microarrays. A Medline search was conducted to review published articles from Jan 2000 to Dec 2011. The search was restricted to English language articles reporting human studies. Citation and reference lists of retrieved articles were checked to ensure sensitivity of the search strategy. Due to the exceedingly high number of publications retrieved, only those that more strictly adhere to the criteria reported in Sect. 3 and that tentatively convey new messages were included.

2 Epithelial Ovarian Cancer

2.1 Pathogenesis

EOC remains one of the most challenging areas of cancer research; in fact, it is a highly heterogeneous disease that from molecular and etiological points of view can be considered a general term for a series of distinct diseases that simply share a common anatomical location [1]. EOCs are classified by their histopathologic traits in serous (SC), endometrioid (EC), mucinous (MC), clear cell (CCC), mixed, and undifferentiated subtypes. Additionally, fallopian tube and primary peritoneal cancers morphologically and clinically resemble EOC. The traditional view of ovarian carcinogenesis derives these tumors from the ovarian surface epithelium, via inclusion cysts that, by subsequent metaplastic changes, lead to the development of the four main histological types (for a review see [2]). The correlation of clinicopathological features with genetic studies has suggested a new paradigm for the pathogenesis and origin of EOC based on a dualistic model of carcinogenesis that classifies EOC in two types [3] as summarized in Fig. 10.1. Type I tumors comprise low grade serous (LGS), low grade endometrioid (LGE), clear cell (CCC),

Fig. 10.1 ***The new classification of ovarian cancer.*** The heterogeneous nature of EOC is encompassed by the dualistic model that classifies the major histological EOC types in two groups (Type I and Type II) according to their distinctive clinicopathologic, molecular, and genetic features

and mucinous (MC) carcinomas that develop in a stepwise fashion from well-defined precursor lesions. They are also indolent and relatively genetically stable. In contrast, type II tumors comprise high-grade serous (HGS) and endometrioid (HGE) carcinomas, malignant mixed mesodermal carcinomas, and undifferentiated carcinomas; they grow rapidly and are highly aggressive. Type II tumors are chromosomally unstable (for a review see [4]).

The introduction of the low malignant potential (LMP) category, lacking destructive invasive growth with significantly better outcome than the invasive carcinomas, was another important step in refining the morphologic classification of (EOC). Furthermore, the lack of coexistence of LMP tumors with invasive carcinomas suggests that their origins were unrelated in agreement with the Singer dualistic model of serous EOC carcinogenesis, in which LMP tumors are the precursors of LGS carcinomas, whereas HGS carcinoma is a genetically distinct entity that does not simply represent a transition from a low- to a high-grade phenotype [5]. At the biological level, the most relevant need is for a new molecular classification of EOC that would enable identification of targetable pathways and predict outcome of disease.

2.2 Molecular Landscape

It is by now well-accepted that the different EOC histotypes are characterized by peculiar molecular alterations. The molecular landscape of EOC is excellently described in the seminal reviews by Bast [6] and Despierre [7], and briefly summarized herein.

HGS carcinoma is mainly characterized by somatic mutation of TP53 (97% of cases); in most cases, the loss of function of p53 is due to missense mutations which lead to protein accumulation in the nucleus; in some cases, it is due to null mutations that lead to the complete absence of protein. The mutation of TP53 fails to eliminate cells with chromosomal instability [8]. Fifty percent of HGS are characterized by loss of function of BRCA mainly due to mutations in the BRCA1/2 tumor suppressor genes or to hypermethylation of the BRCA1 promoter and concomitant loss of heterozygosity (LOH). At least 10% of all EOCs are familial, with germline mutations in BRCA1/2 accounting for approximately 90% of cases [9]. Most of the remaining 10% are caused by germline mutations in DNA mismatch repair (MMR) genes, primarily MLH1 and MSH2, which are responsible for Lynch syndrome. The predominant cancers in families with a mutation in one of these genes are colon and endometrial cancer, but retrospective studies have shown that women in such families also have an increased estimated lifetime risk of EOC (4–12%) [10]. Alterations in BRCA1/2 cause chromosomal rearrangements increasing chromosomal instability. Furthermore, the combination of p53 mutation and loss of BRCA1/2 causes the survival of cells with DNA damage, which fails to be repaired, leading to a generalized chromosomal instability characterized by copy number variation (CNV) and chromosomal rearrangements.

These alterations frequently affect oncogenes that regulate proliferation (MYC, CCNE1, NOTCH3 and CCND1) [11]. The high proliferation rate causes the rapid progressive accumulation of genetic alterations and considerable intratumoral heterogeneity.

Three percent of EOC can be classified as LGS carcinomas, which exhibit a high genomic stability, only rarely mutated in p53 and have an altered MAP-kinase pathway; in fact, in 65% of LGSC mutations occur in either KRAS or BRAF (mutually exclusive), with another 6% in ERBB2 [3, 12].

The EC subtype has characteristic mutations in genes of PTEN/PI3K and Wnt/CTNNB1 pathways. Indeed, 65% of EC have an activating mutation of CTNNB1 [13], the gene encoding for β-catenin, which is normally located in the submembrane where it consolidates the adhesion between cells. In 85% of EC with squamous differentiation areas, CTNNB1 is mutated, and in the same areas aberrant nuclear accumulation of β-catenin is often observed [14]. The PTEN/PI3K pathway is abnormally activated by mutations in PTEN or PIK3CA, in 19 and 12% of cases, respectively [15]. This pathway is required for homeostasis, while the downstream mTOR complex is essential for protein translation.

The CCC presents mutated PIK3CA or ARID1A in 33 and 50% of cases, respectively [16]. ARID1A is involved in chromatin remodeling and confers chromosomal stability to CCC, but with aberrant configurations [17]. The MC histotype, similar to LGS carcinoma, exhibits high chromosomal stability and frequent activating mutations in KRAS (50% of cases) [18], which constitutively activate the MAPK pathway, while in 18% of cases the MAPK pathway is activated by ERBB2 amplification [19]. Homozygous deletion of CDKN2A is present in 38% of cases [20].

2.3 Clinical-Pathological Aspects

EOC is the fifth leading cause of cancer-related deaths among women, and the leading cause of death from gynecological cancer [21]. In spite of progress in diagnosis and treatment of EOC, the incidence and mortality rates have remained unchanged over the past decade. Early detection is paramount to increase survival. In fact, the survival rate for patients with disease confined to the ovary (Stage I) is 90%. Although many patients experience symptoms, these are shared with common gastrointestinal, genitourinary and gynecological conditions and are not useful for early diagnosis [22]. At present, there is no screening strategy with proven efficacy for the early detection of EOC in the general population [23]. Overall, only 25% of all EOC are found at early stage, while for the majority of patients the disease is diagnosed after it has metastasized. For EOC, unlike cancers at many other sites, no anatomical barrier exists to widespread metastasis throughout the peritoneal cavity; small clusters of cancer cells are shed by the primary tumor and implant on the peritoneal surface, forming numerous nodules difficult to remove by surgery [24] and as a consequence, prognosis is extremely poor.

Standard treatment for advanced-stage EOC is aggressive debulking surgery followed by platinum-taxane chemotherapy, with response rates of over 80% [25]. However, most of these patients will eventually relapse, with a median time-to-relapse (TTR) of 18 months. Several drugs are available to treat recurrences; however, clinical responses remain short-lived and lead to only marginal improvements in survival of patients with platinum-resistant disease [26]. The difficulty to diagnose the disease at an early stage and the persistence of dormant, drug-resistant cancer cells, are the primary reasons for the high mortality rate in ovarian cancer patients, with an the overall 5-year survival rate for advanced stage patients approximately 30% [23]. At the clinical level, the open questions are:

- early detection of disease;
- early identification of patients with drug-resistant cancers so that alternative therapeutic modalities can be offered.

3 High-Throughput Technologies, Data Analysis and Interpretation

3.1 High-Throughput Technologies

Microarray-based technologies are powerful tools that may potentially help in understanding the relationship between clinical features of cancers and their underlying biological alterations by measuring the simultaneous structural alteration/expression of thousands of genes.

The genomic landscape in EOC refers to genomic [analysis of DNA by array comparative genomic hybridization (aCGH), copy number analysis, single nucleotide polymorphism (SNP) analysis], functional genomic [analysis of RNA level by whole genome gene expression], and epigenomic [DNA methylation and microRNA analysis] studies. Few exceptions were made to the above mentioned criteria.

3.2 General Criteria for Data Analysis and Interpretation

Theoretically, high-throughput technologies offer the opportunity of improving risk prediction or optimizing treatment selection for individual patients. However, discrepancies among reported data are commonly observed in microarray research and, to obtain results that are clinically relevant, different sources of variability have to be taken into account. Biological sources of variability can be attributed to differences in cellularity of tumor specimens and patient populations, while technical variabilities reside in different commercial and home-made microarray

platforms along with an ever-growing number of pre-processing, statistical and bioinformatic methods applied to the analyses. The Minimum Information About a Microarray Experiment (MIAME) project defines a standard for reporting microarray experiments to allow data exchange and interpretation, and to improve reproducibility of experiments. Following MIAME guidelines [27], each experiment should include laboratory and data processing protocols, raw and final processed data, and annotations regarding the arrays and sample. From 2001 and 2008, for functional genomics and microRNA, respectively, data published in major journals should be deposited in web repositories (GEO, ArrayEpress) applying the MIAME rules.

3.3 Criteria for Correctly Use of Data for Prognosis and Prediction

A molecular signature is a characteristic that allows classification of a tumor based on the expression levels of the features (e.g. genes, miRNAs) present in the signature. Usually, a molecular signature provides a continuous score rather than a binary class identifier, and in order to use this tool as a "predictor" cutoff thresholds have to be defined. At present, there are many studies reporting on the development of classifiers with remarkable accuracy in predicting outcome, but none is currently used in clinical practice. Microarray experiments provide the assessment of a large number of variables compared to the low number of samples that are analyzed in the vast majority of the studies. This problem might lead to generate overfitting predictive models with an overestimated performance in cohorts of patients where the models are developed. To avoid finding spurious associations, signatures should be defined using an initial set of samples (training set), and then the relevance of these signatures should be confirmed in a separate, independent validation set. Some studies split their datasets in training and validation. However, when cohorts that are too small are investigated, it may represent an inefficient use of the data leading to the development and validation of predictive models without sufficient prediction accuracy.

If the study is correctly planned, an internal validation of the classifier within the training set should be carried out through cross-validation based on the repeated model development within k-partitions of the training set. The main aims that should be accomplished using the training set are identification of the features entering into the classifier and the development of a model that combines defined features to stratify a new patient including, if necessary, the assessment of a threshold. Eventually, the model should be confirmed on independent data that are external to the study used to develop the classifier. After a signature has been identified, it is important to assess, by using multivariate analysis on validation sets, whether it confers independent and robust information compared with standard clinical criteria, thus representing an improvement in clinical management [28].

Specific concerns should be considered in the case of methylation analysis. Although alterations in DNA methylation, including global hypomethylation of heterochromatin and specific CpG island methylation could be promising markers for molecular diagnosis and can be detected in body fluids (serum and peritoneal fluid, as in the case of EOC patients), to correctly determine if a gene is methylated only in neoplastic cells it is important to define the correct and more representative normal counterpart to evaluate whether a gene is imprinted and has tissue specific and/or age-related methylation.

3.4 Potential Limitations in Data Interpretation in EOC

One of the major limitations in EOC studies is related to the diverse choice of normal reference. In fact, sources of normal ovaries as control reference include whole ovary samples, ovarian surface epithelium (OSE), short-term culture of OSE or immortalized OSE. Since the use of different controls strongly influences the genes identified as differentially expressed in ovarian cancer specimens, the generalization of these findings might be limited [29]. Furthermore, the new theory shifting the early events of ovarian carcinogenesis to the fallopian tube and endometrium besides the ovary [30] opens a further debate concerning the correct choice of an ovarian normal control. Another challenge is related to the tissue-processing protocols that may include variable amounts of surrounding non-tumor tissues thereby confounding the interpretation of microarray results.

Other important limitations are the clinic-pathological complexity of the disease along with its intrinsic heterogeneity and relatively low incidence. Accordingly, many studies have a relatively small sample size and heterogeneous histological composition.

Finally, specific limitations for each type of genomic approach can be observed. For example, in the case of methylation, at variance of other cancer types, such as prostate cancer where GSTP1 is known to be methylated in more than 90% of tumors [31], no gene has been found to be methylated in more than a small fraction of ovarian cancers. If a new genome-wide approach can contribute to the discovery of gene alterations, a challenge remains in the identification of markers that take into account disease heterogeneity and commonly found altered in patients with each different EOC type.

4 Genomic Landscape of EOC

In 2006, a large-scale collaborative effort, coordinated by the National Cancer Institute (NCI) and the National Human Genome Research Institute (NHGRI) and named The Cancer Genome Atlas (TCGA) was initiated, whose aim was to systematically characterize the genomic changes that occur in cancer. Information

about TCGA and the investigators and institutions that constitute the TCGA research network can be found at http://cancergenome.nih.gov. The pilot project focused on three tumor types: glioblastoma multiforme, lung (squamous) carcinoma and HGS EOC. This project is the most comprehensive examination of EOC genomics ever undertaken [32]. TCGA microarray analyses produced high-resolution measurements of mRNA and miRNA expression, DNA copy number and DNA promoter methylation for almost 500 clinically annotated HGS carcinoma samples; massively parallel sequencing provided whole exome DNA sequence information for about 300 of these samples.

Beside this comprehensive study, numerous independent research groups analyzed several aspects of the EOC genomic landscape and the data published in the last 10 years are summarized in the following subsections:

- subtype classification and early detection
- prognosis and prediction

4.1 EOC Subtype Classification and Early Detection

4.1.1 Genomic Data

The development of metaphase CGH in 1992 [33] opened a new window into EOC genomics, and by 2000 the most common amplicons and deletions, such as gains of 8q, 3q, 1q and 20q and losses of 4q, 5q, 8p, 22q, 18q and 17p, were well documented (see citations in [20]). Conventional metaphase CGH technology, despite its low resolution, provided the basis for classification of EOC according to DNA aberrations (see Sect. 10.1). The subsequent advent of CGH and SNPs arrays strongly increased the resolution and further contributed to the classification of different histotypes and the separation between sporadic and BRCA mutated cases (see Table 10.1). However, the results obtained with conventional CGH analysis were only partially confirmed [20]. Recently, a high-resolution CGH analysis in 23 cases of primary LMP tumors identified a subset of tumors with detectable imbalances or karyotypic aberrations, suggesting that this subgroup could evolve in a more malignant phenotype [34].

4.1.2 Functional Genomic Data

Gene expression profiles may allow classification of tumors in an organ-specific manner by identifying genes expressed in a putative tissue specific manner for each tumor type [35]. For instance, comparisons between endometrial and ovarian cancers, and serous and endometrioid tumors, showed expression patterns reflecting their origin, whereas clear cell tumors showed remarkably similar expression patterns regardless of their origin (endometrium or ovary) [36].

Table 10.1 Genomic contributions to disease classification

Author (publication year)	Methodology	Platform	No. samples	Main conclusion
Nowee (2007) [104]	CGH array	2464 BACs (UCSF Comprehensive Cancer Center)	28 SC cases: 14 arising from fallopian tube and 14 from ovary	Carcinomas arising from fallopian tube or ovary surface epithelium, despite sharing several genomic aberrations involving a few predominant pathways, show differences in genomic profiles
Gorringe (2009) [20]	SNP array	250K Sty 250KNsp SNP 6.0 arrays (Affymetrix)	106 EOC cases with paired normal lymphocytes	Subtype specific differences in LOH frequency were noted, particularly for MC
Yoshihara (2011) [105]	SNP array	SNP 6.0 arrays (Affymetrix)	105 EOC cases: 71 BRCA1 mutated, 34 sporadic, 47 healthy controls	CNV in BRCA1 carriers are qualitatively distinct from those in sporadic EOC patients

This observation may have implications for therapeutic decisions that should be based on clear cell histology rather than the anatomic site of origin of the tumor.

Numerous gene profile studies published in the last 10 years addressed questions related to the molecular classification of EOC (see [37, 38]). The main conclusions are reported in Table 10.2. Microarray technology has been used to compare gene expression profiles of ovarian cancers *vs.* surface epithelium of normal ovaries with the aim to identify genes encoding proteins that can be detected in serum which are differentially upregulated in EOC. However, many of these studies used a small number of samples or were performed on EOC cell lines rather than on surgical specimens [39–42]. Microarray studies describing potential new markers to be used alone or in combination with CA125 for EOC early detection are listed in Table 10.2. Although the results of these studies support the concept that gene profiling can identify new tumor markers for early detection of disease, the clinical utility of such markers remains uncertain.

4.1.3 Epigenomic Data

Methylation Data

Similar to other malignancies, aberrant DNA methylation, including global hypomethylation of heterochromatin and specific CpG island methylation, characterize the development of EOC.

To date, the vast majority of studies have focused on identification of single candidate hypermethylated tumor suppressor genes such as: classical tumor suppressor (BRCA1, p16, MLH1), putative tumor suppressor (RASSF1A, OPCMLI), imprinted (ARH1, PEG3I) and proapoptotic (LOT1, DAPK and PAR4) genes (see [43] for a comprehensive review of the literature). Although the reported frequency of methylation often varies in different studies, methylation patterns in these genes have been frequently associated with EOC molecular and clinical characteristics, whereas no clear association of a specific hypomethylated gene has been described to date.

By comparing the methylation profiles of the major EOC types at the tissue level, studies reported in Table 10.3 generally suggest that increased altered methylation patterns accompany disease progression, and that methylation profiles can distinguish between different molecular subtypes of disease [44–46].

Specific methylated DNA markers can also be detected in body fluids (serum and peritoneal fluid) of EOC patients, and therefore represent a potential, minimally invasive tool for early detection of both high risk and sporadic cancer as demonstrated by a feasibility study showing that promoter hypermethylation is detectable in the serum DNA from early-stage ovary-confined tumors with cytologically negative peritoneal fluid [47]. In addition to proof of principle, these data suggest that circulating ovarian tumor DNA could be more readily accessible for early diagnosis in the bloodstream than in the peritoneum, consistent with previous studies [48]. To date, only two studies have performed a genome wide approach to evaluate the DNA

Table 10.2 Functional genomic contributions to disease classification and early detection

Author (publication year)	Platform	Training set No. samples	Validation set No. samples	Main conclusion
Disease classification				
Schwartz (2002) [106]	Oligonucleotide arrays	113 tumors	None	Gene expression profiling can distinguish MC and CCC from serous tumors, regardless of tumor stage and grade
Schaner (2003) [107]	DNA arrays based on IMAGE clones	44 tumors and 12 cell lines	None	Identification of a distinctive profile of gene expression for CCC
Gilks (2005) [30]	High-density spotted cDNA	23 tumors	None	A relatively small gene set can distinguish among serous tumors the LMP from invasive ones
Bonome (2005) [108]	U133 Plus 2.0 (Affymetrix)	80 tumors and 10 normal OSE	None	LMP tumors and LG carcinomas may represent a distinct group of tumor rather than early precursors in the development of the advanced high-grade malignancy
Anglesio (2008) [109]	U133 Plus 2.0 (Affymetrix)	90 tumors	150, in silico [57]	The molecular events underlying the development of serous LMP tumors are distinct from those leading to HGS carcinoma. There is an overall redundancy of RAS-MAPK pathway in LMP tumors
Tothill (2008) [57]	U133 Plus 2.0 (Affymetrix)	285 tumors	119, in silico [110]	Identification of six EOC molecular subtypes. Two subtypes represented serous LMP and LGE subtypes. The remaining four subtypes represented HG and advanced stage of serous and endometrioid tumors
TCGA (2011) [32]	U133A (Affymetrix)	489 tumors	285, in silico [57]	Identification of at least four robust expression subtypes in HGS carcinoma: differentiated, immunoreactive, mesenchymal and proliferative

Early detection				
Schummer (1999) [111]	cDNA	10 tumors 6 normal tissues	None	Identification of HE4 over-expressed in EOC. HE4, together with CA125, are at present the only new markers approved for EOC
Mok (2001) [112]	cDNA	3 EOC cell lines 3 normal OSE cell lines	64 patient sera 137 normal subject sera	Profiling for identification of genes coding for secretory proteins to be used as potential serum markers: identification of prostasin as overexpressed in cancer
Meinhold-Heerlein (2003) [113]	Oligonucleotide array	67 tumors 9 normal ovarian tissues	67 patient sera 67 healthy control sera	Identification of 275 genes predicted to encode secreted proteins with increased/decreased expression in EOC. Two of these markers (osteopontin or kallikrein 10/matrix metalloproteinase-7) in combination with CA-125 could discriminate early-stage disease from healthy controls

Table 10.3 DNA methylation: contributions to classification and detection

Author (publication year)	Methodology	Platform	No. and type of samples	Main conclusion
Makarla (2005) [44]	MS-PCR	Panel of eight preselected loci (tumor suppressor genes)	23 invasive EOC (all stages/histotypes), 23 LMP, 23 benign cystadenoma and 6 normal tissues	Pattern of RASSF1A, APC, GTSP1 and MGMT methylation profiles can differentiate between LMP and EOC
Su (2009) [45]	MS-PCR	Platform of seven preselected genes	126 EOC, 14 LMP, 75 benign or normal ovarian tissues; serum samples form: 26 EOC; 20 benign tumors	Increased methylation of 6 of 7 loci was observed in tumor tissue; tissue and serum signature were correlated. Combined methylation pattern of three of these loci seemed to have also diagnostic and prognostic power
Montavon (2011) [46]	MS-PCR	Platform of ten preselected genes	80 EOC and 12 OSE samples	HOXA9 was methylated in 95% of tumors, combination of two-gene methylation pattern discriminate normal vs. tumor; 1 of 10 genes has independent unfavorable prognostic relevance
Watts (2008) [114]	Two-color microarray	6.5K CpGs islands. Custom platform	104 EOC 23 LMP and 10 normal ovarian samples	Progressive alterations of CpG methylation patterns with disease progression; identification of a CpG methylation pattern able to distinguish advanced-stage EOC from LMP or normal tissue
Houshdaran (2010) [115]	DNA probe array	1.5K CpGs Illumina islands	27 primary EOC samples (all stages/histotypes); 15 EOC cell lines	EOC cell lines significantly different from primary tumors. 90 CpG sites (68 genes) subtype specific methylation pattern able to distinguish SC from EC and CC histotypes

Yoon (2010) [116]	DNA probe array	1.5K CpGs Illumina islands	40 advanced stage EOC samples (all histotypes); 5 normal ovarian tissue samples	Identification of 6 genes commonly hypermethylated in all histotypes (ALOX1, DAB2IP, HOXA9, MOS, SPARC) and identification of histotype-specific hypermethylated genes
Gloss (2011) [117]	MS-PCR followed by MassARRAY system	MassARRAY sequenom	2 EOC cell lines 54 type II EOC and 10 OSE	On the basis of DNA methylation analysis in 2 EOC cell lines, identification of promoter hypermethylation of 6/15 preselected genes as a potent discriminator of cancer vs. normal tissue (AUC 0.98).
Teschendorff (2009) [49]	Genome wide DNAm profiling	Methylation beadchip array (Illumina)	Blood samples: 113 pre-treatment EOC, 148 age-matched healthy women, 112 post-treatment EOC	Identification of two DNAm signatures able to predict presence of EOC or presence of active cancer in post-treatment samples with 0.8 and 0.7 accuracy, respectively (355 overlapping CpGs in the two signatures). The presence of cancer lead to an increase of the myeloid skewing in peripheral blood CpG methylation pattern normally caused by aging
Campan (2011) [50]	Genome wide DNAm profiling	Methylation beadchip array (Illumina); MethyLight PCR	Blood samples: 41 EOC samples and 16 age-matched healthy patients. Within subjects, longitudinal analysis in independently selected 16 advanced-stage EOC patients	Identification of 554 putative tumor-specific markers (see Fig. 10.2). Among the top 15 loci selected for verification, serum levels of IFFO1 methylation showed a post-resection kinetic correlated with CA125 levels and significantly associated with tumor burden

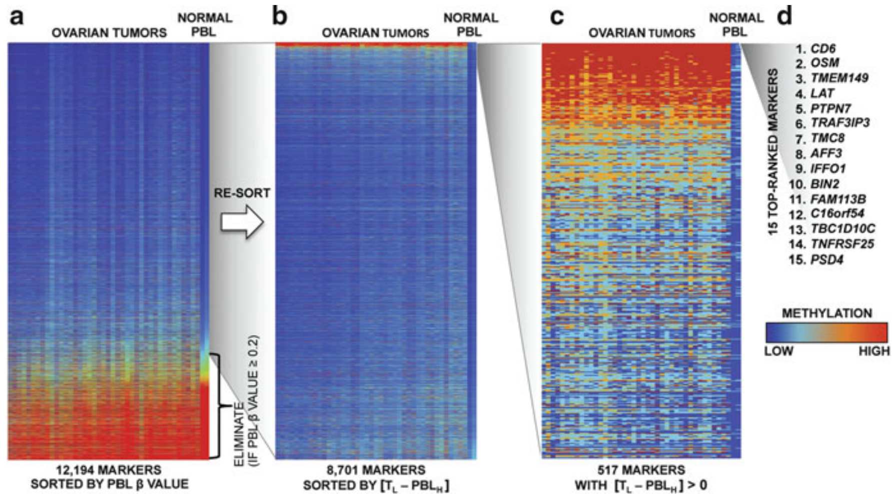

Fig. 10.2 ***Heat map representation of the marker selection process.*** (**a**) The 12,194 markers remaining after the elimination of the probes that failed in any of the samples, and of the probes containing SNPs or repetitive elements. Markers are ranked in an ascending order based on the mean DNA methylation β value of PBL samples. (**b**) The 8,701 markers remaining after eliminating probes with DNA methylation β values ≤0.2 in any of PBL samples. Probes were ranked in a descending order based on the difference in DNA methylation between the tumor with the lowest β value (T_L) and the PBL sample with the highest b value (PBL_H). (**c**) The 517 markers with higher DNA methylation values in any of the tumor than in any of the PBL samples. The markers are ranked in a descending order based on the difference between the tumors and the PBL DNA methylation values. (**d**) The top-ranked 15 markers that were transitioned to the MethyLight platform for further verification (Legend modified from Campan et al. [50])

methylation profile in peripheral blood [49, 50] (Table 10.3). Since the two studies differed in study design, especially in marker selection strategy, general conclusions cannot be drawn. Figure 10.2 shows the complexity of the marker selection process as reported by Campan et al. [50].

miRNA Data

In normal cells miRNAs regulate the balance of various processes, including proliferation, differentiation and cell death by regulating multiple mRNAs, and as such their dysregulation can have profound cellular consequences. miRNAs are aberrantly expressed in cancerous tissues, and/or body fluids from cancer patients suggesting that miRNAs are novel cancer genes and biomarkers [51]. Given the stability of miRNA, i.e. resistance to degradation, and the relative abundance of target, these molecules have been used to accurately classify human cancers and have been shown to be more informative than a mRNA-based tissue classifier for the segregation of tumors by developmental origin (see [52, 53]).

Several studies have been performed using microarrays in the attempt to identify markers suitable for early detection of EOC (Table 10.4). In this perspective, a recent study analyzing the miRNome profile in blood samples from patients suffering from relapsing EOC (mostly of serous origin) and age- and sex-matched healthy controls, identified multiple miRNAs (n = 147) whose expression was significantly dysregulated in EOC patients [54] and the miRNA profile was able to discriminate between blood samples of EOC patients and healthy controls with an accuracy of 76%. However, only eight of these miRNAs have been previously described in the context of EOC, although the list also included miR-29a and miR-155, previously shown to be significantly dysregulated in 28 EOC patient sera compared to 15 healthy controls using a qRT-PCR-based microarray method [55].

Considering the general biological and technical limitations already underlined in other contexts, it is still not possible to draw any general conclusions, and the most consistently deregulated miRNA in EOC compared to normal tissue are those of the miR200-family (frequently up-regulated) and let-7 family (frequently down regulated). In particular, using data from an independent dataset including 900 tumor samples, the downregulation of let-7 was recently described to be specifically associated with a particular serous subtype [56] previously identified by functional genomic analysis [57].

4.1.4 State of the Art

On the basis of the genomic landscape here described the following can be affirmed:

- All approaches have contributed to the identification of tumor subtypes, but none of the proposed signatures has been sufficiently confirmed or validated;
- The clinical question of an early identification remains unanswered. In fact, even if there are promising data from epigenetic-based analysis of blood samples from EOC patients, their predictive power is still too low for population-based screening, and improvement in terms of accuracy and further confirmation of their reliability as specific markers of early detection in adequately sized prospective studies are needed.

4.2 Prognosis and Prediction

EOC that appear similar based on traditional clinical and histopathologic features may respond very differently to therapy; indeed, it is not possible to predict who will progress or recur during or after chemotherapy. The development of predictive molecular markers may facilitate the identification of subpopulations of patients who may respond more favorably to different therapeutic modalities [58]. Since chemotherapy resistance is likely to be a multifactorial mechanism, it is no surprise that individual molecular markers are not very accurate predictors of response.

Table 10.4 miRNA contributions to classification and detection

Author (publication year)	Methodology	Platform	No., type of samples	Main conclusion
Iorio (2007) [118]	Microarray chip	Ohio State University OSU.CCC v.2; 260 probes for human miRNA	69 EOC; 15 normal ovarian tissue samples	Identification of miRNAs signature associated with different histotypes and poorly differentiated tumors
Zhang (2008) [119]	Microarray chip	Ohio State University OSU.CCC version 3; 1100 miRNA probes (326 human)	106 EOC, 4 immortalized OSE cell lines, 18 EOC cell lines	Identification of 44 miRNAs significantly different between early- and late-stage EOC, most of them downregulated (miR15a, miR34a, miR34b). 13 miRNA significantly down regulated in HG vs. LG. 10/13 commonly downregulated with stage or grade advancement. Possible involvement of epigenetic mechanisms in miRNA downregulation
Nam (2008) [120]	Two color miRNA custom microarray	314 probes for human miRNA	20 EOC, 8 benign uterine disease	miRNA signature differentially expressed in tumor samples; identification of prognostic miRNAs
Dahiya (2008) [121]	LNA array	Exiqon MiRCURY miRNA array covering 92.3% of miRNAs in miRBase 9.0	34 EOC, 1 immortalized OSE, 10 EOC cell lines	miRNA signature differentially expressed in tumor samples
Wyman (2009) [122]	Massively parallel pyrosequencing	Not applicable	33 EOC, 4 OSE samples	Identification of a set of 124miRNAs differentially expressed in normal vs. tumor samples and 38 miRNAs differentially expressed across histologic subtypes
Hausler (2010) [54]	Microarray chip	Geniom biochip miRNA homo sapiens; 904 miRNAs	24 blood samples form relapsed EOC patients and 15 healthy controls	Identification of miRNA profile associated with EOC with accuracy of 76%

4.2.1 Genomic Data

Conventional metaphase CGH has shown that patients with chromosomal instability and microsatellite stability or low instability have shorter overall survival and poorer prognosis [59]. Subsequently, data from CGH arrays, as summarized in Table 10.5, suggested some association between specific genomic alterations and chemoresistance or survival. A single study exploiting SNP arrays was retrieved in our search [60] and reported in Table 10.5. While promising, the data were obtained in a limited number of tumors and further studies are needed in a larger group of patients. Interestingly, at variance from the in vivo data, when cell line model systems were compared to paired primary tumor samples to investigate genomic changes, an extensive and non-linear genetic divergence between treatment-sensitive and treatment-resistant clones cultured from the same individual was observed [61]. The authors concluded from their observations that, at least in vivo, one clone is strongly dominant at the time of presentation which renders finding genetically different subclones at a single time-point difficult.

Although much of the literature on germ-line SNPs has focused on their association with susceptibility of disease, more recently evaluations of the relationships between these variants and clinical outcome after cancer diagnosis have been described. In fact, pharmacogenomics is now defined as the study of genetic/genomic differences underlying inter-individual variability in drug responses. A systematic review of the published literature in EOC SNPs [62] found 50 studies evaluating the association between polymorphisms and outcome or response to chemotherapy. The majority of these studies were either case series or cases that formed part of an existing case-control study, and only few study designs included cohort observational studies or secondary analyses of randomized clinical trials. Most studies reported polymorphisms in multiple genes, as well as different polymorphisms within the same gene. Overall, the broader studies did not identify any association with response to treatment and the reported association with overall survival was marginal without a clear validation in independent studies [62].

The data on SNPs in the ABCB1 and in the ERCC1 genes and in other genes involved in various pathways, such as TP53 and FGFR4, are summarized in Table 10.6. The large number of prognostic and predictive studies evaluating the impact of SNPs in the VEGF pathway are reviewed by Diaz Padillas [62].

In Table 10.7 are reported the main characteristics of large studies in which the impact of polymorphisms in genes involved in drug metabolism or drug response or toxicity is evaluated.

4.2.2 Functional Genomic Data

Numerous studies have used microarray analysis to identify gene-expression profiles associated with clinical outcome, the most relevant of which are reported in Table 10.8 (see also [37, 38]).

Table 10.5 Genomic contributions to prognosis and prediction

Author (publication year)	Methodology	Platform	No. of samples	Main conclusion
Birrer (2007) [123]	CGH array	60-mer 22 K array (Sigma-Genosys)	42 advanced stage, HGS	4p16.3 and 5q31-5q35.3 correlated with overall survival
Osterberg (2009) [124]	CGH array	BAC arrays, 38 K BAC clones (SCIBLU Genomics Center)	40 stage III pts preselected for sensitivity (20 pts) or resistance (20 pts) to paclitaxel/carboplatin	Statistically significant higher frequency of altered genome in resistant cases (38% vs. 26%); some genetic alterations might be potential predictive markers of chemotherapy resistance
Osterberg (2010) [125]	CGH array	BAC arrays 38 K BAC clones (SCIBLU Genomics Center)	30 stage III pts preselected for sensitivity (24 pts) or resistance (6 pts) to docetaxel/carboplatin	Specific genomic alterations in resistant cases; different genetic alteration profiles were found by comparing these results to those previously reported for a different dataset [124]
Etemadmoghadam (2009) [60]	SNP array	50 K *XbaI* SNP array (Affymetrix)	85 advanced stage EOC	Amplifications, involving two loci, each with highly relevant biological candidates within or near the mapped *peak* regions, *CCNE1* and *NCOA3*, associated with chemoresistance
Cooke (2010) [61]	CGH array	CGH 44 k array (Agilent)	six pairs of primary tumors taken before and after treatment	Neo-adjuvant treatment with 3–6 cycles of chemotherapy, even when a clinical response was observed, did not provide significant enrichment for a substantially different genetic subclone

Table 10.6 Pharmacogenomics: impact on prognosis

Author (publication year)	Gene (SNP)	No. (type of study)	Main conclusion
Polymorphisms in genes involved in response to Platin derivatives			
Marsh (2007) [126]	ERCC1 (19007T > C)	904 (SCOTROC1 study: phase III trial)	No association with overall survival
Smith (2007) [127]	ERCC1 (19007T > C)	178 (observational case-series: subgroup analysis)	103 pts treated with platinum without paclitaxel: C/C genotype associated with greater risk of death 75 pts treated with platinum plus paclitaxel: C/C genotype and high ERCC1 expression were not associated with worse overall survival
Krivak (2011) [128]	ERCC1 (ˆ118C > T)	280 (phase III clinical trial)	Association with reduced overall survival
Polymorphisms in genes involved in response to taxanes			
Marsh (2007) [126]	ABCB1(2677G > T/A) CYP2C8 (R139K K399R) CYP3A4(CYP3A4*1B)	904 (SCOTROC1 study: randomized phase III trial)	No association with overall survival.
Polymorphisms in genes involved in tumor relevant pathways			
Galic (2007) [129]	TP53 (Arg72Pro)	181 (observational case-series)	Reduced overall survival for Pro allele
Bartel (2008) [130]	TP53 (SNP309)	107 (observational case-series)	Patients with a weakened p53 pathway (either the *G*-allele of SNP309 or a *TP53* mutation) had increased overall survival compared to those wild-type for both *TP53* and SNP309
Paige (2010) [131]	WWOX (T1497G)	554 (observational case-series)	Significant association with progression-free survival, not confirmed in the SCOTROC1 study [126]
Marme (2011) [132]	FGFR4 (Gly388Arg)	242 (observational case-series: subgroup analysis)	In multivariate analysis, the 388Arg genotype was associated with platinum sensitivity, prolonged progression-free and overall survival

Table 10.7 Pharmacogenomics: impact on drug toxicity, response and progression-free survival

Author (publication year)	Gene (SNP)	nNo. (type of study)	Main conclusion
Polymorphisms in gens involved in response to Platin derivatives			
Saldivar (2007) [133]	XPD (3507G > C)	125 (observational case series)	No association with response
	XPA (228A > G)		
Marsh (2007) [126]	ERCC1 (19007T > C)	904 (SCOTROC1 study: phase III trial) [126]	No association with response
Smith (2007) [127]	ERCC1 (19007T > C)	178 (observational case-series)	Subgroup analysis in 103 patients treated with platinum without paclitaxel: C/C genotype associated with greater risk of disease progression
Steffensen (2008) [134]	ERCC1 (^118C > T)	159 (phase III clinical trial)	No association with response
Kim (2009) [135]	GSTP1 (313A > G)	118 (observational case-series)	Higher risk to develop grade 3–4 hematological toxicity
Khrunin (2010) [136]	ERCC1 (19007T > C)	104 (observational case-series)	Associated with nephrotoxicity
	GSTP1 (313A > G)		Strong association with progression-free survival
Polymorphisms in genes involved in response to taxanes			
Marsh (2007) [126]	CYP2C8 (R139K, K399R)	904 (SCOTROC1 study: phase III trial) [126]	No association with response
	CYP3A4 (CYP3A4*1B)		No association with response
	ABCB1 (2677G > T/A)		No association with response
Johnatty (2008) [137]	ABCB1 (2677G > T/A)	309 (population registry: subgroup analysis)	Longer progression free survival; confirmed in patients (281/904 patients) with no macroscopic residual disease from the SCOTROC1 study [126]
Gréen (2008) [138]	ABCB1 (2677G > T/A)	38 (observational case-series)	Clearance of paclitaxel is influenced by the SNPs G2677T/A in ABCB1 and neurotoxicity correlates with the exposure to paclitaxel. Patients with TT genotype have reduced clearance of paclitaxel
Kim (2009) [135]	ABCB1 (2677G > T/A)	118 (observational case-series)	Higher risk to develop grade 3–4 gastrointestinal toxicity

Table 10.8 Functional genomic contributions to prognosis

Author (publication year)	Platform	Training set	Validation set	Main conclusion
Spentzos (2005) [72]	U95Av2 (Affymetrix)	24 tumors (most advanced-stage HGS) selected for pathological response to therapy at second look	36 tumors (most advanced-stage HGS)	The comparison of gene expression between patients with or without residual disease at second look, through pattern-recognition analysis resulted in 134 multigene patterns containing 176 unique genes. Class prediction (compound covariate algorithm) and leave-one-out cross validation were performed in order to select the most accurate profile associated to response and a 93-gene profile was developed achieving 91% of correct classification: the Chemotherapy Response Profile (CRP)
Hartmann (2005) [73]	Custom 30K nylon cDNA microarray	51 advanced-stage HGS selected for time to relapse (TTR).	28 advanced-stage HGS	By generating a binary model to predict recurrence imposing a threshold at 21 TTR months, a 14-gene signature succeeded in correctly categorizing 86% of samples with late relapse and 86% samples with early relapse in the validation set
Jazaeri (2005) [74]	Custom 40K cDNA microarray	21 chemosensitive tumors and 24 chemoresistant tumors selected for TTR	15 post-chemotherapy tumors	By class comparison between the two groups of samples, a restricted list of the 9 most significantly differentially expressed genes was identified as able to predict clinical outcome in almost 80% of samples in the validation set
Dressman (2007) [110]	U133A (Affymetrix)	83 advanced-stage HGS	36 EOC	Using a binary logistic regression model and a stochastic regression model, gene expression profiles were developed that predicted response to platinumThe platinum response was predicted with an overall accuracy of 84.3% in validation set

(continued)

Table 10.8 (continued)

Author (publication year)	Platform	Training set	Validation set	Main conclusion
Helleman (2006) [139]	Custom 18K cDNA microarray	24 tumors: 5 non-responders, 19 responders	72 tumors: 9 non-responders, 63 responders	A discriminating signature of 69 unique genes was identified by merging two lists obtained by class prediction and SAM able to classify patients according to response to therapy. A final list of 9 genes significantly discriminated between responders and non-responders in the training set. Considering the non-responders as a reference group, a threshold expression level in the training set was calculated for each of the 9 genes entering the predictive model whose performance was assessed on the validation set as able to classify classified 8 of 9 non-responders and 37 of 63 responders
Konstantinopoulus (2011) [71]	Not applicable	61 EOC from public dataset [140]: 34 BRCA1/2 mutated; 27 sporadic	70 sporadic EOC: 35 with negative family history for breast or ovarian cancer [72] [141]	An unsupervised hierarchical clustering analysis identified three major groups representing the BRCA1, BRCA2 mutated and non-mutated conditions. After elimination of samples that do not cluster correctly, a binary split was performed defining BRCA-like (BRCA1 and BRCA2 cases) and non-BRCA-like (the sporadic cases) phenotype by generation of a 60 genes predictor. BRCAness profile succeeded in stratifying the validation set patients based on TTR

Interestingly, four of these studies identified prognostic profiles in EOCs through supervised models wherein patients were categorized in two classes based on survival time, as short- *vs.* long-term survivors [63–66]. Spentzos et al. [63] applied a pattern recognition algorithm to discover gene expression patterns associated with the two binary phenotypes to a training set of samples, and the pattern giving the most significant class assignment was chosen. A definitive predictive signature (OCPP signature) confirmed its prognostic power in the validation set and retained independent significance in both the training and validation sets. However, at present, independent validation of the OCPP signature is still lacking. Using a similar approach, Berchuck et al. [65] developed a classifier that outperformed the predictive ability of clinical variables to correctly classify short- and long-term survivors. In the same paper, a different predictive method involving linear discriminant models was applied to the genes able to cluster Spentzos's dataset [63]. A significant difference in outcome was observed between the two major clustering classes, suggesting a potential prognostic role of these genes. It is worthwhile mentioning that the genes in the two models were different. Therefore, after the two models were equally weighted, the best linear discriminant model was validated on an independent dataset of 101 cases [66]. The tested seven genes signature correctly predicted the majority of short- and long-term survivors; furthermore, by classifying early stage and LMP tumors as long-term survivors, it confirmed a shared underlying biology between advanced stage tumors with favorable outcome and early stage and LMP tumors [66].

Three other studies [67–69] developed prognostic models of gene expression using a semi-supervised approach [70]. Genes whose expression was significantly associated with survival time by univariate Cox proportional hazard regression entered into the predictive model. The complexity of the expression patterns inherent in the selected gene list was reduced through principal component analysis (PCA). The resulting principal component(s) explaining the largest amount of variance in the data is (are) used in the proportional hazard model to predict survival, providing a regression coefficient (also called "weight") for each principal component. Finally, an index for each sample was calculated considering the expression of the genes entering into the model and their "weights". Finally, Konstantinopoulos et al. [71] integrated four previously generated microarray raw data from different institutions and run on different platforms to identify a reproducible predictor of survival. The identified predictors are not simply a compilation of prognostic genes, but appear to track true molecular phenotypes of good- and poor-outcome, suggesting that integration of previously-generated cancer microarray datasets may lead to robust and widely applicable survival predictors (See Fig. 10.3).

Several microarray studies have attempted to identify gene signatures able to accurately predict response to first-line platinum and taxane chemotherapy in EOC using different strategies for patient selection (Table 10.9). Spentzos et al. [72], on a subset of patients used to generate the OCPP [63] identified a gene profile (CRP) through its association with pathological complete response to therapy defined at

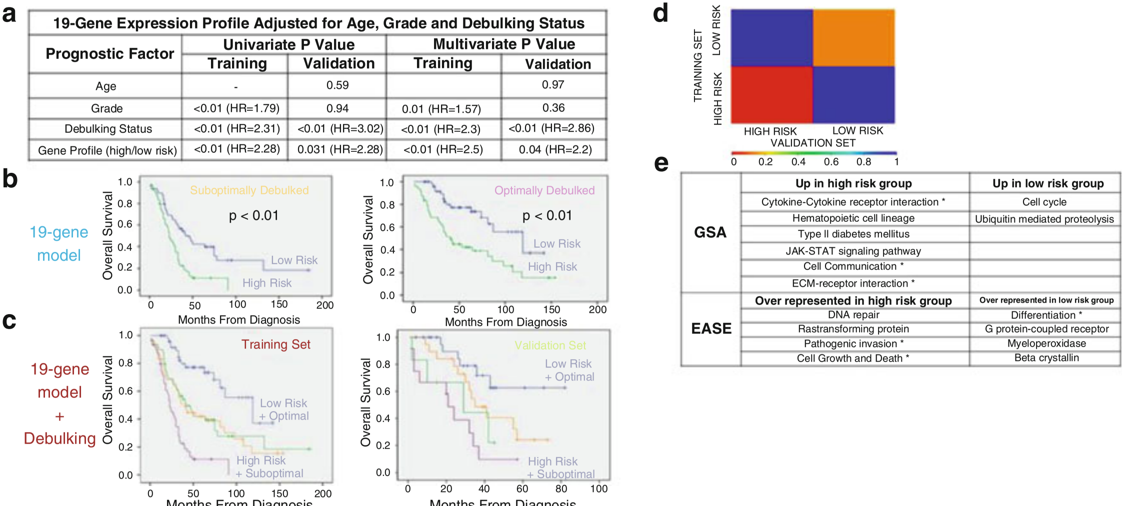

Fig. 10.3 ***Integrated analysis of multiple microarray datasets identifies a reproducible survival predictor in EOC.*** (**a**) Prognostic value of the 19-Gene expression profile adjusted for known prognostic factors by Cox Proportional Hazards Regression in the training and 1st validation sets. (**b**) Kaplan-Meier analysis for OS as a function of the 19-gene profile for homogeneous subsets of patients with optimal and suboptimal debulking status in the training set. (**c**) The combination of optimal debulking and low-risk 19-gene profile was associated with a median OS of 119 months in the training set and not-yet-reached in the validation set, while the combination of suboptimal debulking and high-risk 19-gene profile was associated with a median OS of 23 months in the training set (HR = 7.3, 95% C.I. 3.4–13.5) and 21 months in the 1st validation set (HR = 5.8, 95% C.I. 2.1–16). (**d**) SubMap analysis of genome-wide correspondence (similarity) between respective high and low risks groups in the training and 1st validation set. The *legend* shows the relationship between color and FDR-adjusted p-values. *Red color* denotes high confidence for correspondence; *blue color* denotes lack of correspondence. (**e**) Gene set analysis (*GSA*) over a wide range of differentially expressed genes revealed eight pathways that were consistently statistically significantly differentially expressed. (Efron-Tibshirani GSA, $p < 0.05$). Selected pathways-gene sets are shown that were overrepresented among high-risk and low-risk tumors by functional representational analysis using EASE (within-system FDR $\leq$0.01). *Asterisks* (*) denote pathways that were similarly expressed in corresponding prognostic groups in the 2nd validation set. (Legend modified from Konstantinopoulos et al. [71])

Table 10.9 Functional genomic contributions to prediction

Author (publication year)	Platform	Training set	Validation set	Main conclusion
Spentzos (2004) [63]	U95Av2 (Affymetrix)	34 tumors (most advanced-stage HGS): 14 as outlier for survival; 20 as test set	34 tumors (most advanced-stage HGS)	A multistep approach starting from14 samples chosen based on survival time with 7 samples from short-term survivors (<2-years) and 7 long-term survivors (>5 years), enabled developing and testing a 115-gene signature named Ovarian Cancer Prognostic Profile (OCPP)
Lancaster (2004) [64]	Hu GeneFL (Affymetrix)	31 advanced-stage serous tumors selected as outlier for survival	None	Using a training set selected based on survival with 17 samples from short-term survivors (<2-years) and 14 long-term survivors (>7 years), a list of 43 differentially expressed genes prognostic of survival was identified, but no predictive model was developed
Berchuck 2005 [65]	U133A (Affymetrix)	54 advanced-stage HGS selected as outlier for survival and 11 early stage patients	In silico Spentzos's dataset [63]	A training set including 30 short-term survivors (<3-years) and 24 long-term survivors (>7 years), a classifier was generated based on regression tree models and exhibiting a predictive accuracy of 83.3 and 96.7% for long- and short-term survivors, respectively. A different model also considering early stage lesions suggested that advanced stage tumors with favorable outcome might share similar expression patterns with early-stage cases
Berchuck (2009) [66]	U133A (Affymetrix)	In silico 54 advanced-stage tumors Berchuck [65]	101 tumors: 42 advanced stage selected as outlayer for survival; 39 early stage and 20 LMP	The best linear discriminant model containing seven genes (MAL, APMCF1, PRPS2, L3MBTL, HNMT, RANBPM, DGCR2) succeeded in correctly predicting 82 and 78% of short- and long-term survivors, respectively; 97.4% of early stage and 75% of LMP tumors

(continued)

Table 10.9 (continued)

Author (publication year)	Platform	Training set	Validation set	Main conclusion
Mok (2009) [67]	U133 Plus2.0 (Affymetrix)	53 advanced-stage HGS	None	A 200 gene signature was able to significantly stratify patients in high and low risk groups. Among the selected genes, MAGP2 was identified as a survival-associated target
Denkert (2009) [68]	U133A (Affymetrix)	80 primary ovarian or peritoneal carcinoma	In silico Dressman's dataset [110]	Identification of a 300 gene signature referred to as ovarian prognostic index (OPI). Imposing the median of OPI as threshold, the high- and low-risk groups showed a significant difference in clinical outcome. OPI predictor was successfully validated on an independent set of 118 tumors and in a multivariate model OPI and residual tumor retained independent prognostic value
Crijns (2009) [69]	Operon v3.0 Custom-spotted oligo-arrays	157 advanced stage EOC	Dressman's dataset [110]	An 86-gene profile was able to stratify patients in low-risk (median survival 41 months) and high-risk (median survival 19 months), maintained independent prognostic significance in a multivariate model. Although the validation dataset provides expression data for only 57/86 unique genes, a significant difference between low- and high-risk groups was confirmed
Konstantinopoulos (2011) [71]	Meta-analysis of Affimetrix arrays (U95v2, U133A, U133 Plus2.0, 0.6 K custom GeneChip)	239 advanced-stage tumors from 4 microarray datasets	61-patient cohort and in silico on a 229-array dataset [57]	The prognostic models identified by supervised principal component survival analysis were independently validated in two datasets. A 19-gene model showed optimal performance in the training set, 1st and 2nd validation sets maintaining independent prognostic power in multivariate analysis (See Fig. 10.3)

Bonome (2008) [108]	U133 Plus2.0 (Affymetrix)	185 advanced-stage HGS selected for debulking status	In silico Berchuck's dataset [65]	Independent analyses were performed in training set on the optimally (90 cases) and suboptimally (95 cases) debulked patients. A significant signature of 572 probe sets able to discriminate patients with good and poor prognosis was identified for the suboptimal cases. No significant predictive classifiers were found for the optimally debulked tumors
Sabatier (2011) [142]	Human Exon 1.0 ST Array (Affymetrix)	35 EOC	In silico 366 cases from 5 public datasets [57] [65] [66] [68] [143]	A Cox regression analysis was applied to disclose the gene expression patterns significantly associated to progression-free survival and a list of 94 genes was identified. An Optimal Prognostic Model eventually retained a 7-gene model validated in a large meta-analysis of 5 microarray studies as able to significantly classify the patients in favorable and unfavorable cases

second look, which achieved 91% of correct classification of a validation set of patients. No genetic overlap between the CRP and the OCPP was observed, and the combination of the two profiles yielded better prognostic discrimination than either profile alone [72]. Selection of patients according to TTR has been applied by other two groups. Hartmann et al. [73] imposed a threshold at 21 months and found a 14-gene signature that correctly categorized patients in a validation set according to TTR. Jazaeri et al. [74] categorized patients as chemosensitive (TTR >13 months) or chemoresistant (TTR <6 months), and by class comparison identified a restricted list of the most significantly differentially expressed genes that were able to predict clinical outcome in the 89% of cases.

Taking into account that the most effective strategy to improve outcomes is to personalize therapy, the effect of PARP inhibitors in patients with inactivating germline mutation of BRCA1 or BRCA2 has proven to be one of the best examples of personalized therapy in EOC [75]. Recent data suggest that both genetic and epigenetic mechanisms can account for a BRCA1 or BRCA2 dysfunction to generate the so-called BRCAness phenotype (see [76]). With the aim to identify patients with this phenotype, Konstantinopulos et al. [77] developed a BRCAness signature correlated to chemotherapy response and able to identify a subset of sporadic patients with improved outcome. A further strategy used *in-vitro* models to disclose the molecular patterns associated with sensitivity to chemotherapeutic drugs to identify specific oncogenic pathways that could predict the response in an EOC validation set of patients [78–81].

An important criticism was pointed out by Gavaert and coauthors who failed to validate a model for predicting response to platinum-based chemotherapy [82] obtained by profiling a training set of patients which included stage I patients without recurrence, platinum-sensitive, and platinum-resistant advanced-stage patients [83]. The authors concluded that existing results based on gene expression patterns of EOC need to be thoroughly scrutinized before these results can be accepted to reflect the true performance of microarray technology.

4.2.3 Epigenomic Data

Methylation Data

Assessment of epigenetic alterations holds promise as valuable prognostic indicator. The methylation status of individual genes has been investigated in past years as a potential prognostic marker for EOC. For example, BRCA1 is one of the most extensively studied epigenetically regulated genes in ovarian cancer, and a population-based study showed that BRCA1 hypermethylation in 10–15% of sporadic EOC cases is associated with poor patients outcome [84]. Hypomethylation, a less frequent epigenetic aberration, has also been found to have prognostic relevance [85].

As already described, determining the methylation status of multiple genes rather than individual genes can provide a more sensitive and specific assay for prognosis assessment of EOC patients. Indeed, combining IGFBP3 methylation, already described to have prognostic relevance, with methylation in the promoter regions of other 3/8 preselected relevant loci, a sevenfold increase risk of disease progression and death was found in patients with at least 3 methylated genes [86]. In this perspective, by using methylation-specific PCR or genome-wide array based approaches, the potential prognostic power of methylation status of multiple genes was shown to reliably predict patient outcome. The main studies using different approaches are summarized in Table 10.10. Using differential methylation hybridization, a first pilot study in 2002 [87], whose conclusion were validated in 2006 using the same approach [88], identified two distinct subgroups of advanced-stage EOC patients with significantly different outcomes according to diverse methylation profiling of 112 CpGs containing loci including known tumor suppressor genes (WWOX) and genes related to survival signaling. The same approach was used to examine the prognostic relevance of promoter CpG island methylation in the specific context of the Wnt pathway [89]. One independent study [90] evaluated the global DNA methylation profiles of 20 EOC tissue samples, identifying genes related to telomere organization and immune regulation whose methylation profile significantly associated with longer progression-free survival. However, even in this case, it was not possible to identify common loci with an altered methylation profile as described in previous studies. The sensitivity and specificity of the panel of markers analyzed were relevant in each single study, pointing out the potential prognostic power of this approach. However, the number of the markers evaluated varied among studies, with single or no overlapping genes in different studies due to the above-mentioned biological and technical limitations. Therefore, no general conclusions can be drawn.

It is now recognized that epigenetic changes (e.g. DNA methylation and histone modifications) play a prominent role in intrinsic and acquired drug resistance. The potential utility of epigenetic markers in predicting response to chemotherapy has so far been approached by evaluating in advanced stage EOC patients the methylation profile of a relatively small number of different genes through MS-PCR (see Table 10.10). For example, the maintenance of a $>90\%$ MCJ methylation correlated with poor response and decreased survival [91], whereas demethylation of the FANCF gene (BRCA pathway) has been proposed as a model of resistance to cisplatin therapy in EOC [92]. However, as in many other cancer types, the knowledge of methylation status of multiple genes may be a more powerful indicator for platinum response [93]. Chemotherapy itself can exert a selective pressure on subpopulations of cells, and EOC resistance to platinum-based drugs often occurs in initially responsive tumors. Although the precise mechanism underlying the development of platinum resistance has yet to be fully understood, a number of in vitro studies suggest that CpG island methylation and consequent gene inactivation may contribute to the development of chemoresistance and disease relapse. For example, matched cell lines models of acquired resistance to cisplatinum have shown that chemotherapeutic treatment was associated with selected patterns of

Table 10.10 DNA methylation contributions to prognosis and prediction

Author (publication year)	Methodology	Platform	No. and type of samples	Main conclusion
Su (2009) [45]	MS-PCR	Platform of seven preselected genes	Tissue samples: 126 EOC, 14 LMP, 75 benign or normal ovary; serum samples: 26 EOC, 20 benign tumor	An increased methylation of 6 of 7 loci was observed in tumors vs. normal tissue with tissue and serum signature correlations. Combining the methylation pattern of three of these loci had diagnostic and prognostic power
Montavon (2011) [46]	MS-PCR	Platform of ten preselected genes	80 EOC, 12 OSE samples	HOXA9 was methylated in 95% of tumors, combination of two-gene methylation pattern discriminated normal-to tumor; 1 of 10 genes had independent unfavorable prognostic relevance
Wei (2002) [87]	Differential methylation hybridization	Custom array of 7.7K CpGs island from Human Genome Mapping project genomic library	19 advanced stage EOC tissue samples (5 relapsed)	A higher degree of CpG island methylation associates with early relapse; identification of 220 loci predicting TTP
Wei (2006) [88]	Differential methylation hybridization	Affymetrix custom array from 112 to 220 preselected loci [87]	40 EOC, 7 normal tissue samples	Validation of the Wei study [87] using SAM and PAM algorithms, with identification of 112 loci highly prognostic of early relapsing disease
Dai (2011) [89]	Differential methylation hybridization	Custom array; 302 CpGs island	111 type II EOC for screening study; 61 EOC for validation study	Among 302 loci (189 genes) belonging to the Wnt pathway and epigenetically regulated, 7 were independent predictors of disease progression

Bauerschlag (2011) [90]	Genome wide DNAm profiling	Human Methylation Beadchip Array; 27K CpG sites (Illumina)	20 advanced stage type II EOC	Identification of altered methylation pattern in specific CpG sites, classifying patients according to TTR (cut-off 28 months)
Teodoridis (2005) [93]	MS-PCR	24 preselected CpG loci	106 advanced-stage EOC	Increased methylation of at least one among BRCA1, GTSP1 and MGMT genes was associated with improved response to platinum and taxane-based chemotherapy ($p = 0.01$). Factors affecting DNMT genes may contribute in favoring tumors to acquire aberrant CpG island methylation
Chaudhry (2009) [96]	MS-PCR	5 preselected CpG loci	35 advanced stage EOC	BRCA1 gene promoter methylation is associated with chemosensitivity to front line therapy
Gifford (2004) [95]	MS-PCR	hMLH1 promoter	138 paired plasma samples from EOC (all stages/histotypes) patients at presentation and at relapse from the SCOTROC1 Phase III clinical trial [126]	Methylation of hMLH1 increased at relapse, and acquisition of hMLH1 methylation in plasma DNA at relapse predicted poor overall survival of patients, independent of TTP and age

CpGs island methylation of loci related to cell-growth promotion and cell cycle progression. Moreover, methylation of genes involved in apoptosis, including the DNA mismatch repair (MMR) gene hMLH1, has been linked with acquired resistance to chemotherapeutic drugs in ovarian cancer cell line models [94]. These in vitro observations are now supported by evidence from clinical studies showing that patients with a high degree of specific CpG loci/pattern methylation had a worse response to second line-line treatment, suggesting that they were more prone to develop resistance to chemotherapy [87, 95, 96].

miRNA Data

Expression of miRNA may offer prognostic and predictive information, and in recent years some groups have successfully identified robust miRNA profiles that can predict overall and relapse-free survival in different tumor types (see [97]). The possible role of dysregulated miRNA expression as a potential prognostic or predictive marker in EOC has been recently reviewed in detail [53, 98]. The main recent studies performed using microarray methods are summarized in Table 10.11. However, once again due to heterogeneity of the biological material analyzed in terms of relative frequency of histotypes or different stages of disease, and due to the different types of platform and approaches used, the results between studies have little in common and may even be contradictory. As already mentioned in the section on detection and classification, general consensus on a specific miRNA signature associated with prognosis and/or resistance to chemotherapy has not yet been reached, with the possible exception of miRNA-200 and Let-7 families (see Table 10.11).

4.2.4 State of the Art

On the basis of the genomic landscape described, the following can be affirmed:

- Genomic and methylation analyses have only recently been carried out on a genome-wide level, and accordingly only a limited number of promising signatures and predictors have emerged;
- In contrast, gene and miRNA expression analyses, being based on more mature technologies, have provided a larger number of promising signatures and predictors.

In any case, not all studies used the same platform and techniques for data analysis and the signatures are often non-overlapping and share only a small number of genes. These differences do not invalidate the predictive ability of signatures, but they make it imperative to confirm gene signatures in large, independent sets of patient samples.

Table 10.11 miRNA contributions to prognosis and prediction

Author (publication year)	Methodology	Platform	No. and type of samples	Main conclusion
Zhang (2008) [119]	Microarray chip	Ohio State University OSU.CCC version 3	78 advanced stage EOC (see also Table 10.7)	Downregulation of miRNA cluster at Dlk-Gtl2 domain was associated with poor survival
Hu (2009) [144]	qRT-PCR profiling	96 custom preselected miRNAs	55 advanced stage EOC	Downregulation of 3 miRNAs belonging to the miR-200 family was associated with cancer recurrence and overall survival
Yang (2008) [145]	Microarray chip	Ohio State University OSU.CCC version 3	72 advanced stage EOC	Downregulation of let-7i expression was associated with shorter TTP. PCR-validation on an independent sample set of 62 cases
Yang (2008) [146]	Oligonucleotides array	Custom-based, 515 human and mouse miRNAs probes	10 EOC, 10 immortalized OSE samples; 30 EOC samples as validation set	Identification of 36 deregulated miRNAs; 3 were independently validated. Overexpression of miR-214 associated with chemoresistance in vitro
Eitan (2009) [147]	Two color microarray	Custom preselected 600 human miRNA probes	19 early-stage vs. 38 advanced-stage serous and endometriod EOC samples	Overexpression of miR-23a and miR-27a was associated with poor overall and TTP in late stage EOC patients
Chao (2011) [148]	PCR array	Taqman miRNA assays human panel early access (157 probes for mature miRNA)	Profiling on 7 EOC and 2 OSE cell lines. Validation on 176 EOC and 20 benign ovarian tissue samples.	Higher levels of miR187 and miR-200a were associated with better overall and TTP

(continued)

Table 10.11 (continued)

Author (publication year)	Methodology	Platform	No. and type of samples	Main conclusion
Marchini (2011) [149]	Two color microarray chip	Human miRNA microarray G4470B (Agilent)	93 (51 for training and 38 for test set) stage I EOC samples and 55 independent stage I EOC samples for PCR-validation set	Downregulation of miR-200c is an independent prognostic factor of poor overall and TTP
Shih (2011) [150]	Two color microarray chip	Human miRNA microarray V1.0 (G4770A) (Agilent)	Profiling of 62 advanced stage EOC patients. Validation by PCR in 123 patients	Identification of a signature (high expression of miR410 and miR-645) was associated with poor survival, validated by PCR
Kim (2010) [151]	Oligonucleotides array	miRNA V1 (Illumina)	103 EOC samples (all stages) of SC and MC including 15 benign and 34 LMP	A 3 miRNA signature (miR-519a, -153 and −485-5p) was associated with shorter TTP in the subgroup of advanced stage patients
Bagnoli (2011) [102]	Oligonucleotides array	miRNA V2 (Illumina)	Training set 55 advanced stage EOC; test set: 30 advanced stage EOC. Validation by qRT-PCR on 45 independent EOC and in silico on TCGA's dataset [32]	Identification of an 8 miRNA signature (all on chromosome X), whose low expression was associated with early relapse in advanced stages EOC patients (See Fig. 10.4)

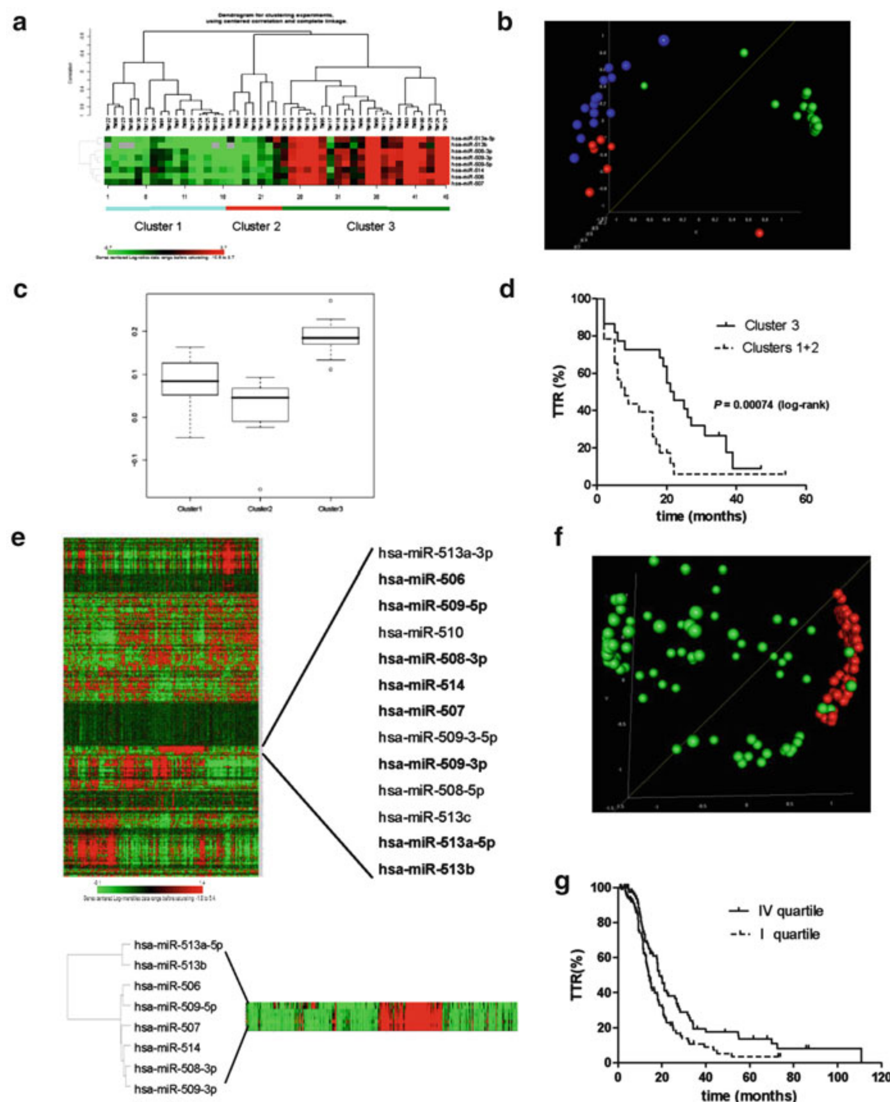

Fig. 10.4 ***Down-regulation of chrXq27.3 miRNAs associated with shorter TTR.*** (**a**) Unsupervised clustering of validation set samples, according to chrXq27.3 miRNA expression by qRT-PCR. (**b**) Multidimensional Scaling (*MDS*) analysis (generated using uncentered correlation) preserving the pair-wise similarities between samples in a three-dimensional graphical representation without forcing the samples into specific clusters as done by hierarchical clustering ($p < 0.001$). (**c**) Principal component analysis showing the eigenvalues for the three clusters Kruskal-Wallis test: *p*-value <0.0001. (**d**) Kaplan-Meier survival curves of patients included in the validation set stratified according to chrXq27.3 cluster classification, clusters 1 and 2 (*dotted line*) and cluster 3 (*continuous line*). Curves were compared using the log-rank test; $p = 0.00074$. Data were i*n silico* validated on TGCA dataset. (**e**) Unsupervised clustering of whole miRNome profile in the TGCA

5 Future Directions and Conclusions

5.1 New Technologies

5.1.1 High-Density SNP Arrays

It was estimated that between 5 and 12% of the human genome is involved in copy number variability. This variability may be either hereditary, involving copy number variations (CNVs) or somatic, involving copy number alterations (CNAs), defined as variants interesting only a subset of somatic cells and eventually, tumor cells. The most suitable platforms to identify both types of variants are high-density SNP (HD-SNP) arrays. These arrays have high resolution and can simultaneously capture copy number and LOH information, thus enabling a near-complete characterization of genome aberrations.

A recent investigation by Ostrovnaya et al. [99] developed a statistical model that can identify both CNVs and CNAs by analyzing the data on HD-SNP arrays available from the TCGA project on glioblastoma. This was chosen because a large amount of copy number data is available from matched tumor and normal samples, hybridized against a common reference, as well as against normal tissues alone. This model was applied to a subset of 38 matched EOC/normal tissue from TCGA data [32], and a prediction accuracy of about 90% was obtained.

Using this new technology, three studies, two of which were applied to TCGA samples, in addition to broadening knowledge in the genomic field, appear to provide new hope in a challenging area of classification for the pathologist, i.e. the discrimination of primary EOC and ovarian metastases after primary breast cancer [100], and in prognosis [101] (Table 10.12).

5.1.2 Whole Exome Sequencing

In the TCGA comprehensive examination of EOC genomics, massively parallel sequencing provided whole exome DNA sequence information for 316 HGS samples and matched normal samples [32]. Two different algorithms identified nine significantly mutated genes and comparison with databases of somatic mutations

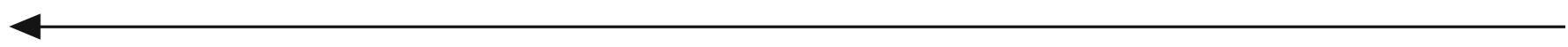

Fig. 10.4 (continued) study for stage III and IV EOC with TTR data revealing a highly correlated cluster of 13 miRNAs all belonging to chrXq27.3 with a magnification of the eight chrXq27.3 miRNAs associated to TTR in our work. (**f**) MDS analysis based on the expression of the 8 chrXq27.3 miRNAs including two groups of patients: first PC1 quartile (*green*) and last PC1 quartile (*red*). The *p*-value of global clustering test by Euclidean distance is <0.001. (**g**) Kaplan-Meier survival curves related to the two sub-groups of patients. First PC1 quartile (*dotted line*) and last PC1 quartile (*continuous line*); curves were compared using the log-rank test; $p = 0.0092$ (Legend modified from Bagnoli M et al. [102])

Table 10.12 New genomic technologies and data integration

Author (publication year)	Methodology	Platform	No. and type of samples	Main conclusion
Gorringe (2010) [101]	SNP array	50 K XbaI, 250 K StyI arrays 500 K arrays, SNP6.0 -1.8 M probe sets (Affymetrix)	398 (including 163) TCGA advanced stage, HGS [32]	Identification of the most frequent aberrations and their interactions. Potential association between CNAs and patient outcome
Meyniel (2010) [100]	SNP array	GeneChip® Mapping 50 K Xba Array or Genome-Wide Human SNP Array 6.0 (for one pair) (Affymetrix)	16 EOC patients with a previous history of breast cancer	SNP array analysis can distinguish primary EOC from breast cancer metastases to ovaries
TCGA (2011) [32]	SNP array	1 M Arrays (Agilent)	489 EOC and matched control (total = 978)	Regional aberrations affecting extended chromosome regions: 8 gains, 22 losses; Focal aberrations: 63 amplifications, 50 deletions (in more than 20% of cases focal amplification of CCNE1, MYC, and MECOM was observed)
Integration				
Etemadmoghadam (2009) [60]	SNP array and gene expression	50K *XbaI* SNP array (Affymetrix)	85 advanced stage EOC	Two subsets were identified within chemotherapy resistant patients, one with a high CCNE1 copy number and the other without CCNE1 amplification but characterized by overexpression of genes involved in ECM structure and cell adhesion
Ramakrishna (2010) [152]	SNP array and gene expression	Human SNP Array 6.0 and Human Gene1.0 ST Array (Affymetrix)	68 primary EOC	Identification of candidate genes as potential dominant drivers of ovarian tumorigenesis by combining the high frequency and high amplitude analyses and targeting the most strongly overexpressed genes

(continued)

Table 10.12 (continued)

Author (publication year)	Methodology	Platform	No. and type of samples	Main conclusion
Wrzeszczynski (2011) [103]	SNP array, methylation and gene expression	High-density oligonucleotide array 85K and 390K CpGs array (Nimblegene), U133 A array (Affymetrix)	421 (including 379) TCGA advanced stage, HGS [32]	156 genes copy number variation (CNV) correlated with changes in expression; 611 potential oncogenes with DNA methylation and expression changes in CNV genes. See Fig. 10.5
Ciriello (2011) [153]	SNP array, methylation and gene expression, whole-exome sequencing	High-density oligonucleotide array 85K and 390 K CpGs array (Nimblegene), U133 A array (Affymetrix)	316 advanced stage HGS from TCGA [32]	The Mutual Exclusivity Modules (MEMo) algorithm identifies mutual exclusivity between BRCA genes and members of the Rb pathway, providing a new hypothesis for the genomic instability in EOC and shedding new light on CCNE1 as a marker for poor prognosis

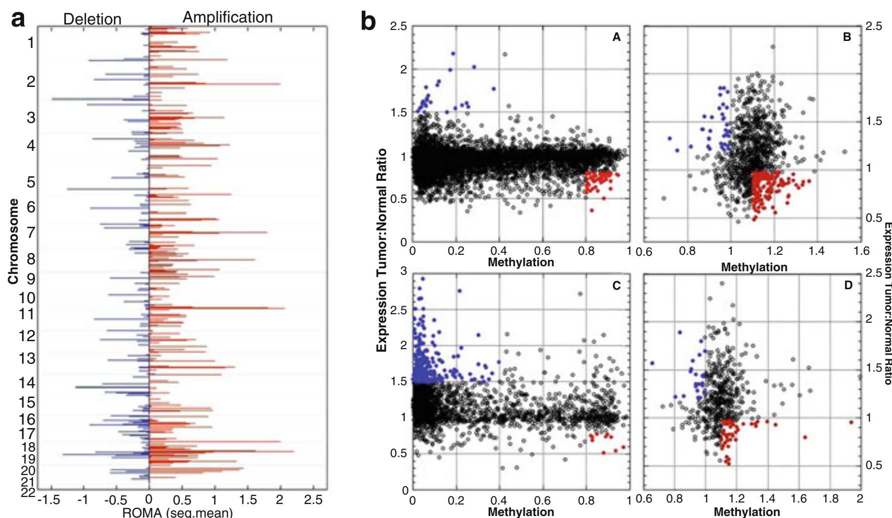

Fig. 10.5 ***Amplification and deletion breakpoint variability among ROMA segments and oncogenic and tumor suppressor features in ovarian cancer*.** (**a**) Breakpoint positions of copy number variability (deletions depicted in *blue*, amplifications depicted in red) in 22 chromosomes are shown as determined from Representational Oligonucleotide Microarray Analysis (*ROMA*) generated segmentation data. The initial altering deletion or amplification genomic position is depicted from all 42 ovarian tumor cancer samples. (**b**) Genes (*all points*) with extreme copy number variation from the TCGA (A and C) and MSKCC (**B** and **D**) data sets. Methylation and tumor to normal expression ratio was then compared for genes at low CNV (**A** and **B**) and high CNV (**C** and **D**). Genes with oncogenic features (*blue ovals*; high expression and low methylation) and tumor suppressor features (*red ovals*; low expression and high methylation) were identified (Legend modified from Wrzeszczynki et al. [103])

provided evidence of other mutations rare but with a driver oncogenic role. Mutation-driven changes in protein function were deduced from evolutionary information. Overall, the most frequently mutated gene was TP53 (mutated in 303 samples (97%)).

5.1.3 Data Integration

A global view of the DNA-RNA relationships, combining the effects of gene alteration and epigenetic control on transcriptional changes, can be obtained only by integration of different genome-wide approaches. At present, only a few such studies in the genomic landscape of EOC have been performed.

The first study exploiting SNP arrays and gene expression is that by Etemadmoghadam et al. [60] (Table 10.12). This LOH survey is the most comprehensive in EOC to date, and the authors, in addition to the association of CCNE1 amplification

with overall survival and treatment resistance, described two subsets within the chemotherapy resistant patients, one with a high CCNE1 copy number and the other without CCNE1 amplification but characterized by overexpression of genes involved in the ECM structure and cell adhesion. Thus, the integration of gene expression data and the localization of high-frequency LOH regions could suggest some promising candidates for further study.

Additional studies have exploited the concept of data integration and their relevant conclusions are summarized in Table 10.12.

5.2 Conclusions and Perspectives

While it appears that much progress has been made in the last 10 years, EOC and its genomic landscape have yet to be fully characterized. Overall EOC landscape is based on analyses performed on total number of patients ranging from 200 to more than 2,000 depending on the area of study. The TCGA project in EOC, whose central goal is the identification of new targets for novel therapeutic approaches, strongly contributed to increase knowledge in this field.

The heterogeneity of the disease, combined with genetic instability of Type II tumors, have limited the deciphering of this pathology also in the larger studies such as that of the TCGA project. Thus, studies in large numbers of clinical samples (tumors or blood), well characterized for both biological and clinic aspects, are still required to better understand EOC. In particular, in the case of early detection, improvement in terms of accuracy and further confirmation of reliability as specific markers in adequately-sized prospective studies are needed; in the case of prognosis and prediction, it is imperative to confirm potential genetic signatures in large, well characterized independent sets of patient samples obtained from multicenter randomized phase III clinical trials.

Another important challenge to take into consideration is not the acquisition of exquisitely detailed genomic information, but making sense of it. In fact, researchers should be able to handle terabytes of data per sample, and also determine the most appropriate bioinformatic and statistical tests to translate these data into meaningful biological interpretations. This aspect requires a strict collaboration between clinicians, biologists, biostatisticians, and bioinformaticians.

In conclusion, the current state of the art is probably summarized best in a sentence by Winston Churchill: “Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.”

The use of homogeneous sample sets adhering to the highest methodological standards, ideally coming from large clinical trials or carefully-annotated prospective observational studies, will allow conducting adequately powered evaluations and pre-defined subgroup analyses. These appropriate sample sets, combined with the introduction of new high-resolution technologies and the integration of data raised by multiple high-throughput genome-wide techniques using sophisticated

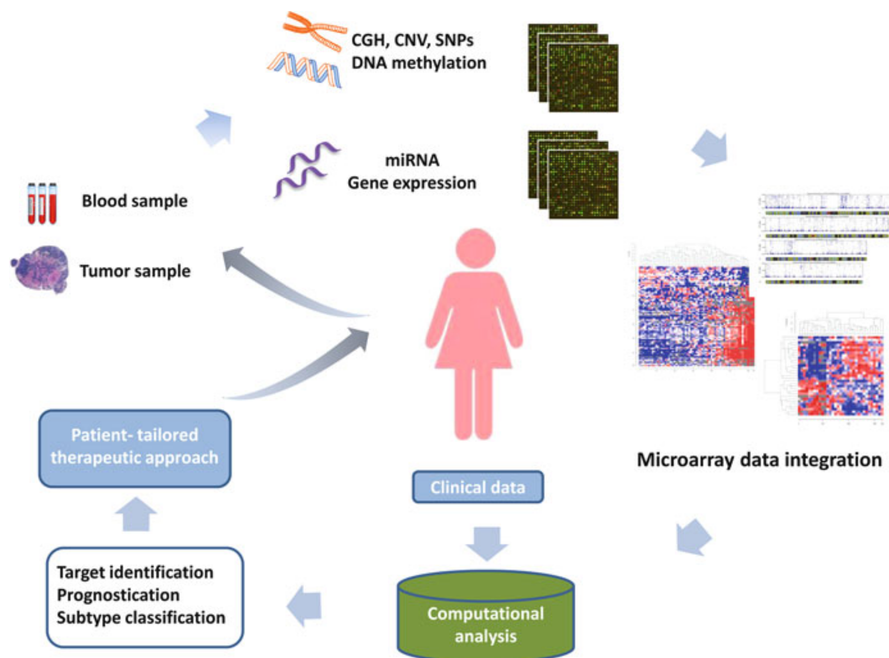

Fig. 10.6 The virtuous circle of EOC genomic landscape: potential translational applications for a personalized approach to disease treatment

bioinformatics analysis, will hopefully enable a global view of the DNA-RNA relationships and ultimately lead to identification of clinically useful biomarkers for patients with EOC, as illustrated in Fig. 10.6.

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Chapter 11
Genetics of Endometrial Carcinoma

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Abstract Endometrial cancer (EC) is the most common gynaecological malignancy in the western world and it comprises a heterogeneous group of tumours with distinct risk factors, clinical presentation, and histopathological features. Two main groups of EC exist, endometrioid endometrial carcinomas (EECs or type I) and non endometrioid endometrial carcinoma (NEECs or type II), which evolve via distinct molecular pathways. The most common molecular alterations associated with EECs affect the phosphoinositide 3-kinase (PI3K)/Akt pathway due to mutations in *PTEN* or *PI3KCA*. Other pathways, such as the RAS-RAF-MEK-ERK, FGF and WNT signalling pathways are also frequently affected by gene mutations or epigenetic changes. In addition, a group of sporadic EECs are characterized by microsatellite instability due to DNA mismatch repair (MMR) deficiency secondary to promoter hypermethylation of *MLH1*. In addition, EC is the second most frequent malignancy in hereditary Lynch syndrome. MMR deficiency in these patients is secondary to germline mutations in *MLH1*, *MSH2* or *MSH6*. Finally, *ARID1A* mutations have been recently described in a subset of EECs.

Endometrial serous carcinoma is the most frequent histological type of NEEC and is characterized by alterations in *TP53* with secondary chromosomal instability, which leads to multiple chromosomal gains and losses, including amplification of oncogenes and loss of important tumour suppressor genes. By contrast, the molecular alterations in clear cell carcinomas, another histological type of NEEC,

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are poorly defined. Differences in genetic and epigenetic alterations between EEC and NEEC tumours are reflected in distinct gene expression profiles observed amongst different EC types. In the near future, careful molecular characterization of ECs must be necessary in order to implement new directed targeted therapies.

1 Introduction

Endometrial carcinoma (EC) is a primary malignant epithelial tumour that arises in the endometrium and that can invade the myometrium in order to spread to distant sites [1]. In developed countries, EC is the most common malignant primary tumour of the female genital tract, and it represents the fourth and fifth most-frequently diagnosed cancer in women in Europe and the USA respectively, although this incidence is lower in Japan and developing countries. Moreover, while the incidence of EC among black women is approximately half that in white women, the proportion of EC-related deaths is greater in black than in white women for reasons that remain poorly understood [2]. The median age at which these tumours are diagnosed is 61 years, although some specific subtypes are diagnosed later, such as serous or clear cell carcinoma.

EC falls into two broad categories based on epidemiological, clinical, pathological and molecular features. Type I EC is oestrogen-dependent and accounts for 80–85% of ECs. These tumours tend to be low grade endometrioid subtype carcinomas associated with unopposed oestrogenic stimulation, and they coexist with or are preceded by endometrial hyperplasia. Obesity, diabetes, hypertension, nulliparity, late menopause and other estrogenic factors are usually associated with this tumour type. Elevated serum oestrogen levels have also been demonstrated in patients with type I EC [3]. By contrast, types II EC are non-oestrogen-dependent and they occur in older postmenopausal women. While the most common type II EC is the serous subtype, other high grade histological types are also considered type II ECs, such as clear cell carcinoma, and they are independent of oestrogenic stimulation [4–6].

For both EC types, the most frequent clinical manifestation is abnormal uterine/postmenopausal bleeding or menometrorrhagia in younger women. Occasionally tumours are asymptomatic and they are reported as incidental findings in surgical specimens or biopsies submitted for other reasons, such as analyses of infertility or uterine prolapse. A solid exophytic mass growing in the uterine corpus is the most common presentation regardless of the histological subtype, although different clinical and histological features have been described in cases originating in the lower segment of the uterus (see EC in Lynch Syndrome) [7]. Extension into the myometrium may be well-defined or grossly unidentifiable and in advanced cases, the tumour may penetrate the serosa or extend into the cervix.

A description of all the histological subtypes and variants of EC is beyond the scope of this chapter, although a brief description of the main histological types is provided to understand the basic pathological/molecular association. In addition, the different prognostic factors related to tumour progression are discussed.

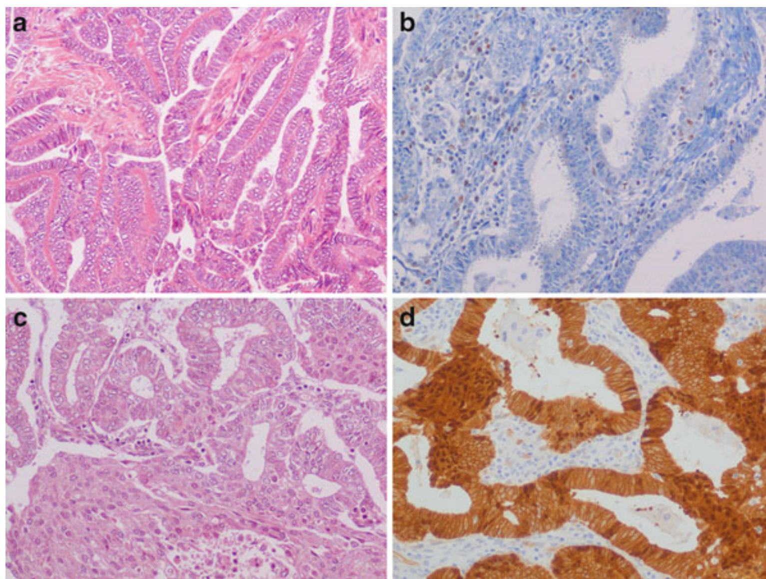

Fig. 11.1 Endometrioid Endometrial Carcinomas. Grade 1 endometrioid carcinoma (**a**) with loss of MLH1 expression (**b**). Endometrioid carcinoma with squamous morular metaplasia (**c**) and nuclear expression of β-catenin (**d**)

Endometrioid endometrial carcinoma (EEC) is characterized by a malignant glandular proliferation resembling the proliferative phase of the endometrium. The histological grade of EEC is based on both its architectural pattern and nuclear features. Grade 1 (G1) tumours are composed of less than 5% of solid areas and exhibit only mild to moderate nuclear atypia. By contrast, grade 3 (G3) EECs exhibit over 50% solid growth and/or very atypical nuclei. EEC may include different epithelial components such as squamous/morular, ciliated, mucinous, clear or eosinophilic cells (Fig. 11.1).

The most frequent non-endometrioid endometrial carcinomas (NEECs) are serous and clear cell carcinomas. Endometrial serous carcinoma (ESC) is histologically similar to its ovarian counterpart, presenting papillary architecture and high grade nuclei. By definition, ESC is considered a high grade adenocarcinoma and thus, it is not graded. Patients are often of advanced age and they may present evidence of disseminated disease, including lymph node metastasis. The overall 5-year survival rate ranges from 40 to 60% [5, 8, 9] (Fig. 11.2).

Clear cell carcinoma (CCC) of the endometrium is composed of clear or hobnail cells growing in different architectural patterns. CCC is the second most frequent type II EC, representing about 1–6% of all ECs [5, 8], and it is a high grade neoplasm with aggressive clinical behaviour. The mean age at diagnosis is 68 years and the 5-year survival rates vary from 20 to 65% [1, 5, 8].

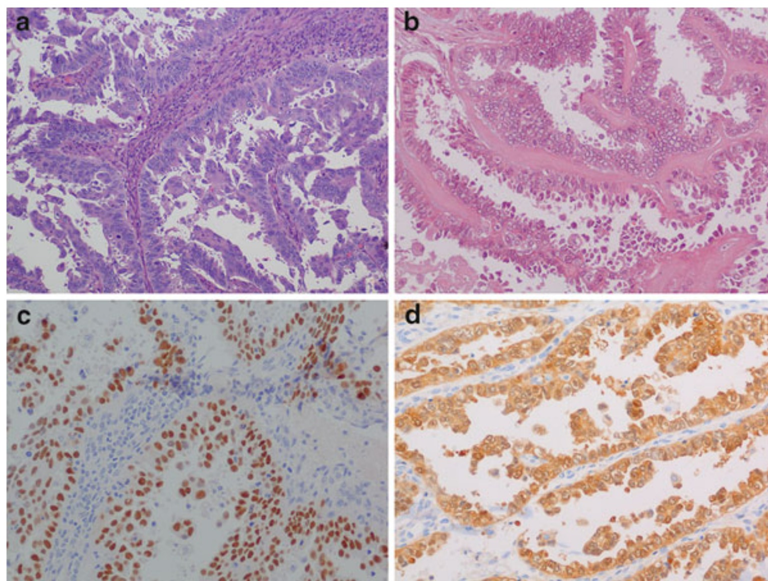

Fig. 11.2 Non-endometrioid endometrial carcinomas. Endometrial serous carcinoma (**a**). Endometrial clear cell carcinoma (**b**). Positive expression of p53 in an endometrial serous carcinoma (**c**). Positive expression of p16 in an endometrial serous carcinoma (**d**)

A subset of ECs exhibits areas of type I (endometrioid or mucinous) and type II ECs (serous or clear cells), with the minor component representing at least 10% of the neoplasm. These tumours are classified as mixed carcinomas and when the type II EC component represents at least 25% of the tumour volume the prognosis is commonly poor [1, 5].

Both uterine and extrauterine factors have been implicated in the recurrence of ECs. Different multivariate analyses have been proposed in order to calculate an accurate prognosis for each patient. Indeed, while tumour type, histological grade, depth of myometrial invasion and nodal involvement appear to be the most important risk factors [1], age, race and socioeconomic status are also indicators of prognosis. Women younger than 45 years of age have a better prognosis than those over 45, due to the significantly higher proportion of early-stage disease at diagnosis. There is a low prevalence of EC in African-American women, although the proportion of high-grade tumours is higher among this racial group and they develop less favourable histological sub-types than white women [5, 10].

Surgical stage is the most important uterine factor and it is a useful predictive factor to plan appropriate treatment. Patients with no extrauterine disease, cervical involvement or vascular invasion have a low risk of recurrence. Indeed, myometrial invasion is one of the indicators of surgical stage and independently of tumour grade, the recurrence rate increases from only 1% in tumours without myometrial infiltration to 15% in those with outer-third invasion of the myometrium. The

frequency of lymph node metastasis is also related to the depth of myometrial infiltration [5]. According to the International Federation of Gynaecologists and Obstetricians (FIGO) staging system (2009), tumours with cervical stroma involvement are categorized as stage II and thus, this is considered a risk factor. However, when other risk factors are considered, its prognostic significance is unclear. Venous or lymphatic invasion is also considered a strong predictor of recurrence and extrapelvic metastasis, irrespective of the histologic grade, deep myometrial invasion or other variables [11, 12]. Among the extrauterine risk factors, the involvement of para-aortic lymph nodes is the most important prognostic predictor, and the disease-free survival (DFS) at 5 years falls from 85% in patients with no para-aortic lymph node involvement to 36% for patients in whom nodes are affected [13].

2 Genetics of Endometrial Carcinoma

2.1 Endometrioid Endometrial Carcinoma

2.1.1 Mismatch Repair Deficiency

Repetitive DNA sequences, known as microsatellites, are widely dispersed throughout the genome and due to their repetitive nature these sites are susceptible to slippage errors of DNA polymerase during replication, resulting in deletions or insertions. Mismatch repair defects secondary to *hMLH1, hMSH2, hMSH6* and *hPMS2* genes cause variations in the size of microsatellites throughout the genome, this phenomenon known as microsatellite inestability (MSI) is a molecular hallmark of DNA MMR deficiency. In endometrial cancer, MSI occurs in two distinct clinical settings: in 15–20% of sporadic tumours, secondary to *hMLH1* methylation; and in Lynch syndrome due to inherited germ-line mutations in the *hMLH1, hMSH2, hMSH6* or *hPMS2* genes.

Studies analyzing MSI in sporadic ECs due to *hMLH1* promoter hypermethylation reported an endometrioid phenotype in over 95% of cases [14, 15]. In these patients, the mean age of presentation was 66.8 years, as opposed to that of 63.6 years in microsatellite stable (MSS) carcinomas. In addition, a lower body mass index was associated with MSI-positive EECs rather than MSS EECs. Based on these findings, it was hypothesized that hyper-oestrogenism may play a less important role in endometrial carcinogenesis with MMR defects.

Analyses of mutations in microsatellites located in non-coding regions, such as *BAT25* and *BAT26,* are used for diagnostic purposes (the Bethesda panel). Mutations involving microsatellite foci in coding regions of certain growth regulatory genes are implicated in tumour progression, such as those in *PTEN*, *BAX*, *IGFRII*, *TGFβRII*, *hMSH6* and *hMSH3* [16–18]. Mutations in *PTEN* are frequent in MSI-positive EEC (see below). *TGFβRII* contains a 10-microsatellite sequence that is targeted by frameshift mutations and that is frequently mutated in MSI-positive colorectal

cancer and in 0%–24% of MSI-positive EECs. Mutations in *BAX* (in 16%), *IGFRII* (in 55%), *MSH3* (in 0%–14%) and *MSH6* (in 0%–17%) have also been reported [16–22].

Several studies have analyzed the morphological features associated with MSI, irrespective of the sporadic or hereditary nature of the tumours. Frequently MSI-positive EECs tumours have peritumoural lymphocytic infiltration and tumour-infiltrating lymphocytes (40 tumour infiltrating lymphocytes/10 high-power fields) [23], and some MSI-positive EECs exhibit areas of dedifferentiation. The hMLH1 and hPMS2 proteins are frequently absent from sporadic MSI-positive EECs and the *hMLH1* gene promoter is often hypermethylated. Unlike MSI-positive colorectal cancer, BRAF mutations are very infrequent in MSI-positive EECs.

Several studies have analyzed the possible association between MSI and specific clinicopathological variables, such as grade, myometrial infiltration, lymphovascular invasion, stage and prognosis, producing quite contradictory results (for a review, see [24]). Thus, additional studies are required to determine whether MSI *status* modulates the therapeutic response in EEC.

2.1.2 Alterations in the Phosphoinositide 3-Kinase (PI3K)/Akt Pathway

In EEC, the constitutive PI3K-AKT pathway is frequently activated in response to alterations to certain genes, such as those inactivating *PTEN*, mutations or amplifications of *PIK3CA* and somatic missense mutations within AKT kinases. *PTEN* is a tumour suppressor gene located at chromosome 10q23.3, which plays an important role in regulating the cell cycle and apoptosis, and it regulates cell survival and proliferation through its antagonism of the PI3K/Akt pathway. The PTEN protein exhibits both lipid and protein phosphatase activities, each fulfilling different functions.

The inactivation of *PTEN*, induced by somatic mutations and/or loss of heterozygosity (LOH), leads to a loss of protein expression and is the most frequent alteration in ECs [25, 26], with somatic mutations of *PTEN* found in 37–61% of EECs and loss of heterozygosity (LOH) in 40% (see Table 11.1). These alterations do not always coexist, suggesting monoallelic inactivation of *PTEN* in a number of cases [27–31]. The most frequent types of mutation are missense substitutions, which account for 33% of all *PTEN* mutations, followed by frameshift deletions and nonsense substitutions (24% and 14%, respectively). Mutations are most frequent in exon 5, mainly in the arginine encoding codon 130, which is essential to catalyse the dephosphorylation of specific PTEN substrates. Several studies reported that 60% of mutations that affect codon 130 are missense substitutions (389G>A and 388C>G) and 30% nonsense substitutions (388C>T), while the remainder involve deletions of the G at position 389 [29–32]. In addition, exon 7 frequently accumulates nonsense substitutions (697C>T in codon 233), while exon 8 was frequently affected between codons 314 and 321 (codons 319 and 318 were affected by the deletion of 4 nucleotides, ACTT and CTTA, respectively).

Table 11.1 Frequency of mutations in different histological types of Endometrial Carcinoma

	Type I	Type II	
Gene	EEC (%)	ESC (%)	CCC (%)
PTEN	37–61	13	8
PIK3CA	36	31	46
PIK3R1	41	9	20
AKT1/2/3	4–12	4–12	4–12
KRAS	10–30	5	10
BRAF	0–21	0	0
FGFR2	10–16	4	10
CTNNB1	12–30	0	0
ARID1A	40	0	0
TP53	34	90	9

Based on www.sanger.ac.uk and references in the text

CCC clear cell carcinoma, *EEC* endometrioid endometrial carcinoma, *ESC* uterine serous carcinoma

Several groups have described a higher frequency of *PTEN* mutations in EC with MMR deficiency and MSI, with *PTEN* mutations detected in up to 86% of MSI-positive tumours as compared with 30% of MSS tumours. A higher frequency of multiple *PTEN* mutations in MSI-positive ECs has also been reported (60% in MSI-positive vs 25% in MSS) [27, 29, 31, 33, 34]. These defects in the *PTEN* gene have been attributed to defects in the DNA repair mechanism, as several analyses of MSI-positive EECs identified small deletions or insertions in short coding mononucleotide repeats ((A)6 in exon 3 and exon 8), two direct repeats (TACT/TACT and ACTT/ACTT) and a palindromic structure (AGTA-NN-TACT) in exon 8 [30, 33, 34]. However, other studies reported that somatic *PTEN* mutations may precede alterations in MMR in up to 50% of cases, as frameshift mutations in the (A)6 tracts occurred with the same frequency in MSI-positive and MSS tumours [29, 30, 33, 34].

The prognostic significance of *PTEN* mutations remains unclear. It is claimed that *PTEN* mutations may be associated with low rates of recurrence and better overall survival (OS), as defined by favourable pathological features such as low grade tumours confined to the endometrium [35]. However, higher incidences of *PTEN* mutations have also been reported in advanced tumours at FIGO stage Ic (up to 72%) as opposed to FIGO stage Ia (56%), as well as in less differentiated versus well-differentiated carcinomas (81% in G2 vs 44% in G1 ECs) [30]. Furthermore, mutations outside exons 5–7 of *PTEN* may be molecular predictors of favourable survival, independent of the clinical and pathological characteristics of the tumours [36]. Indeed, patients with MSI-positive and *PTEN* mutations were diagnosed at more advance stages of progression and exhibited a worse prognosis than patients with *PTEN* mutations alone [33, 37].

PI3K is a heterodimer comprised of a catalytic subunit (p110α) encoded by *PIK3CA*, which is located at chromosome 3q26.32, and a regulatory subunit (p85α)

encoded by *PIK3R1*. In quiescent cells, p85α binds to p110α and inhibits the stabilization and catalytic activity of p110α. PI3K activates AKT and a wide range of downstream effectors that regulate multiple cellular activities, including cell proliferation, survival and migration [38]. A high prevalence of mutations in the *PIK3CA* gene has been reported in EECs (up to 36%) [32, 38–42], with most studies focusing on exons 9 and 20, that encode the C-terminal helical and kinase domains of p110 α [40, 41]. Codons 542 and 545 in exon 9, and 1047 in exon 20 [40, 42, 43] are those most commonly affected. Mutations within exons 1–7, in which the N-terminal domains of p110α that include the p85/adaptor binding domain (ABD), ADB-RBD linker region and C2 domain are encoded, are as frequent as mutations within exons 9 and 20. Indeed, R88, R3 and K111 within the ABD, and E453 and L45 within the C2 domain have been identified as recurrent mutation hotspots [32, 42]. Recently, mutations within the *PI3K* regulatory subunit have been reported in up to 43% of EECs, preferentially localized in the p85α-iSH2 domain that mediates binding to p110α [41]. A significant association between *PIK3CA* and *PTEN* mutations has also been observed, suggesting an additive effect of these alterations in the activation of the PI3K/AKT pathway [40–42]. *PIK3CA* and *KRAS* mutations appear to be mutually exclusive alterations [39, 42, 43]. However, their association with other genetic defects, such as *CTNNB1* mutations or MSI characteristic of EEC, remains to be established [40, 41]. The prognostic significance of *PI3K* mutations is controversial and while *PIK3R1* mutation *status* was reported to have no prognostic implication in some studies [41], link between *PIK3CA* mutations and adverse clinicopathological parameters, such as grade and stage, has been described elsewhere [42]. Moreover, grade and stage have been linked with mutational type, as mutations in exon 20 are observed more frequently in high-grade than in low-grade EECs (67 vs 33%), while grade 1 ECCs are more frequently associated with exon 9 mutations (up to 57%) [40]. *PI3KCA* amplification has also been reported in 12% of EECs, occurring independently of mutational events at the same *locus*, and they are strongly associated with age, suggesting a role of *PIK3CA* amplification in the initiation and progress of ECs in older women [42].

The AKT serine/threonine kinases regulate diverse cellular processes (survival, proliferation, invasion and metabolism) and they are activated by direct recruitment to the plasma membrane via the pleckstrin homology (PH) domain. A missense mutation in the PH domain of *AKT1* (E17K), previously described in other tumours [44], was demonstrated in 2% of EECs [45]. Interestingly, the two cases that displayed *AKT1* mutations did not exhibit any mutations or LOH in *PTEN*, nor mutations in *PIK3CA* or *KRAS*. Subsequently, *AKT1* mutations were demonstrated in 4–12% of EECs [46, 47], while additional mutations in other AKT family members were described in the regulatory C-terminal (D399N), catalytic kinase domain (R368C) and pleckstrin homology (D32H) domains of *AKT2*, and in the regulatory C-terminal domain of *AKT3* [47]. Three of these mutations were identified in tumours harbouring mutations of *PTEN* and/or amplification or mutation of *PIK3CA*. However, in agreement with the reports previously mentioned, the E17K mutation was not associated with alterations in *PTEN* or *PIK3CA*. It is therefore possible that these AKT family mutations have different functional effects to those

of *PTEN* or *PIK3CA* mutations. The prognostic significance of *AKT* mutations remains unclear as *AKT* mutations have been associated with low-grade estrogen receptor-positive early-stage tumours [45, 48], while in other studies they were only reported in high-grade, advanced stage tumours [46].

The tumour suppressor gene *PAR-4 (PAWR)* maps to chromosome 12q21 and it encodes a WT1-interacting protein that acts as a transcriptional repressor. This protein contains a putative leucine zipper domain that binds to the zinc finger DNA binding domain of WT1 and interestingly, PAR-4 is specifically upregulated during apoptosis of prostate cells [49–51]. Based on previous reports describing the development of ECs in *PAR-4*-deficient mice, *PAR-4* was shown to be downregulated in up to 40% of a subset of EECs [52]. Although only one mutation in a single carcinoma was identified by a mutational analysis, *PAR-4* promoter hypermethylation was detected associated with low levels of PAR-4 protein in 32% of the tumours and it was more common in MSI-positive carcinomas. Moreover, low levels of PAR-4 were associated with ER positivity, and poorly differentiated EECs displayed similar PAR-4 levels as those with low and intermediate grades. No *PAR-4* gene abnormalities were associated with mutations in *PTEN*, *KRAS* or *CTNNB1* [52].

2.1.3 Alterations in the RAS-RAF-MEK-ERK Signalling Pathway

The RAS-RAF-MEK-ERK signalling pathway plays an important role in the development and progression of ECs. The *RAS* gene family consists of three closely related genes that encode proteins with GTPase activity, which are localized at the inner plasma cellular membrane and involved in several signal transduction pathways. Single point mutations in codon 12 and 13 of the *KRAS* gene result in a loss of GTPase activity and activation of the corresponding gene product. The frequency of *KRAS* mutations in EECs ranges from 10% to 30% [53, 54] and all of the mutations described are missense substitutions affecting codons 12 and 13, in 70% and 30% of cases, respectively [54, 55]. Moreover, the prevalence of KRAS mutations appears to be higher in MSI-positive EECs (42% vs 11%) [53, 54], yet this was not demonstrated in all studies [56]. Several reports found no significant differences in histological grade or stage among EECs associated with *KRAS* mutations [53, 56, 57], although tumours with *KRAS* mutations included early-stage tumours that have been associated with increased DFS [54].

BRAF encodes a tyrosine kinase involved in mitogenic signalling in the RAS-RAF-MEK-ERK MAP kinase pathway. An activating point mutation in *BRAF* causes unregulated constitutive activation of the tyrosine kinase, facilitating cell proliferation via the MAP kinase pathway. Contrary to the high frequencies of *BRAF* mutations described in colorectal cancer, only a few studies have reported *BRAF* mutations in EECs. Although in one study *BRAF* mutations were identified in 21% of EECs with no preference for grade or stage [55], in the majority of studies *BRAF* mutations are either found at a low incidence or not at all [56, 58].

2.1.4 Alterations in the Fibroblast Growth Factor (FGF) Signalling Pathway

Several findings suggest that the fibroblast growth factor (FGF) signalling, which is involved in many biological processes including embryogenesis, adult tissue homeostasis and cell proliferation, is implicated in EC. In 10–12% of ECs, and particularly in EECs (16%), somatic mutations in the tyrosine kinase receptor *FGFR2* have been reported that are identical to the germline mutations associated with craniosynostosis and skeletal dysplasia syndromes [54, 59, 60], the most common being S252W and N549K. *FGFR2* mutations are associated with enhanced FGF signalling and downstream activity, predominantly through the RAS-MAPK pathway. Interestingly, while mutations in *KRAS* and *FGFR2* are mutually exclusive events, *FGFR2* and *PTEN* mutations frequently coexist [28]. When evaluating the hypothetical correlation between clinicopathological variables and outcome, and the mutations in genes frequently altered in EEC in a large cohort of unselected EECs, *FGFR2* mutations were significantly less common in high *versus* low grade tumours (3% vs 11%), but they were associated with reduced DFS. When analysed in early stage tumours, *FGFR2* mutations were associated with decreased OS and DFS [54].

2.1.5 Alterations in the WNT Pathway

The β-catenin protein, encoded by the *CTNNB1* gene, is a component of the E-cadherin-catenin unit implicated in the maintenance of normal tissue architecture and the regulation of diverse developmental processes such as proliferation, differentiation, motility and survival and/or apoptosis. Depending on its cellular localization, β-catenin fulfils different functions: at the plasma membrane it mediates cell-cell adhesion by linking E-cadherin to the actin cytoskeleton along with α-catenin; in the nucleus, β-catenin acts as the main effector of the canonical WNT signalling cascade, interacting with members of the Lef-1/Tcf pathway and promoting the transcription of various genes implicated in growth control and cell cycling (*MYC, cyclin D1*), cell survival (*inhibitor of DNA binding-2 and MDR1*) and tumour invasion and metastasis (*matrilysin and VEGF*). AXIN 1 and 2 are multidomain proteins that contain binding sites for glycogen synthase kinase 3 beta (GSK-3β), β-catenin, adenomatous polyposis coli (APC) protein and PP2A, and they act as negative regulators of the WNT pathway by blocking the GSK-3β dependant turnover of β-catenin [61]. Together with GSK-3β, APC induces the phosphorylation of serine-threonine residues encoded by exon 3, inducing β-catenin degradation through the ubiquitin-proteasome pathway. Mutations in exon 3 of β-catenin, often missense mutations affecting the NH2 terminal regulatory domain (codons 32–45), have been described almost exclusively in EECs, and they were identified in 12–30% of EECs studied [31, 61–65]. Interestingly, these mutations appear irrespective of the presence of MSI or the mutational status of *PTEN* and *KRAS* [54, 61]. In fact, in EECs that display morule formation and/or squamous foci (up to 25% of cases) there is greater nuclear accumulation of β-catenin (84% in cases with morule formation and 45% in cases with squamous differentiation) and

more frequent mutations in *CTNNB1* (up to 54% of cases with morule formation), supporting a role for β-catenin in the morphological alterations in the tumour [65]. Nonetheless, the presence of cytoplasmic and nuclear β-catenin in up to 30% of EECs analyzed when specific mutations were not demonstrated (~25% of cases) [66], indicates that alterations in other genes in the WNT/β-catenin/Lef-1 pathway may be responsible for the effects of β-catenin on stabilisation and transcriptional activity in EEC. ECs do not usually harbour mutations in *APC* [61–63], although a number of studies have described LOH at the *APC locus* in up to 24% of ECs and hypermethylation of the *APC* gene promoter in 20–45% of ECs, effects that were significantly more frequent in type I carcinomas [61, 67]. Moreover, 12 single-nucleotide polymorphisms (SNPs) in *AXIN 1* have been observed but no mutations [61].

The prognostic significance of mutations in *CTNNB1* for EECs remains controversial. While a significant association between *CTNNB1* mutations and age, grade or stage was not identified in one study [61], an association with early onset and the absence of lymph node metastasis has been proposed [68]. A recent study demonstrated a significant association between *CTNNB1* mutations and tumour grade, with the mutation percentage dropping from 24% in grade 1 to 6% of grade 3 EECs [54], consistent with previous studies [64]. However, no association was identified with other clinicopathological features or patient outcome.

2.1.6 *ARID1A* Gene Mutations

ARID1A is a recently identified tumour suppressor gene located at chromosome 1p36 that encodes a large nuclear protein, BAF-250a. This protein is a key component of the multi-protein SWI/SNF complex involved in chromatin remodelling that plays an integral role in controlling gene expression and regulating widely diverse cellular processes, from differentiation during development and proliferation, to DNA repair and tumour suppression [69, 70]. *ARID1A* mutations were recently described in 43–56% of ovarian CCCs, 30% of ovarian low-grade endometrioid carcinomas and in one case of atypical endometriosis, a putative CCC precursor, suggesting that *ARID1A* loss is a relatively specific event in the genesis of these tumours [71, 72]. Interestingly, most *ARID1A* mutations are insertion/deletion mutations, leading to the generation of premature stop codons due to a frameshift, and giving rise to truncated proteins prone to degradation. A number of studies have demonstrated that the loss of BAF-250a protein is correlated with *ARID1A* mutation *status* [72, 73] and moreover, a high incidence (up to 40%) of *ARID1A* mutations have been reported in low-grade EECs [74]. ARID1A expression was recently analyzed in a subset of tumours arising at different sites, demonstrating that its loss is not common in non-gynaecological malignancies. Moreover, EECs exhibit the highest frequency of BAF-250a loss, which occurs in 29% of G1 and 2 EECs and in 39% of G3 EECs [75]. Interestingly, a recent study reported that in both G1 and G3 EECs, *ARID1A* mutations are significantly associated with concurrent mutations in *PTEN* and *PIK3CA*, suggesting a cooperative role of these pathways in EEC tumorigenesis [76].

2.2 Endometrial Serous Carcinoma

TP53 is a tumour suppressor that either induces apoptosis or prevents a cell from dividing when DNA damage occurs. Mutations in *p53* reduce a cell's ability to repair damage to DNA, consequently increasing the likelihood that mutations will remain in the genome and be passed on to successive generations. Mutations in *TP53* at mutational hotspots (exons 5–8 or 4–10) are the best characterized alterations in SC, with over 90% of cases harbouring a mutation in *TP53* [77–79]. Most commonly, these mutations are missense, resulting in *TP53* inactivation [78]. However, overexpression of p53 has been identified in about 76% of ESCs and its concordance with the mutational status has been demonstrated [80, 81], approximately 84% of cases with *TP53* mutations exhibiting significant protein overexpression [77]. Moreover, *TP53* mutations without protein overexpression may reflect the absence or instability of the protein product of the mutant gene [78].

TP53 mutations (again often missense mutations) have also been reported in up to 34% of EECs [40, 82, 83], primarily affecting exons 4–8, the coding domains responsible for *TP53* binding to DNA, or exon 10. *TP53* mutations and/or overexpression have been associated with clinicopathological features of these tumours, including high-grade, lymphovascular invasion and advanced FIGO stages [40, 82]. Moreover, several studies have investigated the prognostic significance of *TP53* overexpression and/or gene mutation, which is mostly associated with a high risk of recurrence and DFS, disease-specific and OS [83–87].

A common diagnostic problem is to draw a distinction between high-grade (G3) EECs and ESCs. To better differentiate these two entities, target enrichment sequencing was performed on 393 endometrial carcinomas from two large cohorts, sequencing exons from *ARID1A*, *PPP2R1A*, *PTEN*, *PIK3CA, KRAS*, *CTNNB1, TP53*, *BRAF* and *PPP2R5C* [76]. Each endometrial carcinoma subtype exhibited a distinct mutational profile and a high frequency of mutations of *PTEN*, *ARID1A* and *PIK3CA* was reported in grade 3 EECs (90%, 60% and 56% respectively). Mutations in *TP53* and/or *PPP2R1A* were detected in 75% of ESCs, accounting for the majority of aberrations in this subtype. Comparing G3 EECs and ESCs revealed a significant difference in the mutation frequencies for *ARID1A*, *PTEN*, *PIK3CA*, *CTNNB1*, *PPP2R1A* and *TP53*, and from the mutational profiles subtype outliers were identified, *i.e.,* cases diagnosed morphologically as one subtype but with a mutational profile suggestive of another. Careful review of these diagnostically challenging cases suggested that the original morphological classification was incorrect in most instances. While this nine-gene panel does not permit a purely molecular classification of endometrial carcinoma, it may serve as an adjunct to morphological classification and an aid in the classification of problematic cases. This may improve diagnostic reproducibility and help stratify patients for targeted therapeutics [76].

Several lines of evidence suggest that the pRB pathway (p16INKA/Cyclin D-CDK/pRb-E2F) is deregulated in ESC. Although p16, a cyclin-dependent kinase-4 inhibitor, is inactivated in many malignancies, this protein may occasionally accumulate in the cell during some neoplastic processes due to the loss of the negative feedback provided by functional pRB. It has been demonstrated that 92–100% of ESCs display diffuse p16 expression, which is significantly higher than the immunoreactivity seen in other histological subtypes of endometrial cancers tested to date [88, 89].

Recently, *PIK3CA* mutations were described in up to 31% of ECSs [40, 76, 90], being restricted to exon 20. In some cases, *TP53* and *PIK3CA* alterations coexisted, albeit rarely in conjunction with *PTEN* mutations. Moreover, while alterations in PI3K-AKT signalling alone did not influence OS, survival was reduced in patients with a dysregulated PI3K-AKT pathway (PIK3CA and/or PTEN alterations) and with *TP53* alterations, when compared with patients with *TP53* alterations alone [40].

One of the principal features of ESCs as opposed to EECs is the high level of chromosomal instability (losses and gains that involve large chromosomal regions and specific genes). This characteristic probably results from the mutations in *TP53* at early tumour stages, and it is associated with the amplification of genes like *CCND1*, *CCNE1*, *HER2*, *MYC* and *PIK3CA*, and with gene loss affecting suppressor genes such as *PTEN* and *CDH1*. The evidence for these alterations is principally derived from CGH and array-CGH based studies, as only a few studies have investigated alterations in specific genes.

HER2 is a member of the receptor tyrosine kinase family and its implication in cell proliferation is due to its influence on two signalling pathways: the RAS/RAF/MAP kinase and PI3K/AKT pathways. Santin et al. reported that 80% of ESCs at the II or III stages expressed HER2 and 42% exhibited *HER2* amplification [91]. Furthermore, it was demonstrated that serous carcinoma cells are sensitive to herceptin-mediated antibody-dependent cell cytotoxicity *in vitro*, but that they are chemoresistant *in vivo* [91], supporting the hypothetical use of trastuzumab in patients with serous carcinomas, particularly in chemoresistant cases. Overexpression of the HER2 protein product has been reported in 9–30% of ECs, with the highest frequencies in ESCs, and it was also associated with advanced-stage disease and worse progression-free and OS [92, 93]. Although previous studies found inconsistencies regarding HER2 overexpression and amplification, the recent Gynaecological Oncology Group (GOG) phase II trial of trastuzumab in advanced and recurrent endometrial cancer, found that in 28% of ESCs as opposed to 7% of EECs *HER2* was amplified, demonstrating a correlation between HER2 overexpression and *HER2* amplification [94]. However, no objective responses to trastuzumab therapy alone were reported in tumours displaying either overexpression or amplification of *HER2*.

In terms of genes that control the cell cycle, *CCND1* amplification was reported in 2.1% of EECs and in 26.3% of NEECs, a statistically significant difference [95]. Similarly, *CCNE1* was amplified in 16% of NEECs but in less than 3% of EECs [96]. Interestingly, *CCND1* and *CCNE1* amplification was mutually exclusive in

these studies. An approach combining expression profiling and SNP arrays for copy-number variation recently demonstrated that 3q26.32 amplification, predominantly in NEEC and high grade tumours, leads to aggressive tumour phenotypes via *PI3KCA* overexpression [97, 98]. Samuelson and coworkers [99] studied two regions of chromosomes 2 and 7 previously highlighted by qRT-PCR in a subset of 13 ECs, revealing the amplification of the *SDC1*, *MYCN*, *POMC*, *CDK6*, *TAC1* and *MET* genes in >50% of tumours, with amplification levels ranging from 5 to 20 copies per cell.

Classical (type 1) cadherins are transmembrane components of the cellular adherens junctions that predominantly mediate homotypic cell-to-cell adhesions. The cytoplasmic domains of cadherins are connected to the intracellular actin cytoskeletal network through their interaction with catenins, and thus, they are involved in a variety of cell signalling pathways. Epithelial cadherins (E-cadherins) are generally considered suppressors of tumour progression and invasiveness, and reduced E-cadherin expression in endometrial carcinomas is correlated with the serous histological type and with advanced stages. The underlying molecular basis for this downregulation or inactivation remains unclear, however, loss of heterozygosity of the *CDH1* (the gene coding for E-cadherin) is more common in NEECs (57%) than in EECs (22%) [100–102]. In addition, transcription factors involved in the epithelial to mesenchymal transition (EMT) suppress E-cadherin expression, such as the zinc-finger E-box-binding homeobox 1 (ZEB1), which are expressed in high-grade and type II endometrial carcinomas, and they increase the migratory and invasive properties of endometrial cancer cell lines [103]. Moreover, cadherin switching has been reported in serous carcinomas, with upregulation of P-cadherin accompanying the downregulation of E-cadherin [101, 102].

2.3 Clear Cell Carcinoma

As opposed to the serous type, alterations in *TP53* play a relatively minor role in endometrial CCC [104]. Given that mutations in *TP53* are also rarely observed in ovarian clear cell adenocarcinomas [105], CCC in the female genital tract may arise through a unique pathway [106]. *HER2* amplification has been reported in 16%–38% of CCCs, a frequency between those reported for EECs and ESCs [93, 94], while *PIK3CA* mutations have been described in 46% of CCCs [40]. As previously mentioned, *ARID1A* mutations have been described in a relatively high percentage of ovarian CCCs and in cases of atypical endometriosis, regarded as a precursor of CCC [71, 72]. Moreover, it was recently demonstrated that BAF-250a, was lost in 26% of endometrial CCCs. Interestingly, the authors reported a disproportionate increase in BAF-250a negative cases within a group of higher stage endometrial CCCs, suggesting a worse prognosis for CCC patients with BAF-250a negative tumours [75].

3 Ploidy and Chromosome Alterations

Over the last 20 years, DNA ploidy and DNA ploidy-related parameters have been used as a measure of gross genomic alterations in endometrial cancer. A clear correlation appears to exist between DNA ploidy and the histological subtypes of endometrial carcinoma. Accordingly, while most EECs are diploid, exhibiting few gross genomic/chromosomal aberrations, ESCs and CCCs are mostly aneuploid [11, 107, 108]. Many studies have investigated DNA ploidy as a potential parameter for better triage of patients with uncertain disease progression and to determine adjuvant treatment. Moreover, DNA ploidy has been proposed as a prognostic factor for patients with EC. Several studies have described worse outcomes in patients with aneuploid tumours, although multivariate analyses did not always identify DNA ploidy as an independent predictive parameter [109–113]. In a recent study [110], stage I and II endometrioid aneuploid tumours were subdivided into three subgroups based on the DNA index (DI): near diploid/aneuploid tumours with a DI ≤ 1.2; near tetraploid/aneuploid tumours with a DI > 1.2; and tetraploid tumours. In terms of recurrence rates, progression-free survival and overall survival, the worst outcome was found in patients with aneuploid tumours and a high DI, whereas aneuploid tumours with near-diploid DI were associated with intermediate recurrence and survival rates. Patients with diploid tumours had the best survival and lowest recurrence rates, similar to that of patients with tetraploid tumours. Taken together, these and other findings from both retrospective and prospective studies suggest a correlation between aneuploidy and disease progression/course [114, 115].

The chromosomal abnormalities found in EECs are normally restricted to hyperdiploid karyotypes, with a clear association between grade and karyotype complexity, supporting the view that tumour-phenotype can be altered by the accumulation of genomic imbalances [108, 116–122]. The most recurrent aberration reported in most studies is the gain of chromosome 1q in both EECs and NEECs, probably reflecting the presence of the isochromosome +(1) (q10) (see Table 11.2) [118, 123]. Genomic changes involving the long arm of chromosome 1 have been proposed as the primary changes in ECs, as these abnormalities are often the sole chromosomal aberration observed [118, 124]. Gain of chromosome 10 may also constitute an early event in tumour progression, as this has been observed among distinct ploidy groups of endometrioid adenocarcinomas [124]. Other recurrent chromosomal aberrations in EEC include gain of chromosome arms 8q and loss of Xp, 9p, 9q, 17p, 19p and 19q [117, 118].

NEEC often exhibit more complex imbalances than EECs. In addition to gains of 1q, frequent aberrations include gains of 8q and 20q, while a specific genomic change, gain of 5q, has been detected in ESC [116, 118]. Genomic changes involving gains of 3q and 11q have also been described in NEECs, where *PIK3CA* and *CCND1* are located, respectively [95, 98].

Table 11.2 Recurrent chromosomal aberrations in endometrial carcinoma

Chromosome alteration	Chromosome region	Abnormality	Cases
Balanced	7p11	t(7;9)(p11;q12)	2
	9q12	t(7;9)(p11;q12)	2
Unbalanced	1p13	del(1)(p13)	5
	1p21	del(1)(p21)	3
	1p36	add(1)(p36)	3
	1q10	der(1;16)(q10;p10)	3
	1q10	i(1)(q10)	14
	5p11	add(5)(p11)	3
	6p10	i(6)(p10)	2
	6q21	del(6)(q21)	4
	6q25	del(6)(q25)	4
	7q22	del(7)(q22)	2
	8p21	del(8)(p21)	2
	8q10	i(8)(q10)	4
	9q22	add(9)(q22)	2
	11p14	add(11)(p14)	3
	14p11	add(14)(p11)	2
	16p10	der(1;16)(q10;p10)	3
	16p13	add(16)(p13)	2
	17q10	i(17)(q10)	2
	18p11	del(18)(p11)	2
	19p13	add(19)(p13)	2
	22q13	add(22)(q13)	3
	Xp22	add(X)(p22)	3
Numerical	1	Trisomy/Polisomy	<6
	2		13
	3		3
	5		2
	6		4
	7		17
	8		3
	10		31
	11		2
	12		9
	20		5
	X		4
	1	Monosomy	2
	2		3
	4		3
	5		4
	7		3
	8		4
	9		3
	10		3

(continued)

Table 11.2 (continued)

Chromosome alteration	Chromosome region	Abnormality	Cases
	11		3
	12		5
	13		2
	14		2
	15		4
	16		5
	17		3
	18		4
	19		4
	20		4
	21		6
	22		5
	X		9

Described according to the ISCN. Data were extracted from the Mitelman database [123] by searching for adenocarcinoma tumors in uterine corpus

4 Gene Expression Profiles

Microarray platforms have been used to examine the gene expression profiles of different histological types of endometrial adenocarcinoma. The results of these analyses suggest distinct gene expression profiles for EEC, ESC and CCC, and they have identified new genes and pathways involved in the development of EC.

Among the genes upregulated in EECs are those involved in cell secretion (*MGB2*, *LTF*, *END1* and *END3*), adhesion (*CTNNA1*), extracellular matrix remodelling (*HSPG2* and *MMP11*), transcription (*NFYC*, *HOXB5*, *CHD3* and *REST*), and other basic cellular functions (*PPAP2C*) [125]. Interestingly, the most strongly dysregulated genes in EECs are secretory proteins, some of which are hormonally regulated, supporting the idea that EEC is a hormonally driven neoplasia. Genes overexpressed in NEECs relative to EECs include those involved in the control of the cell cycle and mitosis (*STK15, BUB1, CCNB2, PCNA,CDKN2A/p16/p14ARF*), metabolism (*MDH1, PGK1, GLDC*), transcription (*CREG*, *TCEB3* and *SMARCA3*), and/or transport (*RAB*). Table 11.3 shows some genes repeatedly modulated in specific histological types [125]. Interestingly, several high grade EECs exhibit gene expression profiles similar to those of NEECs [98].

The gene expression profiles of endometrioid, serous and clear cell carcinomas of the ovary and endometrium have been compared [106], and whereas serous and endometrioid carcinomas formed distinct ovarian and endometrial groups, CCCs did not group according to their organ of origin. Thus, clear cell tumours exhibited similar expression patterns regardless of their origin, even when compared with renal clear cell carcinomas. The profile specific to CCCs included genes involved in enhancing apoptotic signalling (*ASK1/GLRX*), inhibiting cellular proliferation (*TFP12*), and increasing resistance to chemotherapeutic agents (*ANXA4*

Table 11.3 Genes differentially expressed among different histological types of endometrial cancer

Endometrial tumour	Histological type	Genes overexpressed	Gene definition	**Function**
Type I	EEC	*AGR2*	Anterior gradient homolog 2 (Xenopus laevis)	Cell secretion
		MGB2	Mammaglobin 2	
		LTF	Lactotransferrin	
		END1	Endothelin 1	
		HSPG2	Heparan sulfate proteoglycan 2 (perlecan)	Extracellular matrix remodelling
		TFF3	Trefoil factor 3	Cell adhesion
		NCOR1	Nuclear receptor co-repressor 1	Transcription
		MSX2	Msh homeobox 2	
		ElF5	E47-like factor 5	
		FOXA2	Forkhead box A2	
		GAD1	Glutamate decarboxylase 1	Metabolism
		PPAP2C	Phosphatidic acid phosphatase type 2C	Basic cellular function
		LTB	Lymphotoxin beta (TNF superfamily, member 3)	Immune response/cytokines
Type II	ESC	*STK15*	Serine/Threonine kinase 15	Cell cycle
		BUB1	Budding uninhibited by benzimidazoles 1; Mitotic checkpoint serine/threonine protein kinase	
		CCNB2	Cyclin B2	
		CCNE2	G1/S-specific cyclin-E2	
		CDK2A/p16/p14ARF	Cyclin-dependent kinase inhibitor 2A/p16/p14ARF	
		PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	Regulates angiogenesis in endothelial cells
		MAL	Myelin and lymphocyte protein	Cell death
		IGF2	Insulin growth factor II (somatomedin A)	Cell growth

	HMMR	Hyaluronan-mediated motility receptor	Cell motility and migration
	FOLR1	Folate receptor 1 (adult)	Metabolism
	STAT1	Signal transducer and activator of transcription 1-alpha/beta	Transcription related
	UCHL1	Ubiquitin COOH-terminal esterase-like 1	Basic cellular function
	EPHA1	Ephrin receptor A1	Receptor tyrosine kinase
CCC	*TFP12*	Tissue factor pathway inhibitor 2	Inhibition of cellular proliferation
	GLRX	Glutaredoxin	Apoptosis
	MAP3K5/ASK1	Mitogen activated protein kinase k 5/apoptosis signal-regulating k 1	
	UGT1A1	UDP glycosyl-transferase 1 family, polypeptide A1	Resistance to chemotherapeutic agents
	ANXA4	Annexin A4 (ion channel)	
	KIAA1922	KIAA1922 protein (ciliary rootlet coiled-coil, rootletin pseudogene 3)	Unclear
	CXADR	Coxsackie virus and adenovirus receptor	Homophilic cell adhesion molecule
	RFX5	Regulatory factor X, 5	Influences HLA class II expression

From references [106, 125, 128, 197–200]
CCC clear cell carcinoma, *EEC* endometrioid endometrial carcinoma, *ESC* endometrial serous carcinoma

and *UGT1A1*), consistent with the slow growth and chemoresistance of clear cell tumours [106]. These findings suggest that a common treatment could be used for ovarian and endometrial clear cell tumours targeting differentially expressed genes and activated pathways, which could potentially replace the current organ-based approach.

Several genes implicated in endometrial carcinogenesis have been identified by gene expression analysis. In one study, *STK15* (*AURA*) was identified as one of the genes most strongly upregulated in NEEC [125], and a subsequent FISH analysis demonstrated a high rate of *AURA* amplification in NEEC, a molecular alteration that is very infrequent in EEC and which may contribute to the high degree of chromosomal instability observed in NEEC. Comparing samples of normal proliferative endometrium, atrophic endometrium and EECs [126], the *RUNX1/AML1* oncogene was shown to be one of the most intensely upregulated genes in EEC. This gene regulates the expression of many genes involved in hematopoietic cell development, and it was specifically upregulated more strongly in tumour stages associated with myometrial invasion. In the same study *ERM/ETV5* upregulation was described in EEC and correlated with that of *RUNX1/AML1*. As a result, a co-operative role of *ERM/ETV5* and *RUNX1/AML1* was proposed during the early stages of endometrial tumorigenesis, which may be associated with a switch to myometrial infiltration [127]. From gene expression profiles associated with different stages and prognoses for EEC and NEEC, *KIF14* was proposed as a marker of advanced stages and *NME3* as a metastasis suppressor in EEC [128]. The lysophosphatidic acid receptor 2 (*LPAR2*) was also proposed as an important extracellular signalling molecule that mediates cell proliferation, cell survival, migration, adhesion and angiogenesis, and as a potential indicator of late stage ESC responsible for aggressive tumour behaviour [128].

5 Epigenetic Alterations

5.1 Aberrant Methylation

As for many other cancers, the development of EC cannot only be explained by genetic mutations and it probably involves epigenetic changes. Indeed, epigenetic changes results in aberrant gene expression and are dynamic and modifiable features of many cancer types. Recent developments in the field of epigenetics, particularly studies of DNA methylation, have provided valuable insights into the role of epigenetic alterations in normal cellular processes and the abnormal changes that leading to endometrial carcinogenesis [129]. Indeed, aberrant DNA methylation appears to be more frequent than genetic alterations in ECs, with epigenetic abnormalities described in genes encoding tumour suppressors, apoptosis inhibitors, cell cycle regulators, steroid receptors, transcription factors, angiogenesis modulators and oncoproteins [129, 130].

There is evidence that aberrant DNA methylation is an early event in endometrial tumourigenesis and indeed, a recent study of methylation profiles in endometrial tumours revealed that the number of methylated promoter *loci* increased as the disease progressed from normal endometrium to complex hyperplasia [131]. For example, in carriers of MMR gene mutations, methylation defects appeared up to 12 years before EC.

A high frequency of promoter methylation in EC tumours and cell lines was also reported for *APC, CASP8, CDH1, ERα-promoter-C, hMLH1, progesterone receptor (PR), RASSF1A* and *THBS2*, accompanied by a much lower frequency (∼15%) of promoter hypermethylation of *p16INK4A (CDKN2A), p14ARF, p73* and *PTEN* [129, 132–134]. Regarding hormone receptor, silencing of both the ER and PR by aberrant DNA methylation frequently occurs in EC [129, 135, 136]. When the expression of ERα (three isoforms; ERα-A, ERα-B and ERα-C) and ERβ was analyzed in endometrial cancer cell lines, ERα-C expression was lacking and it was restored by treatment with 5-aza-2′-deoxycytidine. Furthermore, *ERα-C* promoter methylation was observed in 94% of EC tissues. By contrast, there was no association between the loss of ER expression and *de novo* methylation of the cursiva gene in other studies [137] and thus, further studies will be required to define whether methylation of the *ERα* isoforms promoter is altered in EC.

The *progesterone receptor (PR)* gene encodes two receptor subtypes with distinct functions, PR-A and PR-B. Previous studies have demonstrated abnormal ratios of PR-A to PR-B in EC, producing an inappropriate response to progesterone. Consistent with the altered transcription of the two PR isoforms, one study reported that the *PR-B* promoter was methylated in over 70% of EECs analyzed, whereas *PR-A* was unmethylated in both cancerous and normal endometrial samples [136].

A number of studies have demonstrated *PTEN* promoter methylation in about 20% of sporadic EECs, linking this methylation with metastatic disease and the MSI phenotype [67]. Promoter methylation of the *p16* gene has also been described in up to 15% of sporadic endometrial cancers [138] although much lower frequencies (0.7%) were reported elsewhere [139]. However, the data correlating *p16* promoter hypermethylation and clinicopathological features is limited and inconsistent [138].

The *RASSF1A* gene maps to chromosome 3p21.3 and acts as an inhibitor of the RAS-RAF-MEK-ERK MAP kinase pathway. *RASSF1A* inactivation by promoter methylation has been reported in up to 80% of ECs and it is more frequent among EECs [140, 141]. *RASSF1A* methylation is strongly associated with microsatellite instability and *hMLH1* promoter methylation, and it is inversely correlated with *KRAS* mutations in MSS carcinomas [140]. Interestingly, the frequency of *RASSF1A* promoter methylation increases with increasing pathological stage, lymph node involvement, high histological grade and worse outcomes [141, 142].

Hypermethylation of the *APC* promoter is not observed in normal endometrium or in endometrial hyperplasia, although it is detected in atypical hyperplasia and early endometrial cancer. *APC* gene promoter methylation has been demonstrated in around 20–45% of ECs, and it is more frequent in tumours with MSI and endometrioid histology [61, 143]. No significant associations have been observed between *APC* promoter methylation and clinicopathological factors, recurrence or

distant metastases [144]. In addition, no association between *APC* methylation and aberrant β-catenin expression has been observed [61], suggesting a limited role of this alteration as a modulator of the WNT pathway.

Several studies have evaluated the methylation of the *CDH1* promoter in EC, with contradictory results. In one study hypermethylation of *CDH1* was shown to reduce E-cadherin expression in EC, influencing clinical and pathological progression and 5-year survival rates [145]. *CDH1* promoter methylation was also described in 21% of a cohort of ECs, although this alteration was not associated with the histological type or other clinicopathological variables, including E-cadherin expression [101]. By contrast, no *CDH1* promoter methylation was identified in another study of ECs, although E-cadherin expression was not detected and there was an association with the development of distant metastases [146].

DNA hypomethylation may also contribute to endometrial carcinogenesis and as an example, the *PAX2* transcription factor is methylated and silenced in normal adult tissue but not in malignant endometrial cells [147]. Expression levels of *DNMT1* and *DNMT3B* have been analyzed in different histological types of EC and these genes are upregulated in type I cancers but they are downregulated in type II cancers [148]. Decreased *DNMT1* and *DNMT3* expression may result in global hypomethylation in type II EC and thereby contribute to the histological differences.

5.2 MicroRNAs (miRNAs) Expression

Post-transcriptional gene regulation by small (19–24 base pairs), non-coding microRNAs (miRNAs) is a relatively recently discovered epigenetic phenomenon that is implicated in endometrial carcinogenesis. A number of recent studies have analyzed the expression profiles of miRNAs in EC [149–155], identifying aberrant expression of specific miRNAs that suggests that specific miRNA signatures may distinguish histological types, stages and patient outcomes. To date, most miRNA profiling studies have focused on EEC and the most remarkable similarity between them is the upregulation of the miR-200 family observed in EC tissues [152–157]. Moreover, significant overexpression of miR-205 and miR-210 has been reported in EC [149–155].

The miR-200 family includes five miRNAs located in two genomic clusters: miR-200a/b and miR-429 are located on chromosome 1, and miR-200c and miR-141 on chromosome 12 [157]. Both the miR-200 family and miR-205 have been implicated in the epithelial-to-mesenchymal transition (EMT), tumour invasion and metastatic growth [158]. While analyses of the miR-200 family expression in endometrial cancer have generated consistent results, these findings differ from studies in other tumour types regarding the tumour suppressor role of the miR-200 family and its involvement in EMT. Several studies have demonstrated a link between EMT and a downregulation or loss of miR-200 family members [158, 159], and one possible explanation is that Type I ECs may retain many epithelial characteristics when compared to the normal endometrium. Thus, a recent review

proposed that the regulation of the miR-200 family may be influenced by oestrogen receptor-α (ERα) and estradiol [160]. It is possible that the less aggressive nature of ERα-positive ECs is related to upregulation of the miR-200 family, which maintains an epithelial phenotype and resists EMT.

Upregulation of other miRNAs, such as miR-182 and miR-183, has been described in ECs in several studies [150, 151, 153–155], and this has been linked with tumour progression and lymph node metastasis. Moreover, expression of the tumour-suppressor gene *FOXO1* is repressed in EC by miR-182 and miR-183 [161]. Increased or decreased miRNA expression may affect the expression of target genes (Table 11.4), for example, miR-205 targets the critical tumour suppressor gene *PTEN*. Moreover, an inverse correlation between miR-205 expression and PTEN protein expression has been described in EEC, with no changes in *PTEN* mRNA expression [162]. These results support the hypothesis that loss of PTEN expression in some EECs occurs due to post-transcriptional mechanisms. Furthermore, miR-205 expression was seen to be associated with cancer patient survival, whereby patients with low levels of miR-205 expression tended to have higher survival rates than those with stronger expression. Several studies have demonstrated that miR-133a and miR-133b are significantly underexpressed in EEC and the down-regulation of these miRNAs is associated with the overexpression of target genes, including the oncoproteins pyruvate kinase type M2 (PKM2), fascin 1 (FSCN1) and MET.

Altered expression of 120 miRNAs was demonstrated in ESC when compared with normal endometrial tissue [155], and decreased expression of miR-10b*, miR-29b and miR-455-5p was associated with vascular invasion, as well as down-regulation of miR-101, miR-10b*, miR-139-5p, miR-152, miR-29b and miR-455-5p with poor overall survival. In addition, decreased expression of miR-101 and miR-152 was identified as an independent risk factor for DFS, while down-regulation of miR-152 alone was an independent risk factor for overall survival. Interestingly, restoring the expression of these microRNAs in serous endometrial cancer cell lines by transfection led to diminished cell proliferation. Moreover, a correlation was demonstrated between miR-101 downregulation and strong positive immunoreactivity for cyclooxygenase-2.

Several mechanisms that affect miRNA expression have been identified and include aberrations in miRNA coding sequences, dysregulation of miRNA biogenesis and epigenetic regulation [163–165]. At least half the miRNA encoding genes are located at chromosomal *loci* that are unstable in cancer and that frequently undergo genomic alterations, such as deletions, insertions, inversions and translocations. DNA copy number alterations may therefore contribute to the miRNA dysregulation observed in malignancies. While such correlations have been demonstrated for specific miRNAs in distinct tumours types, no such studies have been performed in endometrial cancer.

Altered expression of genes implicated in miRNA biogenesis, such as *Drosha* and *Dicer*, has been proposed to account for the differences in miRNA profiles observed between normal and tumour tissues [166, 167]. A decrease in the expression of Dicer and Drosha transcripts was recently described in EEC samples

Table 11.4 Main dysregulated miRNAs in endometrial carcinoma

miRNAs	Chromosome localization	Potential targets	Mechanisms of regulation	Consequences for tumour progression
miR-200a/b/429 (upregulated)	1p36.33	ZEB1, ZEB2, TCF8, BAP1, GATA4 and PTEN	Transcriptional repression	Modulation of invasion and metastasis
miR-141/200c (upregulated)	12p13.31			
miR-203 (upregulated)	14q32.33	_JPH4, ZIC1, CDK6, VIM, ABCE1, SMYD3_ and _p63_	DNA hypomethylation	Modulation of cancer stemness
miR-205 (upregulated)	1q32.2	_PTEN, E2F1, ERBB3, JPH4_*, _S100A2_*, _ZEB1_ and _ZEB2_	Unknown	Modulation of invasion and metastasis
miR-210 (upregulated)	11q15.5	_DCHS1_*, _ENPP2_*, _MYH11_*, _KCNMB1_*, _MNT_, _BDNF_ and _PTPN1_	Hypoxia and DNA hypomethylation.	Oncogenic signalling and cell migration
miR-182/miR-183 (upregulated)	7q32.2	_FOXO1, FOXO3, CASP3, CASP2_ and _Fas_	Copy gain	Dedifferentiation, reduced apoptotic potential. Strongly associated with tumour progression and lymph nodes metastasis
miR-10a (upregulated)	17q 21.32	_HOXA1, HOXD10, HOXB1, HOXB3_ and _RB1CC1_	Hypoxia and retinoic acid target	Modulation of invasion and metastasis
Let7 family (downregulated)	Various locations	_KRAS_, _c-Myc_, _HMG2A_ and _IL-6_	Unknown	Oncogenic activation, drug resistance and cancer stemness
miR-29b (downregulated)	7q32.3	_IGF1_, _Mcl-1_ and collagens	Unknown	Inhibition of migration and invasion. Correlated with poor disease-free survival
miR-129-2 (downregulated)	11p11.2	SOX4*	Promoter methylation	Migration and invasion
miR152 (downregulated)	17q21.32	_ENPP2_*, _SNCAIP_*, _LTBP4_*, _MLH1_ and _Bcl2_	Promoter methylation	Apoptosis inhibition, metastasis and microsatellite instability. Correlated with poor disease-free survival
miR-455-5p (downregulated)	9q32	_PP1R12A_, _KDR_, _SUZ12_, _FOXN3_ and _PTPRJ_	Copy loss	Cancer stemness and oncogenic signalling. Associated with vascular invasion

when compared with healthy controls. In addition, down-regulation of Dicer was significantly correlated with Drosha downregulation, and a correlation between high-grade tumours and Drosha downregulation was also described [164]. While these alterations in Dicer and Drosha expression remain poorly understood, chromosomal aberrations may underlie these effects. Dicer is located at the subtelomeric region of chromosome 14 (14q32.13), which is affected by allelic deletion in various tumours. Interestingly, loss of heterozygosity (LOH) at chromosome 14q was reported in a high proportion of ECs, defining a minimal region of deletion for these tumours at 14q32 [168].

One-third of all human miRNAs contain a CpG island in their upstream region and they can be regulated by DNA methylation [169]. Therefore, epigenetic regulation of miRNA encoding sequences may constitute another possible regulatory mechanism in human cancer. In ECs, a link between the loss of miR-129-2 expression and hypermethylation of the miR-129-2 CpG island has been described, which in turn correlates with poor overall survival [170]. Significantly, miR-129-2 expression was restored in EC cells following histone acetylation.

The importance of miRNAs in the onset and progression of cancer has prompted the development of miRNA-based therapeutic approaches, with the ultimate goal of modulating dysregulated miRNAs by reintroducing the miRNAs that are lost in cancer using miRNA mimics, or by inhibiting oncogenic miRNAs using anti-miRNA oligonucleotides. The use of miRNA mimics was described in an *in vitro* model of EC, where miR-145 upregulation reduced the expression of OCT4 and induced the differentiation of Ishikawa cells, both *in vitro* and *in vivo*, closely resembling normal endometrial epithelium [171]. Furthermore, these authors reported that miR-145 successfully inhibited tumour growth.

6 Genetics of Precursor Lesions in Endometrial Cancer

The existence of a precursor lesion prior to the development of endometrial carcinoma was proposed many years ago, although the terminology and histological definitions have been widely disputed [172]. As we previously mentioned, endometrial adenocarcinomas fall into two main categories with distinct aetiologies, histological features and biological behaviours. Similarly, their corresponding precursor lesions also differ as endometrial hyperplasia develops into endometrioid adenocarcinomas and variants (type I), while endometrial intraepithelial carcinoma represent the pre-invasive lesion of type II EC [9, 173].

6.1 Endometrial Hyperplasia

In 1994 the WHO proposed a classification that although now widely accepted, remains difficult to reproduce [172]. The term endometrial hyperplasia (EH) covers

a progressive spectrum of endometrial glandular alterations classified according to the degree of architectural complexity and cytological atypia, *i.e.,* simple hyperplasia, complex hyperplasia, simple atypical hyperplasia (a very unusual pattern), and complex atypical hyperplasia (CAH). This latter category combines glandular complexity with cellular atypia, and it is the most common atypical pattern that is associated with an increased risk of progression to invasive EEC. Based on correlative histomorphometric analyses and some molecular studies, monoclonal precursor lesions of EEC have been termed endometrial intraepithelial neoplasia (EIN), while polyclonal lesions with low risk of progression are described as endometrial hyperplasia [174].

PTEN mutations have been detected in endometrial hyperplasias with and without atypia (19 and 21%, respectively) [175, 176], although higher incidences (up to 33%) have been reported [30]. Most of the mutations described were frameshift deletions and nonsense substitutions clustered in exons 5, 7 and 8, similar to those found in EECs. Interestingly, a study analyzing *PTEN* mutations and MSI in endometrial hyperplasia failed to detect an MSI phenotype in any of the CAHs studied without associated invasive carcinoma, suggesting that *PTEN* mutations represent an early event in the pathogenesis of EECs and that they may precede the development of the MSI phenotype in a subset of cases [176].

Few studies have investigated the incidence of *PIK3CA* mutations within CAHs, and while *PIK3CA* mutations were reported in up to 7% of CAHs [177], in other studies no mutations were identified in cases of atypical hyperplasia [42]. The former study demonstrated identical missense mutations in exon 20 in 2 out of 29 CAHs. The lack of coinciding *PTEN* and *PIK3CA* mutations and the lower frequency of *PIK3CA* mutations observed in CAHs *versus* EECs indicates that *PIK3CA* mutations represent a late event in endometrial cancer pathogenesis [177].

Similar *KRAS* mutation rates have been described in carcinomas and endometrial hyperplasias, with no specific distribution regarding complexity and atypia, suggesting that this molecular alteration represents an early event in tumorigenesis in a subset of EECs [178]. Nonetheless, lower incidences (up to 4%) or the absence of *KRAS* mutations associated with atypical hyperplasias were reported elsewhere [53, 55].

Atypical endometrial hyperplasias exhibit *CTNNB1* mutations with frequencies similar to those found in EECs (14%) [65, 101, 179], indicating that abnormalities in β-catenin may represent important early events during the endometrial hyperplasia-carcinoma sequence in some patients. Interestingly, *CTNNB1* mutations are common in CAH with squamous morules, a form of hyperplasia associated with lower rates of *PTEN* and *KRAS* mutations, and MSI. Furthermore, this morphologic pattern appears to correlate, at least partially, with the clinical course of the disease, and it is associated with less aggressive behaviour than CAH with *KRAS* and *PTEN* mutations but without squamous morules [179].

6.2 Endometrial Intraepithelial Carcinoma

Endometrial intraepithelial carcinoma (EIC) has been proposed as the precursor lesion of ESC based on molecular genetic evidence. It is characterized by the replacement of the surface epithelium or part of the endometrial glands with proliferated cells of malignant appearance similar to those of serous invasive adenocarcinoma. Overexpression of p53 protein, loss of heterozygosity of chromosome 17p and *TP53* gene mutations have been described in many EICs, similar to the defects observed in serous carcinoma [173].

Recently, the term "p53 signatures" has been proposed to designate benign-looking endometrial glands with p53 overexpression. Indeed, p53 signatures have been specifically associated with ESC, and they are frequently found in the benign-looking endometrium adjacent to the ESC and rarely detected in either the endometrium adjacent to endometrioid carcinomas or in non-cancerous uteri. Almost 50% of the p53 signature samples analyzed exhibited *TP53* gene mutations. The identification of identical *TP53* mutations in p53 signatures, precancerous regions, and in the uterus of a subset ESCs provides further evidence of a common lineage between these lesions and suggests that epithelia displaying these p53 signatures are probably latent ESC precancerous regions [180, 181].

7 Hereditary Endometrial Carcinoma

7.1 Lynch Syndrome

Lynch syndrome (LS), or hereditary non-polyposis colorectal cancer, is the most common hereditary colorectal cancer syndrome, accounting for approximately 2–5% of all newly diagnosed cases of colorectal cancer. Patients with LS have a greater risk of developing colorectal cancer (52.2% in women and 68.7% in men), and can develop tumours in the small intestine, stomach, endometrium and upper urinary tract, and sebaceous tumours of the skin. EC is the second most common type of cancer in patients with LS. However, the risk of EC in women with LS surpasses the risk of colon cancer. For individuals with documented *hMLH1* and *hMSH2* germline mutations, the lifetime risk of EC is estimated between 40 and 60%. For women with *hMSH6* mutations a cumulative risk of EC ranging from 16 to 71% at 70 years of age has been reported [182–184].

Population-based prevalence studies have identified a 1.8% prevalence of germline mutations in *hMLH1*, *hMSH2* or *hMSH6* among unselected EC patients [185]. However, studies evaluating EC patients of less than 50 years of age reported a 9% prevalence of germline mutations in these genes [186]. In recent years, specific pathologic features associated with colon cancers have been evaluated as potential predictors of LS, and several studies have attempted to identify specific pathologic factors in Lynch-associated endometrial cancers. The prevalence of LS

among women with endometrial cancer of the lower uterine segment (LUS) is up to 29% [187], suggesting that screening for LS should be considered in cases of pathologically confirmed EC originating in the LUS. As previously mentioned, the reliability of routinely assessed morphologic features as predictors of MSI status (and by extension, Lynch status) in EC has been investigated in several studies. As seen in CRC, tumour infiltrating lymphocytes (TIL) and peritumoral lymphocytes (PL) in EC were demonstrated as independent predictors of MSI [188, 189]. In addition, a distinctive "undifferentiated" subtype in MSI-H endometrial cancer (both sporadic and associated with LS) has been identified [189]. Based on these findings, an algorithm has been proposed to detect EC patients at high risk of developing LS based on tumour morphology and epidemiological factors. In this algorithm, immunohistochemistry for MMR proteins is performed on: (1) all ECs in patients younger than 50 years of age; and on (2) ECs with TILs and PLs (suggestive of MSI), and those originating in the lower uterine segment [189]. Other authors have proposed that all ECs [190], or those in women of any age with at least one first degree relative with an LS-associated cancer [191] should be tested for MMR deficiency.

Given the higher rate of *MSH6* mutations in endometrial cancer associated with LS and the lower predictive value of MSI detected by molecular techniques in *MSH6*-associated LS, IHC rather than MSI should be considered as the primary screening strategy for LS in patients with endometrial cancer [190].

7.2 Cowden Syndrome

Cowden Syndrome (CS), an autosomal-dominant disorder with incomplete penetrance and variable expression that is caused by inactivating germline mutations in the *PTEN* gene, is characterized by a number of benign conditions and an increased risk of malignancies of the breast, thyroid and endometrium [1]. The lifetime risk of EC associated with this syndrome is estimated between 5 and 19%, compared with 2.6% in the general population. Germline PTEN mutations are observed in up to 80% of patients with CS [192, 193]. Mutations include missense, nonsense and splice-site mutations, and small deletions and insertions, and they are dispersed throughout the gene with a clustering in exon 5. Indeed exon 5, which encodes the lipid phosphatase core at residues 122–132, harbours 43% of these mutations [194–196]. Nonetheless, genotype-phenotype correlations have not been described [194–196].

8 Concluding Remarks

EC is the most common gynaecological malignancy in the western world and it comprises a heterogeneous group of tumors, with distinct risk factors, clinical presentation, and histopathological features. Two main groups of EC exist, EECs

(type I) and non-EECs (NEECs-type II), which evolve via distinct molecular pathways. The most common molecular alterations associated with EECs affect the phosphoinositide 3-kinase (PI3K)/Akt pathway due to mutations in *PTEN* (phosphatase and tensin homologue deleted from chromosome 10) or *PI3KCA*. Other pathways, such as the RAS-RAF-MEK-ERK, FGF and WNT signalling pathways are also frequently affected by gene mutations or epigenetic changes. In addition, a group of sporadic and hereditary EECs are characterized by MSI due to DNA MMR deficiency. ESC is characterized by alterations in *TP53* with secondary chromosomal instability, which leads to multiple chromosomal gains and losses, including the amplification of oncogenes and the loss of important tumour suppressor genes. By contrast, the molecular alterations in CCC are poorly defined. Differences in genetic and epigenetic alterations between EEC and NEEC tumours are reflected in the distinct gene expression profiles observed amongst different EC types.

While early-stage endometrial cancer is often successfully treated with surgical intervention and radiotherapy, treatment of advanced endometrial carcinoma can be difficult and prognosis poor, particularly in the context of metastatic or recurrent disease. To date, standard chemotherapy agents are used for both adjuvant first-line treatment and recurrent endometrial cancer with poor results. This has led to a shift from the use of traditional chemotherapeutic agents and radiotherapy regimens to the promising area of targeted therapy. Although several clinical trials have tested inhibitors of the EGFR, VEGFR and PI3K/PTEN/AKT/mTOR signaling pathways, responses to these targeted therapies were modest. Despite the striking molecular differences between EECs and NEECs, most clinical trials have not taken this diversity into account. To maximize the effects of directed targeted therapy, careful molecular characterization of ECs is warranted.

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Chapter 12
Usefulness of Molecular Biology in Follicular-Derived Thyroid Tumors: From Translational Research to Clinical Practice

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Abstract The development of molecular biology analyses in thyroid pathology is currently active and provides new diagnostic tools with the aim of accurately distinguishing malignant and benign thyroid tumors. This is particularly useful as most of these analyses can be done preoperatively on thyroid fine-needle aspiration biopsy samples. Furthermore, molecular biomarkers may have a promising role on account of their ability to predict the prognosis of thyroid tumors. Moreover, identification of molecular markers as well as a better understanding of thyroid carcinogenesis are attractive prospects for the development of innovative targeted therapies, particularly in patients with metastatic iodo-resistant thyroid carcinoma.

To date, four types of somatic genetic alterations are known to have a potential interest for the diagnosis and/or prognosis of follicular cell-derived thyroid carcinomas: *BRAF* and *RAS* mutations, and *RET/PTC* and *PAX8/PPARγ* rearrangements. Other recent molecular biomarkers have been investigated in thyroid oncology, in particular on different microRNA signatures.

The purpose of this review is to describe the different aspects of ancillary methods, including molecular biology, which are of current interest for the diagnosis, prognosis and/or treatment of follicular cell-derived thyroid carcinomas.

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1 Introduction

The incidence of thyroid cancer, the most common endocrine malignancy, is increasing, particularly in Western Europe and North America [1–3]. However, whether this is due to a real increase in disease prevalence or to improved detection thanks to ultrasound technology, of clinically insignificant tumors is still uncertain [1, 4, 5]. More than 95% of thyroid cancers are derived from follicular cells, while a minority, called medullary thyroid carcinomas, are derived from C-cells. Follicular cell-derived thyroid cancers are commonly divided into well-differentiated thyroid carcinoma (WDTC), poorly-differentiated thyroid carcinoma (PDTC), and undifferentiated, or anaplastic, thyroid carcinoma (ATC). WDTCs include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) types.

While thyroid nodules are very prevalent, the proportion of malignant tumors represents less than 5% of these nodules [6]. In order to avoid inappropriate thyroid surgery, it is vital, preoperatively, to identify malignant tumors among the thyroid nodules. Fine-needle aspiration biopsy (FNAB) is the most accurate and cost-effective method for evaluating thyroid nodules preoperatively [6, 7]. Nevertheless, the Bethesda classification system for reporting thyroid cytopathology identifies numerous situations where FNAB cannot distinguish efficiently between benign and malignant thyroid lesions [7, 8]. In this regard, when FNAB suggests the presence of follicular neoplasm, the latter may be either a follicular adenoma (FA) (70–80% of cases), or an FTC (20–30% of cases) [8]. Additional and novel ancillary methods are therefore needed to accurately identify benign and malignant thyroid tumors, but also to determine, preoperatively, the prognosis of malignant thyroid lesions. The sensitivity and specificity of immunocytochemistry are not sufficient to resolve these issues completely [9]. In this regard, there is a critical need for new diagnostic tools and, particularly, for innovative molecular biology-based assays in thyroid pathology [10].

In the field of thyroid oncology, molecular biomarkers should also predict prognosis in order to adapt the intensity of the treatment to the biological aggressiveness of the tumor [11]. Advances in our understanding of the specific biology of thyroid tumors have also led to the identification of potential molecular targets for the development of new targeted therapies. As molecular targeted therapies come into clinical practice in the treatment of thyroid cancer, particularly for iodo-resistant metastatic tumors, the predictive factors of their efficacy need to be identified. The relevance of molecular biology is obvious in this regard [12].

The aim of this review is to describe the different fields of molecular biology which show potential interest for the diagnosis, prognosis and/or molecular targeted therapy of follicular cell-derived thyroid tumors.

2 Biomarkers in Thyroid Tumors

2.1 Immunohistochemical and Immunocytochemical Markers

Most thyroid tumors can be diagnosed by means of morphological features alone. Nevertheless, in some cases, it may be difficult to differentiate benign from malignant thyroid lesions, particularly on cytological material. There are some well-encapsulated follicular thyroid tumors that could be difficult for the pathologist to characterize, since a morphological approach alone may seriously restrict predictions regarding the outcome of the lesions. Some authors have suggested labeling these thyroid tumors with "borderline" features as well-differentiated tumors of uncertain malignant potential [13]. Given all theses difficulties, immunohistochemical approaches have been adopted to enhance characterization of thyroid lesions [14].

Immunohistochemistry (IHC) and immunocytochemistry (ICC) enable localization of antigens or proteins in tissue sections or cell suspensions. Both techniques use labeled antibodies as specific reagents through antigen-antibody interactions which are visualized by markers such as fluorescent dye, enzyme, or colloidal gold. IHC/ICC may improve the diagnostic accuracy of thyroid nodules [15, 16]. Studies have focused on many IHC/ICC markers belonging to different categories of molecules such as cell adhesion molecules (galectin-3 (GAL3), HBME-1, E-cadherin, fibronectin), membrane receptors (rearranged during transfection (RET)), transcription factors (thyroid transcription factor-1(TTF1)), cell cycle regulators (p27, cyclin D1 or D3), cytoskeletal molecules (cytokeratin-19 (CK19)), enzymes (thyroid peroxydase (TPO)) and secreted molecules (thyroglobulin, calcitonin, carcinoembryonic antigen) [17, 18].

Most studies investigating the role of IHC/ICC markers in thyroid pathology have focused on three thyroid biomarkers: CK19, GAL3 and HBME1 [18–21]. Scognamiglio et al. found that the level of expression of these three markers was significantly higher for PTC than for FA, with HBME1 being the most specific and CK19 the most sensitive marker of malignity [20]. Furthermore, the authors reported that HBME1/CK19 coexpression was 100% specific of malignity. In a study assessing GAL3 expression on 465 preoperative thyroid FNA samples, Bartolazzi et al. found an overall sensitivity and specificity of the GAL3 test of 78 and 93% respectively [22]. Martins et al. reported that although GAL3 immunostaining demonstrated a sensitivity of 93.8% for the identification of cancer, the accuracy of the distinction between benign and malignant tissues was only 77%, and was even lower when GAL3 expression in FA was compared with FTC [23]. Furthermore, expression of CK19, GAL3 and HBME1 has been found by some authors to be increased in thyroid tumors of uncertain malignant potential. However, heterogeneous results have been reported for these borderline tumors [13, 24].

Among the other IHC/ICC markers, Troncone et al. found that cyclin D3 overexpression increased the suspicion of malignancy in 51 FNA samples that were suspicious for Hürthle cell neoplasia [25]. In this study, the diagnostic performance of cyclin D3 immunostaining depended on the cutoff point used and was enhanced further when combined with cyclin D1 [25]. Death-associated protein 3 (DAP3),

one of the constituents of the small subunit of the mitochondrial ribosome, has been found to be overexpressed in human thyroid oncocytic tumors in a study by Jacques et al. [26]. The IHC staining of the DAP3 protein was similar to that observed for a mitochondrial antigen, suggesting that the major pool of DAP3 is localized in the mitochondrion [26].

Finally, IHC markers can improve preoperative diagnostic accuracy for patients with indeterminate thyroid nodules. Many of these markers are commercially available for use in reference laboratories but have not yet been widely applied in clinical practice [7, 27, 28]. It is likely that some combination of IHC markers will be used in the future to optimize management of patients with indeterminate cytology on FNAB specimens.

2.2 Somatic Mutations and Gene Rearrangements

2.2.1 Genetic Alteration of the MAPK Pathway

The mitogen-activated protein kinase (MAPK) signaling pathway plays a major role in several complex cellular phenomena such as division, proliferation, survival, apoptosis, differentiation, adhesion and migration. An aberrant activation of this critical signaling pathway has been described in several human malignancies, including thyroid cancer [29]. In thyroid follicular cells, the MAPK pathway is activated by various hormones and growth factors. These stimuli are able to activate a G protein-coupled receptor on the plasma membrane. The G protein RAS is activated and, in turn, activates the serine/threonine-protein kinase RAF. BRAF is the predominant isoform of RAF proteins in follicular thyroid cells and is a potent activator of the MAPK pathway. After activation, BRAF is recruited to the plasma membrane and phosphorylates MEK1-MEK2. Subsequently, MEK1 and MEK2 activate the MAPK ERK1 and ERK2, leading to the regulation of gene expression, through the phosphorylation of many transcription factors such as elk-1, AP-1, Ets-1, c-myc or CERB [29–31]. Furthermore, BRAF is able to activate the NF-κB signaling pathway which plays a critical role in the regulation of the inflammation process, immune response, cell proliferation and apoptosis [29–31].

In PTC, activation of the MAPK signaling pathway, which is present in about 80% of tumors, constitutes the most important oncogenic mechanism. Three main initiating events, *BRAF* mutation, *RET/PTC* rearrangement and *RAS* mutation, are considered to be mutually exclusive alternative triggers for the activation of this critical pathway (Fig. 12.1) [32]. *NTRK1* rearrangement is also a well-known, but less frequent, mechanism of MAPK pathway activation [33].

BRAF Mutation

Somatic mutations in the *BRAF* gene have recently been described in several types of human cancer, including thyroid cancer. After melanoma, PTC is the second

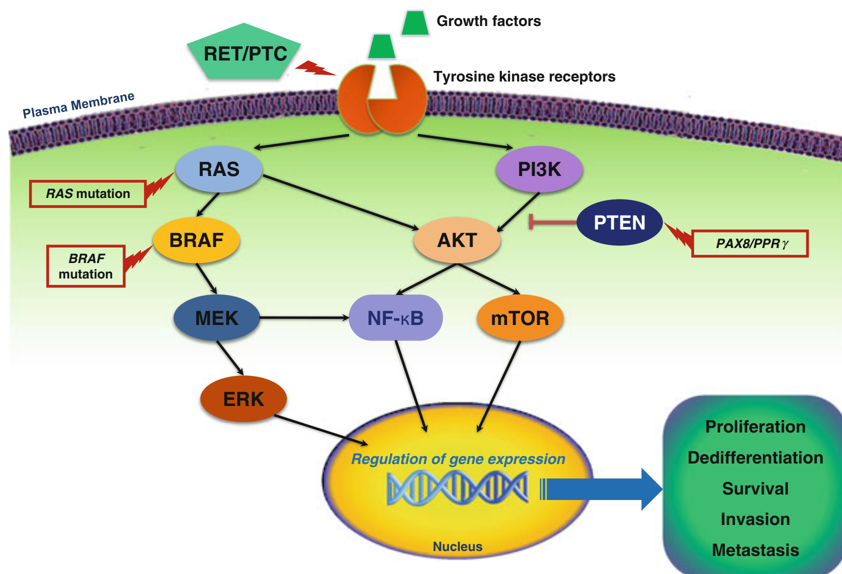

Fig. 12.1 Main signaling pathways and genetic alterations involved in thyroid carcinogenesis

human cancer in which BRAF mutations are especially frequent [29, 34, 35]. More than 30 different types of *BRAF* mutation have been identified and most of them are located on the glycin-rich loop (exon 11) or on the activation segment (exon 15) of the catalytic domain [36]. Mutations in exon 11 of the *BRAF* gene are not encountered in thyroid cancers. The most common *BRAF* mutation, called *BRAF*(*V600E*) mutation, is a thymine-to-adenine transversion at nucleotide position 1799 of *BRAF*, which results in a valine-to-glutamate substitution at residue 600 of the BRAF protein. The mutant BRAF protein is characterized by elevated kinase activity and activates the MAPK pathway independently of RAS. Moreover, in vivo, Knauf et al. demonstrated that thyroid-specific expression of BRAF(V600E) induced invasive PTC, which transitioned to PDTC [37].

BRAF is a more potent activator of the MAPK pathway than the two other isoforms of RAF, ARAF and CRAF. Furthermore, *ARAF* and *CRAF* mutations are rarely encountered in human cancers, unlike *BRAF* mutations. In an interesting study, Emuss et al. showed that the introduction of the equivalent of *BRAF*(*V600E*) mutation into *CRAF* only had a weak effect on kinase activity and did not convert *CRAF* into an oncogene [36]. The authors explained this lack of activation by the fact that CRAF lacks a constitutive charge within a motif in the kinase domain called the N-region. This fundamental difference in RAF isoform regulation explains why *BRAF* is frequently mutated in cancer whereas *CRAF* mutations are rare [36].

The BRAF-induced activation of the MAPK pathway leads to phosphorylation of the retinoblastoma protein, which releases inhibition of E2F-dependent transcription factors, allowing the cell to pass from G1 into S phase, increasing growth

and promoting survival. Furthermore, the *BRAF* mutation promotes methylation-induced silencing of the tissue inhibitor of metalloproteinase 3, leading to overexpression of metalloproteinases, and finally tumor cell invasion and metastasis [29, 34, 35]. Finally, the MAPK pathway regulates diverse cellular programs including cellular communication, cellular division, differentiation, proliferation, apoptosis, and participates in numerous disease states including chronic inflammation and cancer [38, 39].

Recent studies have shown that the *BRAF*(*V600E*) mutation is the most common genetic alteration in PTC, with a prevalence ranging from 29 to 83% [29, 34]. This mutation can be found in the early stages of the development of PTC, such as in papillary thyroid microcarcinomas. Interestingly, the prevalence of *BRAF* mutation depends on the histologic subtypes of PTC and is more common in tall cell PTC, followed by classical PTC, and less frequently in follicular variant PTC [40, 41]. This mutation has also been found in PDTC and anaplastic carcinomas, but is not encountered in FTC or in benign thyroid lesions [34, 35]. Finally, the *BRAF*(*V600E*) mutation is a specific molecular marker of the malignity of thyroid nodules [29, 34].

Other mutations of the *BRAF* gene have been found in PTC, with a variable frequency depending on the series, such as the *BRAF*(*K601E*) mutation. This mutation (lysine-to-glutamate substitution at residue 601 of the BRAF protein) has been found particularly in the follicular variant of PTC, but with an incidence generally not superior to 10% [42, 43]. In vitro, BRAF(V600E) and BRAF(K601E) mutated proteins have a kinase activity 2.5 fold higher than the activity of the non-mutated BRAF protein [29]. Beside punctual mutations, BRAF can be activated by a chromosomal rearrangement, the *AKAP9/BRAF* rearrangement resulting from a paracentric inversion of the chromosome 7 [44]. This rearrangement, involving exons 1–8 of the *AKAP9* gene and exons 9–18 of the *BRAF* gene, leads to an oncogenic fusion protein exhibiting high kinase activity. This rearrangement has been found in 11% of PTC arising in previously irradiated patients [45].

RET/PTC Rearrangement

The *RET* proto-oncogene is structurally related to the growing family of tyrosine kinase transmembrane receptors and is involved in GDNF (glial cell-derived neurotrophic factor) signaling. RET is normally expressed in the calcitonin-producing parafollicular C cells, which are derived from the neural crest, but not in the thyroid hormone-producing follicular cells. The *RET/PTC* rearrangement is the second most common genetic alteration in PTC. The different types of *RET/PTC* chromosomal rearrangements, between the 3′ portion of the *RET* gene and the 5′ portion of an unrelated gene, produce an aberrant RET/PTC protein with ligand-independent activation of its intracellular tyrosine kinase domain. This phenomenon, resulting in constitutive activation of the MAPK pathway, has been frequently described in occult small PTC and would seem, therefore, to be an early event in thyroid tumorigenesis [39, 46, 47]. The oncogenic effects of *RET/PTC* rearrangement require signaling along the MAPK pathway in the presence of functional BRAF

kinase [48]. Transgenic mice expressing the *RET/PTC* rearrangement develop PTC [49, 50]. Furthermore, expression of thyroid specific genes such as *NIS* (sodium-iodide symporter) is decreased in thyroid tumor cells lines harboring the *RET/PTC* rearrangement [51, 52].

Besides the MAPK pathway, *RET/PTC* rearrangement is able to activate other crucial signaling pathways such as the PI3K/AKT and NF-κB pathways [53, 54]. Moreover, it has been demonstrated that *RET/PTC* rearrangement induce nuclear translocation of β-catenin promoting cell proliferation and invasion phenotype [55]. *RET/PTC* rearrangement increases the expression of proinflammatory molecules such as interleukin 24 (IL-24) and CXCR4, the receptor of the chemotactic factor CXCL12/SDF1 [56, 57]. Interestingly, Shinohara et al. demonstrated that *RET/PTC* rearrangement-harboring thyroid tumor cells overexpressed IL-24, which acted as an autocrine growth factor for tumor cells, supporting tumor growth at the early stages of cancer [57].

Among the different types of *RET/PTC* rearrangement, the two intrachromosomal translocations *RET/PTC1* and *RET/PTC3*, involving *RET* on 10q11.2 with, respectively, the *CCDC6* gene (or *H4* gene) on 10q21 and the nuclear receptor coactivator 4 (*NCOA4*) gene on 10q11.2, are the most frequent, with *RET/PTC1* and *RET/PTC3* representing, respectively, 60–70% and 20–30% of all *RET/PTC* rearrangements [46, 47]. *RET/PTC1* is preferentially encountered in conventional PTC and in the diffuse sclerosing variant of PTC, whereas *RET/PTC3* is more frequent in the solid variant of PTC [58, 59]. *RET/PTC1* has been associated with PTC arising in the context of Hashimoto thyroiditis [38]. Indeed, Muzza et al. demonstrated that *RET/PTC1* was more represented in PTC associated with thyroiditis than in PTC alone, and that this rearrangement was also found in 41% of non-neoplastic thyroiditis tissues [60]. The *RET/PTC3* induced-solid variant of PTC prevails in children exposed to radiation from the Chernobyl nuclear power plant disaster.

RET/PTC rearrangement is recognized as an organ- (thyroid) and histotype- (PTC) specific event. However, this genetic alteration can also be found in benign thyroid lesions such as lymphocytic thyroiditis [61–63]. Overall, *RET/PTC* rearrangement is encountered in approximately 20% of sporadic PTC. Nevertheless, its prevalence in different studies is highly variable, probably due to the different detection methods used and to geographic variations [64]. The prevalence of the *RET/PTC* rearrangement is higher in young patients and decreases with patient age [65, 66]. As mentioned previously, several studies have demonstrated that the *RET/PTC* rearrangement was more frequent in PTC developed in some specific conditions, such as patients exposed to radiation from the Chernobyl nuclear power plant disaster or to external beam radiotherapy to the neck, and in patients with Hashimoto's thyroiditis [38, 39, 65, 67]. The distribution of *RET/PTC* rearrangement in the tumor is also heterogeneous. Some tumors exhibit a clonal distribution of *RET/PTC* rearrangement, which is encountered in almost all the tumors cells. However, in other tumors, this rearrangement can be harbored only by a small number of cells [68, 69]. Even if the *RET/PTC* rearrangement has been described in benign thyroid lesions, it is admitted that a clonal distribution of this

rearrangement in the tumor is quasi-specific of PTC. As there is a substantial degree of multiclonality in PTC, several mutations which are generally considered to be mutually exclusive, such as the *BRAF* mutation and the different types of *RET/PTC* rearrangement, can be found as non-clonal changes in different tumor foci in a same patient [64, 70, 71].

RAS Mutation

The RAS family is a group of more than 50 small guanosine triphosphate binding-proteins, which relay signals from tyrosine kinase receptors and G protein-coupled receptors. RAS, associated with guanosine diphosphate, is anchored to the inner-cell membrane. When the extra-cellular ligand binding occurs, guanosine triphosphate replaces guanosine diphosphate and the active form of the RAS protein in turn activates the MAPK and the PI3K/AKT signaling pathways [71]. Three members of the RAS family (H-, K- and N-RAS) are cellular proto-oncogenes. Point mutations resulting in constitutive activation of the RAS protein and leading to the induction of a malignant phenotype, with cell proliferation, resistance to apoptosis, invasion and metastasis, have been reported in several human cancers including thyroid cancer [71]. These mutations are generally located on the GTP-binding domain of exon 1 (codons 12 or 13), increasing the affinity for GTP, or on the GTPase domain of exon 2 (codon 61), impairing the GTPase autocatalytic function [72].

In thyroid pathology, the most frequent *RAS* mutation is a point mutation located in codon 61 of the *NRAS* isoform. *KRAS* mutations and mutations located on codons 12 or 13 of *NRAS* are rare [73–75]. *RAS* mutations are not specific of malignant tumors and can be encountered in benign lesions such as FA [76, 77]. All types of thyroid cancers can harbor *RAS* mutations excepting medullary thyroid cancer. *RAS* mutations are more frequent in FTC (40–50% of cases) than in PTC (10–20% of cases) [78, 79]. If *RAS* mutations can be found in benign thyroid lesions, Vasko et al. demonstrated that their incidence is significantly higher in FTC than in FA [73]. In PTC, the incidence of *RAS* mutations is highly variable depending on the series [74–76]. Interestingly, *RAS* mutation-harboring PTC are, in most cases, well-encapsulated follicular variants of PTC [71, 74]. Indeed, in a study on 97 PTC, Adeniran et al. found that tumors with *RAS* mutations were exclusively follicular variants of PTC and correlated with significantly less prominent nuclear features and a low rate of lymph node metastases [59]. *RAS* mutations can be found in Hurtle cell carcinoma or adenoma, but less frequently than in FTC or FA [80]. *RAS* mutations are also encountered in PDTC and ATC [77, 81, 82]. Indeed, in a study on 65 cases of PDTC, Volante et al. found that *RAS* mutations in codon 61, identified in 23% of cases, were by far the most common genetic alteration [81].

NTRK1 Rearrangement

NTRK1 is a transmembrane receptor tyrosine kinase regulating neuronal development and differentiation. After binding of its ligand, the growth factor NGF (nerve

growth factor), the dimerization and autophosphorylation of NTRK1 lead to the activation of RAS and, consecutively, of the MAPK and PI3K/Akt signaling pathways. NTRK1 promotes cell proliferation of different cell types such as lymphocytes, keratinocytes and prostatic cells. An intrachromosomal inversion of chromosome 1 resulting in the fusion of the 3′ portion of the *NTRK1* gene with the 5′ portion of three different genes, *TPM3* (tropomyosin 3), *TPR* (translocated promotor region) or *TFG* (TRK fused gene) has been found in PTC. The resulting fusion protein displays constitutive tyrosine-kinase activity and leads to in vitro and in vivo transformation. In a study on 33 Polish patients with PTC, Brzezianska et al. detected *NTRK1* rearrangements in four cases (12%), but found no correlation between *NTRK1* rearrangements and patient age, gender, the histopathological variant of PTC and the assignment to a particular stage in clinical staging systems [33].

2.2.2 The PI3K/Akt Pathway

The PI3K/Akt pathway is one of the most frequent activated signaling pathways in human malignancies. This pathway promotes cell cycle progression, cell survival, resistance to apoptosis and metastasis. Its deregulation has been found in several types of tumors including brain, breast, ovarian, renal and thyroid cancers. Among the different isoforms of the catalytic subunit p110 of PI3K, the α (PI3KCA) and β (PI3KCB) isoforms have been particularly implicated in cancer development [83]. Several tyrosine kinase growth factor membrane receptors, such as vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), c-Kit and c-Met, are able to activate the PI3K/Akt pathway [84]. Activation of the serine/threonine kinase Akt leads to phosphorylation and activation of various downstream effectors, including mTOR (mammalian target of rapamycin), which is considered to be a promising therapeutic molecular target in human cancers [85].

Several types of genetic alterations are implicated in the activation of the PI3K/Akt signaling pathway. Among them, *PTEN* mutation and *PAX8/PPARγ* rearrangement have been extensively studied in thyroid tumors.

PTEN Mutation

The protein phosphatase and tensin homolog (PTEN) is an inhibitor of the PI3K/Akt pathway and, therefore, a tumor suppressor protein. Interestingly, patients with congenital mutations of *PTEN* are predisposed to develop tumors and particularly breast and thyroid cancers. *PTEN* somatic mutations can be found in FTC and, more frequently, in ATC, but are rarely encountered in PTC [82, 86, 87]. Indeed, in a study by Hou et al. *PTEN* mutations were reported in 2% of PTC, 7% of FTC and 16% of ATC [79]. Aberrant methylation leading to epigenetic inactivation of the *PTEN* gene has been described in thyroid cancers, particularly in FTC and ATC. Interestingly, Hou et al. demonstrated that *PTEN* methylation became progressively higher from

FA to FTC and to aggressive ATC, which harbored activating genetic alterations in the PI3K/Akt pathway with a corresponding progressively higher prevalence [88]. The authors concluded that the silencing of the *PTEN* gene, which coexisted with activating genetic alterations of the PI3K/Akt pathway, might enhance the signaling of this pathway aberrantly activated by genetic alterations and hence contribute to the progression of thyroid tumors [88].

PAX8/PPARγ Rearrangement

The *PAX8/PPARγ* rearrangement results from the translocation t(2;3)(q13;p25) and leads to the fusion between *PAX8* and *PPARγ* ("peroxisome proliferator-activated receptor") genes. PAX8 is a transcription factor, which regulates the expression of thyroid specific genes (*NIS*, *TPO*, *TSHR* . . .) as well as proliferation and differentiation of thyroid follicular cells. PPARγ is a transcription factor belonging to the hormone nuclear receptor family, lowly expressed in normal thyroid tissue. Because PPARγ is normally an activator of *PTEN* expression, the *PAX8/PPARγ* rearrangement leads indirectly to a decrease in *PTEN* expression and, consecutively, to activation of the PI3K/Akt signaling pathway [89, 90]. Nevertheless, the mechanisms of *PAX8/PPARγ*-induced carcinogenesis are not perfectly understood.

The *PAX8/PPARγ* rearrangement is principally encountered in conventional FTC where it has been demonstrated in approximately 30–40% of cases [91–93]. This genetic alteration can also be found in follicular variants of PTC and, more rarely, in Hurthle cell carcinoma [43]. However, it has not been identified in conventional PTC [94, 95]. In some studies, the *PAX8/PPARγ* rearrangement has also been reported in FA [91, 92]. Nevertheless, the fact that the *PAX8/PPARγ* rearrangement may be found in benign follicular lesion remains controversial [71]. In a study investigating the presence of *PAX8/PPARγ* rearrangement in thyroid tumors by RT-PCR and immunohistochemical analyses, Nikiforova et al. detected this rearrangement in 53% of FTC but also in 8% of FA [95]. Interestingly, in this study, the FA positive for *PAX8/PPARγ* had a trabecular growth pattern and thick capsule but no invasion, and thus may constitute "pre-invasive" FTC. In another study, the same authors demonstrated that *PAX8/PPARγ* rearrangement and *RAS* mutations had to be considered mutually exclusive suggesting that FTC develop through at least two distinct and virtually nonoverlapping molecular events, *RAS* point mutation and *PAX8/PPARγ* rearrangement [80].

Other Genetic Alterations Activating the PI3K/Akt Signaling Pathway

Several mutations of the upstream activators and different components of the PI3K/Akt signaling pathway have been described in thyroid cancer. Mutations of the tyrosine kinase membrane receptors EGFR, PDGFRβ and VEGFR1 have been

found mainly in ATC [96]. Besides the MAPK pathway, *RAS* mutations are also able to activate the PI3K/Akt signaling pathway. This is strongly corroborated by the activation of the PI3K/Akt pathway frequently encountered in FTC and follicular variants of PTC, which also commonly harbor RAS mutations [59, 96, 97]. Activating mutations of *PI3KCA* have been reported in thyroid cancers, and particularly in FTC and ATC, with a frequency of approximately 10–15%. Nevertheless, in thyroid cancers, *PIK3CA* amplification is a more frequent mechanism of activation of the PI3K/Akt signaling pathway than *PIK3CA* mutation [79, 97]. This amplification leads to overexpression of *PI3KCA* and increased-Akt phosphorylation, suggesting a significant role of *PI3KCA* amplification in activation of the PI3K/Akt signaling pathway in thyroid cancers [96, 98]. Accordingly, in a study investigating the presence of PI3KCA copy number gain and mutation, in a large series of primary thyroid tumors, Hou et al. found *PI3KCA* amplification and mutations in respectively 17 and 6% of FA, 12 and 3% of PTC, 28 and 6% of FTC, and 42 and 12% of ATC [79].

2.2.3 Other Somatic Mutations in Follicular Cell-Derived Thyroid Cancer

Mutations of the tumor suppressor gene p53 occur late in thyroid carcinogenesis. The frequency of p53 mutations increases with tumor dedifferentiation, and it is therefore not surprising that p53 mutations have been found principally in ATC [82]. Similarly, mutations of β-catenin gene (CTNNB1) are essentially encountered in PDTC [99, 100].

The main somatic mutations and gene rearrangements encountered in follicular cell-derived thyroid cancers are listed in Table 12.1.

2.3 Gene Expression Profiling: Lessons from High Throughput Methods

Gene expression profiling studies showed that PTC differed from normal thyroid tissue by the high number of upregulated genes, whereas FTC were characterized much more by numerous downregulated genes [101–104]. In an interesting study, Oler et al. showed that three genes (*CST6*, *CXCL14* and *DHRS3*) were strongly associated with PTC and that *CST6*, *CXCL14*, *DHRS3* and *SPP1* were associated with PTC lymph node metastasis, with *CST6*, *CXCL14*, and *SPP1* being positively correlated with metastasis and *DHRS3* being negatively correlated [104]. Finally, the authors found a strong correlation between *CST6* and *CXCL14* expression and *BRAF*(*V600E*) mutational status, suggesting that these genes may be induced subsequent to BRAF activation and therefore may be downstream in the MAPK signaling pathway. In a study investigating the mutation-specific gene expression profiles in PTC by DNA microarray analysis, Giordano et al. defined distinct

Table 12.1 Main somatic mutations and gene rearrangements in follicular cell-derived thyroid cancers

Somatic mutations/ gene rearrangements	Histologic tumor types	Comments
***BRAF* mutation**	PTC, PDTC, ATC	Particularly frequent in tall cell variant of PTC and in conventional PTC
		Specific of malignity (FNAB)
		Prognostic value (controversial)
***RET/PTC* rearrangement**	PTC	*RET/PTC1*: conventional PTC and diffuse sclerosing variant of PTC
		RET/PTC3: solid variant of PTC
		Can be found in benign thyroid lesions such as Hashimoto's thyroiditis
		Associated with younger age and more lymph node metastases
***RAS* mutation**	PTC, PDTC, ATC, FTC, Hurthle cell carcinoma	Principally found in FTC
		For PTC: essentially in follicular variant
		Can be found in FA
		Associated with tumor dedifferentiation
***PAX8/PPARγ* rearrangement**	PTC, PDTC, ATC, FTC, Hurthle cell carcinoma	Principally found in FTC
		For PTC: essentially in follicular variant
		Can be found in FA (controversial)

PTC papillary thyroid carcinoma, *FTC* follicular thyroid carcinoma, *FA* follicular adenoma, *PDTC* poorly differentiated thyroid carcinoma, *ATC* anaplastic thyroid carcinoma

expression profiles for the *BRAF*, *RET/PTC* and *RAS* mutation groups [105]. In particular, *BRAF*-mutated PTCs were characterized by a distinct and easily recognizable gene expression profile. Interestingly, gene expression profiling of *BRAF*-positive PTCs shows a downregulation of thyroid-specific genes such as *TPO*, *TG* and *NIS* [105, 106]. This downregulation of thyroid-specific genes is, however, not specific of PTC [71]. In a recent study, Vriens et al. showed that the extent of disease at presentation and the survival of patients with PTC differed between between patients aged 15–39 years and patients aged ≥40 years. However, the authors found no distinct gene expression profiles that distinguished younger and older patients with PTC [107].

Among the multiple downregulated genes in FTC, Aldred et al. demonstrated, by semiquantitative reverse transcription-PCR, that *calveolin-1* and *-2*, as well as *GDF10*, were downregulated in 79% of cases. In this study, immunohistochemical analysis of 141 thyroid tumors of various histological types showed, unlike PTC and ATC, significantly fewer caveolin-1-positive tumors in FTC in comparison with normal thyroid [108]. This is of particular interest because calveolin-1 is known to interact with the tumor suppressor gene *PTEN*. In a recent study, Arora et al. used gene expression array analysis to identify borderline thyroid tumors, a subset of follicular lesions of the thyroid similarly encapsulated similarly to FA but with partial nuclear features suggestive of PTC [109]. The expression profile

of these histologic borderline tumors overlapped with the benign and malignant groups. Twenty-seven genes were expressed differentially between the benign and intermediate groups, including *CITED1* gene and *FGFR2* gene. Fourteen genes were expressed differentially between the intermediate group and malignant tumors, notably overexpression of the *MET* proto-oncogene and *HMGA2* gene in malignancies [109].

Finally, even though several studies have shown that some genes are differentially expressed in normal thyroid tissue, FA and the different types of thyroid malignancy, none of these genes can, to date, be considered as an ideal biomarker in terms of sensitivity and specificity.

2.4 MicroRNAs Expression Profiling

2.4.1 MicroRNAs Biogenesis and Function

MicroRNAs (miRs) constitute a class of small endogenous noncoding RNAs of 19–23 nucleotides that negatively regulate gene expression. They modulate the expression of many protein-coding genes in multicellular organisms. The first miR (*Lin 4*) was identified in *C. elegans* in 1993. To date, more than 800 miRs have been discovered [110–113]. They are transcribed as a huge double-stranded primary transcript by RNA polymerase II, and subsequently converted by nuclear enzymes belonging to the family of ribonucleases (RNase) III, Drosha and its essential cofactor DGCR8/Pasha into a double-stranded miR precursor of approximately 70 nucleotides [114]. They are then transported into the cytoplasm through a GTP-dependent active transporter, exportine-5. The miR precursor is transformed by Dicer, a RNase III, with the protein cofactor TRBP, into the 22-nucleotide double-stranded miR, which is later unwound [114]. The leading strand is incorporated into the RISC, and then becomes able to bind the 3′ unrelated region of the target mRNAs leading to a block of mRNAs translation or mRNAs degradation, depending on the level of complementarity [115–117]. Schematically, a high level of complementarity between the miR and its target mRNA favors degradation of the mRNA by the RISC, whereas poor complementarity favors a block in the translation of the mRNA [118]. Furthermore, recent studies have shown that, in particular conditions, miRs can also activate the translation of the target mRNAs [119, 120]. To date, the regulation of miR biogenesis is poorly understood but seems to be under the control of the target proteins, the expression of which they regulate [117].

By regulating gene expression, miRs play a critical role in several biological processes such as cell proliferation, differentiation and apoptosis [117]. Furthermore, several studies reported a deregulation of miRs expression in several human malignancies, including thyroid cancer [115, 117]. Interestingly, different variations in the miRs expression profile have been described and correlated to the histologic type and to the mutational profile of thyroid tumors [117, 121].

2.4.2 MicroRNAs Expression in PTC

Several studies have shown a differential expression of miRs in PTC compared with benign thyroid lesions or normal thyroid tissue [117, 121–123]. In PTCs, several miRs including miR-21, -31, -34a, -122a, -146, -146b, -155, -172, -181a, -181b, -187, -205, -213, -220, -221, -222, -223 and -224 are upregulated, whereas other miRs, including miR-1, -26a, -138, -191, -219, -345, -451 and -486, are downregulated [117, 121, 123]. In an interesting study, Nikoforova et al. established a strong correlation between miR expression and the mutational status of PTC [124]. In this study, miR-187 was overexpressed in PTCs harboring *RET*/*PTC* rearrangement, whereas miR-221 and -222 were overexpressed in those exhibiting *BRAF* or *RAS* mutation, or with no known mutations. MiR-146 was found at the highest level in PTCs expressing *RAS* mutation. Finally, unlike *RAS*-positive PTCs, *BRAF* and *RET*/*PTC*-positive PTCs segregated into separate clusters [124]. On tumor cell lines, Ricarte-Fiho et al. showed that down-expression of let-7f could be implicated in *RET*/*PTC3*- induced carcinogenesis [125]. Overexpression of miR-146b has been found in *BRAF*-mutated PTC [126]. However, to date, no clear correlation between the miR expression profile and the histological subtypes of PTC (conventional PTC, Hurthle cell-, tall cell- or follicular-variants) has been demonstrated [117, 127]. Furthermore, the mechanisms responsible for miR overexpression in PTC have not yet been elucidated.

The biological mechanisms linking overexpression of miR-221 and -222 to the development of PTC have been partially identified. These two miRs are grouped into clusters on chromosome 10 and harbor a similar expression profile. They regulate the expression of genes involved in the control of key cellular functions such as cell proliferation, invasion and apoptosis [127]. MiR-221 and -222 overexpression enhances cell proliferation of PTC cell lines [127]. The expression of the *KIT* gene, which encodes a protein kinase regulating cell growth and differentiation, is decreased in PTC. Interestingly, the *KIT* gene is one of the targets of miR-221 and -222 [128]. Indeed, He et al. have demonstrated that, in PTC, overexpression of miR-221 and -222 was associated with down-expression of *KIT* [123]. The *CDKN1B* ($p27^{Kip1}$) gene is also a target of miR-221 and -222 and is recognized as a critical regulator of cell cycle, particularly in cell growth arrest in the G1/S transition [129]. Furthermore, miR-221 and -222 are also able to impair tumor necrosis factor (TNF)-related apoptosis and control cancer cell invasion [130].

Overexpression of miR-146a and miR-146b in PTC has been reported in most studies [122–124, 126]. Like miR-221 and -222, miR-146a and miR-146b are able to control the expression of the protein kinase KIT. They act also as negative regulators of the NF-κB pathway [117]. Accordingly, Bhaumik et al. demonstrated that these miRs downregulated IL-1 receptor-associated kinase and TNF receptor-associated factor 6, two key adaptor proteins in the IL-1 and Toll-like receptor signaling pathway, known to positively regulate NF-κB activity [131]. Interestingly, there is a G/C polymorphism in the pre-miR-146a that is known to affect the predisposition to PTC, with the GC heterozygosity correlated to a higher risk of developing PTC [132, 133]. Several miRs can also be downregulated in PTC and can

therefore be considered as tumor suppressors. Among these miRs, miR-1 is able to target *CXCR4*, an α-chemokine receptor, which is frequently overexpressed in PTC and which plays a major role in the mechanism of lymph node metastasis [117]. On thyroid cancer cell lines, Brest et al. demonstrated that miR-129-5p was involved in the antitumor activity of histone deacetylase inhibitors, highlighting a miR-driven cell death mechanism [134]. Recently, Wiong et al. demonstrated that miR-886-3p was differentially expressed between sporadic and familial PTC and that this miR was also significantly downregulated in sporadic PTC compared to normal thyroid tissue [135]. Furthermore, the authors showed that miR-886-3p regulated genes involved in DNA replication and focal adhesion.

2.4.3 MicroRNA Expression in FTC

Different miR expression profiles have been reported in FTC and FA [117]. Nikiforova et al. showed that miR-155, -187, -221, -222 and -224 were overexpressed in conventional FTC, and that conventional FA and the oncocytic-variant of FTC or FA harbored distinctive miR expression patterns [124]. Weber et al. reported that two specific miRs, miR-197 and -346, were significantly overexpressed in FTC [136]. They also demonstrated in vitro that overexpression of miR-197 or -346 induced proliferation, whereas inhibition led to growth arrest.

2.4.4 MicroRNA Expression in ATC

In comparison with other thyroid tumors, miRs are more frequently down-expressed in ATC. Accordingly, in a study by Visone et al. [137], several miRs, such as miR-26a, -30a-5p, -30d and -125b, which inhibit the expression of key genes for tumor development, have been shown to be downregulated in ATC. In another interesting study, Mitomo et al. found that miR-26a, -138, -219 and -345 were downregulated in ATC and that one of the potential targets of miR-138 was the human telomerase reverse transcriptase (hTERT), overexpression of which has been correlated to dedifferentiation, advanced tumor stage and metastatic and invasive phenotype [138]. Braun et al. showed that two families of miRs, the miR-200 and the miR-30 family, were significantly down-expressed in ATC in comparison with PTC or FTC [139]. This is particularly interesting because the miR-200 family of miRs is known to inhibit epithelial to mesenchymal transition, a process that is viewed as an essential early step in tumor metastasis [140].

However, some miRs are overexpressed in ATC. Nikiforova et al. have reported overexpression of miR-137, -155, -187, -205, -214, -221, -222, -224, and 302c in four cases of ATC [124]. In a study on ATC cell lines, Takakura et al. have found an overexpression of the miR-17-92 cluster (including particularly miR-17-5p, -17-3p, -18a, -19a, -19b, -20a and 92) and of miR-106a and -106b [141]. They also demonstrated in vitro that inhibition of miR-17-3p, -17-5p or -19a induced strong growth reduction. Interestingly, PTEN, an inhibitor of the PI3K/Akt pathway which

can be mutated in ATC, is one of the targets of miR-17-5p and 19a [141]. The NF-κB signaling pathway is also frequently activated in ATC. Accordingly, in a recent study, Pacifico et al. showed that NF-κB contributed to ATC malignant potential through up-regulation of miR-146a [142].

2.4.5 MicroRNA Expression in Borderline Tumors of Uncertain Malignant Potential

Borderline tumors of uncertain malignant potential seem to harbor a distinct miR expression profile compared to PTC or FTC. Lassale et al. recently demonstrated that a small set of miRs (miR-7, miR-146a, miR-146b, miR-200b, miR-221, and miR-222) appeared to be useful, though not sufficient, in distinguishing thyroid tumors of uncertain malignant potential from other well differentiated tumors of the thyroid gland [143]. In the same way, Sheu et al. showed that the expression of several miRs was significantly lower in borderline tumors than in FTC (miR-21) or in the follicular variant of PTC (miR-146b) [144]. Similarly, Chen et al. reported lower expression of mir-146b in borderline tumors compared to conventional PTC and the follicular variant of PTC, whereas, in borderline tumors, miR-221 and -222 evidenced an intermediate expression level between benign and malignant thyroid lesions [122].

The main deregulated miRs in follicular cell-derived thyroid cancers are shown in Table 12.2.

2.5 Molecular and Gene Expression Signatures for Radiation-Induced Thyroid Cancers

Both external and internal exposures to ionizing radiation are strong risk factors for the development of thyroid tumors. Until now, the diagnosis of radiation-induced thyroid tumors has been deduced from a network of arguments taken together with the individual history of radiation exposure. Neither the histological features nor the genetic alterations observed in these tumors have been shown to be specific fingerprints of an exposure to radiation [145].

Among the different types of RET/PTC rearrangements, RET/PTC3 has been implicated in the development of an unusual solid subtype of PTC which prevails in children exposed to radiation from the Chernobyl nuclear power plant disaster. Interestingly, Powell et al., who have generated transgenic mice expressing human *RET/PTC3* exclusively in the thyroid, have shown that these mice also developed solid tumor variants of PTC [49]. In a study on 71 cases of PTC in atomic bomb survivors, Hamatani et al. reported that, contrary to the *BRAF*(*V600E*) mutation, *RET/PTC* rearrangements showed increased frequency with increased radiation dose, and that PTC patients harboring *RET/PTC* rearrangement developed this

Table 12.2 Deregulation of microRNA expression in follicular cell-derived thyroid cancers

Histologic tumor types	Deregulated microRNAs	Comments
PTC	Overexpression: miR-21, -31, -34a, -122a, -146, -146b, -155, -172, -181a, -181b, -187, -205, -213, -218, -220, -221, -222, -223 and -224	Correlation between the miR expression profile and the mutational status of PTC
	Underexpression: miR-1, -19b-1,2, -26a, -30c, 30a-5p, -130b, -138, -145sh, -191, -219, -292-as, -300, -345, -451 and -486	No clear correlation between the miR expression profile and the histological subtypes of PTC
FTC	Overexpression: miR-146b, 155, -183, -187, -192, -197, -221, -222, -224, -328, -339 and -346	Different miR expression profiles in FTC and FA
ATC	Overexpression: miR-9, -10a, -17-3p, -17-5p, 17–92 cluster, miR-106a, -106b, -124a, -127, -129, -137, -146a, -154, -155, -187, -205, -214, -221, -222, -224, -302c, -323 and -370	miRs are more frequently under-expressed in ATC in comparison with other thyroid tumors
	Underexpression: miR-let7d, -let7g, -26a, -26b, -30a-5p, -30b, -30c, -30d, -125b, -138, -141, -200a, -200b, -200c, -219, -345 and -429	

PTC papillary thyroid carcinoma, *FTC* follicular thyroid carcinoma, *FA* follicular adenoma, *ATC* anaplastic thyroid carcinoma, *miR* microRNA

cancer earlier than did patients with *BRAF*(*V600E*) mutation [146]. Similarly, Nikiforova et al. who detected the *PAX8*/*PPARγ* rearrangement in 53% of patients with FTC, also showed that the prevalence of this rearrangement was increased in patients with a history of radiation exposure [95].

Several authors have investigated a specific gene expression signature for radiation-induced PTC. Ugolin et al. recently evaluated the new method EMts_2PCA, in 26 patients with PTC, to differentiate post-Chernobyl tumors from their sporadic counterparts [147]. The EMts_2PCA method identified a molecular signature using a subset of 13 tumors, and this signature was robust enough to classify unambiguously 12 of the 13 remaining tumors in either the sporadic PTC or post-Chernobyl PTC subgroup. The 13th tumor (a sporadic PTC) clustered between the two subgroups and could not be classified, but it was not misclassified as radiation-induced. In another recent study to identify molecular markers that could represent a radiation-induction signature, Ory et al. compared 25K microarray transcriptome profiles of a learning set of 28 thyroid tumors (14 FTA and 14 PTC, either sporadic or consecutive to external radiotherapy in childhood) [145]. They identified a signature composed of 322 genes which discriminated radiation-induced tumors (FTA and PTC) from their sporadic counterparts. The robustness of this signature was further confirmed by blind case-by-case classification of an independent set of 29 tumors (26/29 tumors were well classified regarding tumor etiology, 1 was undetermined, and 2 were misclassified).

3 Molecular Biology and Thyroid Tumors in Clinical Practice: Current Role and Future Directions

3.1 *Methodology and Technical Aspects of Molecular Biology in Thyroid Pathology*

There are a lot of published studies assessing the role of various molecular markers for the diagnosis and prognosis of thyroid tumors [32, 35]. The results of such studies should be interpreted with caution as they are critically dependent on the pre-analytical steps and methodological approaches used to detect these molecular markers. The techniques used to detect the different genetic alterations involved in thyroid oncology may differ greatly from one laboratory to another. For example, concerning the *BRAF*(*V600E*) mutation, most studies have been made on formalin-fixed paraffin-embedded samples, others on fresh-frozen surgical specimens, and some on both. Some studies used singlestrand conformation polymorphism (SSCP) to detect the mutation while others used direct sequencing after PCR amplification. Several reports have demonstrated that SSCP could be less sensitive than direct sequencing [29]. Recently, pyrosequencing, nonelectrophoretic nucleotide extension sequencing has emerged as a new sequencing methodology for various applications, including detection of mutations in tumors and detection of tumor cells among normal cells. Pyrosequencing is a simple and less expensive methodology compared with other approaches such as direct DNA sequencing. In addition, pyrosequencing is much faster and could be more sensitive than dideoxy sequencing for detection of different mutations [29, 148]. These considerations are of critical importance to explain the variations in the results of different studies.

The choice of the technique used to detect each genetic alteration raises a crucial issue. For instance, PCR-based techniques to detect *RET*/*PTC* or *PAX8*/*PPARγ* rearrangements cannot be used on formalin-fixed tissues. In this situation, fluorescent in situ hybridization (FISH) is a more appropriate method. Conversely, point mutations such as *BRAF* or *RAS* mutations can be detected by most methods (RT-PCR, direct sequencing, pyrosequencing) on frozen or formalin-fixed tissues [29, 149–151]. The miR expression profiling of tumors samples can be done both on frozen or formalin-fixed tissues. Detection of miRs in frozen tissue sections can also be accomplished by FISH using locked nucleic acid probes and tyramide signal amplification [117].

3.2 *Role of Molecular Biology for the Diagnosis of Thyroid Tumors*

Detection of the molecular alterations associated with thyroid cancers described above can be performed on cytological material from fine needle aspiration biopsy (FNAB) or on surgical resection specimens.

3.2.1 Molecular Analyses Performed on FNAB

FNAB is the most accurate and cost-effective method for evaluating thyroid nodules [3, 7]. If the nodule is benign on cytology, as is most often the case, further immediate diagnostic studies or treatment are not routinely required. If a cytology result is diagnostic of PTC, which is a relatively rare event, surgery is recommended [3, 7]. Nevertheless, in approximately 20–40% of cases, FNAB cannot efficiently differentiate benign from malignant thyroid lesions. These indeterminate cytological results, reported as "follicular neoplasm" or "Hurthle cell neoplasm", can be found in 15–30% of FNAB specimens and carry a 20–30% risk of malignancy, while lesions reported as "atypia" or "follicular lesion of undetermined significance" are variably reported and have a 5–10% risk of malignancy [8]. In such situations, new diagnostic tools are required in order to avoid unnecessary thyroid surgery.

Many molecular markers have been evaluated to improve diagnostic accuracy for indeterminate nodules. The *BRAF* mutation can be detected in FNAB specimens. Several studies demonstrated that the presence of *BRAF* mutation in FNAB was highly specific of PTC [29, 152]. Interestingly, in these studies, *BRAF* mutation-positive FNABs were regularly encountered in case of indeterminate cytologic results [153–156]. In a study on 111 patients with thyroid lesions and different cytological diagnoses, Marchetti et al. detected the *BRAF*(*V600E*) mutation in 18 over 32 cases (56.2%) with a cytology of suspicious for PTC and in 41 of 56 (73.2%) with PTC [155]. The authors demonstrated that the addition of molecular analysis for the preoperative diagnosis of PTC yielded a 20% increase in sensitivity compared to cytology alone. In another recent study, Marchetti et al. identified the BRAF(V600E) mutation with a high frequency in patients with papillary thyroid microcarcinoma (74%) and showed that the combination of the cytological diagnosis and the molecular analysis was able to identify 82% of all cases of papillary thyroid microcarcinoma, with an increase of 37% compared with a morphological diagnosis alone [157]. In another interesting study on 244 patients with thyroid nodules, Nam et al. found the *BRAF*(*V600E*) mutation for nodules with indeterminate or nondiagnostic cytology in 45% (5/11) of nodules with malignant ultrasonography features and in 8% (2/26) of those without malignant ultrasonography features [156]. All nodules with the mutation were surgically confirmed as PTC. The authors concluded that the application of *BRAF*(*V600E*) mutation analysis in FNAB specimens is more effective for thyroid nodules with malignant ultrasonography features.

The role of other genetic alterations, such as *RAS* mutation, *RET*/*PTC* or *PAX8*/*PPAR*γ rearrangements, has also been explored in FNAB of thyroid nodules. French et al. found the *PAX8*/*PPAR*γ rearrangement in 2 of 24 FNAB samples with different cytological diagnoses [158]. In these two cases, FNAB was suggestive of a follicular neoplasm and the two corresponding surgical specimens were diagnosed as FTC in one case and as follicular variant of PTC in the other. Nikiforov et al., investigating the presence of a panel of mutations in 470 thyroid FNAB samples, identified 32 mutations, including 18 *BRAF*, 8 *RAS*, 5 *RET*/*PTC* and 1 *PAX8*/*PPAR*γ [159]. The presence of any mutation was a strong indicator of cancer because 31

(97%) of mutation-positive nodules had a malignant diagnosis after surgery. Finally, the authors concluded that a combination of cytology and molecular testing showed significant improvement in diagnostic accuracy and allowed better prediction of malignancy in the nodules with indeterminate cytology.

Deregulation of the miR expression profile has been demonstrated in FNAB samples from patients with thyroid cancer. Pallante et al. found, in FNAB samples, miR-221, -222 and 181b overexpression in seven of eight cases of PTC [127]. Nikiforova et al. demonstrated that a set of seven miRs (miR-146b -155, -187, -197, -221, -222 and -224) were differentially overexpressed in thyroid tumors in comparison with hyperplastic nodules with high accuracy of thyroid cancer detection in FNAB samples [124]. Chen et al. established that miR-146b was the most consistently overexpressed miR in PTC and, when applied to FNAB samples of various thyroid lesions, was a useful distinguishing marker for PTC [122].

Finally, the molecular biology indications for the preoperative diagnosis of thyroid tumors in clinical practice have still to be defined. To date, no molecular biomarker can, alone, distinguish malignant from benign thyroid nodules with sufficient sensitivity and specificity. Nevertheless, the detection of a set of molecular genetic alterations including, particularly, *BRAF*(*V600E*) and *RAS* mutations, as well as *RET*/*PTC* and *PAX8*/*PPARγ* rearrangements, appears to be very useful in cases of indeterminate FNAB cytology [124, 152]. If the presence of one these genetic alterations is very suggestive of cancer, at the opposite, their absence is not synonymous with benign thyroid lesion [29, 159]. In practice, the presence of one of these genetic alterations, and particularly of *BRAF* mutation, on FNAB material should lead to thyroid surgery. This strategy has to be evaluated in clinical practice in large series of patients.

3.2.2 Molecular Analyses Performed on Surgical Resection Specimens

BRAF mutation-harboring PTCs generally correspond to conventional or tall cell variant of PTCs, neither of which raises diagnostic difficulties on histological examination. *BRAF* mutation is rare in follicular variants of PTC and is generally not encountered in FTC [29, 40]. Therefore, the detection of *BRAF* mutation in surgical resection specimens provides no benefit in the diagnosis of thyroid cancer.

Even if *RAS* mutations are more frequent in thyroid carcinomas than in benign thyroid lesions, they can be encountered both in follicular variants of PTC, FTC and FA [71]. Therefore, this mutation is not useful in distinguishing, with certainty, malignant from benign thyroid follicular lesions. If detection of the *PAX8*/*PPARγ* rearrangement is generally associated with FTC, it cannot exclude the diagnosis of FA [71]. Indeed, the *PAX8*/*PPARγ* rearrangement can be found in a small proportion of FA. Nevertheless, when this mutation is detected in a thyroid lesion suggestive of FA, the presence of vascular or capsular invasion should be carefully examined in order not to miss the diagnosis of FTC. Although several punctual mutations have been reported in thyroid tumours of uncertain malignant potential, these thyroid lesions do not harbor a specific mutational profile [13].

Finally the impact of molecular biology on the histological diagnosis of follicular cell-derived thyroid tumors in surgical specimens is quite limited. This is due, at least in part, to the fact that histological diagnosis must provide certainty, which, most often, is not available with molecular biology, and not a mere estimation of the risk of cancer, which is the essential goal of preoperative FNAB cytology.

3.3 Role of Molecular Biology for Predicting the Prognosis of Thyroid Cancer

WDTCs, and particularly the small, unifocal and well-localized tumors, generally harbor a good prognosis. With surgery and radioiodine therapy, the recurrence rate is low, between 10 and 20%, and survival of patients treated for WDTC is nearly similar to that of the healthy population [3, 7]. Nevertheless, in some cases, WDTC may behave more aggressively and require more intensive therapy. Molecular biomarkers can provide precious information for predicting the prognosis of thyroid tumors, allowing a more accurate adaptation of the treatment to the aggressive potential of the tumor.

3.3.1 Prognostic Impact of BRAF Mutation

Numerous studies have reported that PTCs harboring the *BRAF*(*V600E*) mutation correlated with a worse prognosis. In a metaanalysis on 1,168 patients with PTC, Lee et al. have reported 49% frequency of the *BRAF* mutation with a significant correlation between *BRAF* mutation and the histologic subtype, the presence of extrathyroidal extension and a higher clinical stage, but not with age, sex, race, or tumor size [41]. Similarly, Kebebew et al. showed that *BRAF* mutation was correlated to recurrence risk and disease-free survival [160]. Recently, Elisei et al. investigated the impact of *BRAF* mutation on overall survival in 102 patients with a median follow-up of 15 years [161]. They demonstrated that *BRAF* mutation was a poor prognostic factor irrespective of other clinicopathological features. The *BRAF* mutation has also been correlated with the loss of radioiodine avidity and tumor dedifferentiation [34].

Nevertheless, these data are still contested and there are also studies that have failed to find any correlation between *BRAF* mutation and prognosis [29, 162]. For example, in a study on more than 600 patients, Ito et al. using multivariate analysis found no significant correlation between *BRAF* mutation and patient gender, tumor stage, massive extrathyroidal extension, lymph node involvement or distant metastasis at presentation [162]. Furthermore, the authors found no difference in 5- or 10-year disease-free or metastases-free survivals.

As *BRAF* mutation can be detected on preoperative thyroid FNAB, it may represent a useful marker in appropriately tailoring the initial surgical treatment for patients with PTC. In a recent study, Xing et al. assessed the *BRAF* mutation status in

thyroid FNAB specimens obtained from 190 patients before thyroidectomy for PTC and showed that the presence of *BRAF* mutation strongly predicted extrathyroidal extension, thyroid capsular invasion, lymph node metastasis and PTC persistence or recurrence [152].

Preoperative detection of the *BRAF* mutation seems particularly interesting in papillary thyroid microcarcinomas. In a study on 500 consecutive cases of PTC, Lupi et al. found that the correlation between the presence of the *BRAF* mutation and the histopathological prognostic factors, such as extrathyroidal invasion, nodal metastases or absence of tumor capsule, was stronger for papillary thyroid microcarcinoma than for larger tumors [163]. In most cases, papillary thyroid microcarcinomas offer an excellent prognosis and can be treated by surgery alone without radioiodine therapy [164]. Furthermore, surgery for papillary thyroid microcarcinomas could be less extensive than for larger tumors, and partial thyroidectomy without central neck dissection could be considered in some cases [3, 7, 165]. Nevertheless, there are some more aggressive papillary thyroid microcarcinomas which should be treated more extensively [166]. Therefore, it would seem to be particularly beneficial to identify the rare cases of papillary thyroid microcarcinoma with a worse prognosis. In this context, in a recent study on 64 cases of papillary thyroid microcarcinomas, Lee et al. found that *BRAF* mutation may be a marker of high tumor aggressiveness [167].

The determination of *BRAF* status could also have an impact on the indication for radioiodine therapy. This is particularly true for patients with T1 larger than 1 cm or T2 N0 disease or with small but multifocal tumors, who are classified in the “gray zone” of indication for radioiodine therapy [3, 7]. Nevertheless, to date, there is no clear evidence that radioiodine therapy will prove helpful for patients with *BRAF*-positive PTC, notably because *BRAF* mutation correlates with a lack of proper membrane sodium – iodine symporter expression [29]. Accordingly, gene expression profiling of *BRAF*-positive PTCs shows downregulation of thyroid-specific genes such as *TPO*, *TG* and *NIS* [105, 106]. However, this downregulation of thyroid-specific genes is not specific of PTC and does not seem to explain the aggressive phenotype of *BRAF*-positive PTC [71]. As both BRAF-positive and BRAF-negative PTCs exhibit mainly non-functional cytoplasmic localization of NIS, this is more increased aggressive biology rather than poorer response to radioiodine therapy that could explain the poorer outcomes of BRAF-mutated PTCs [71, 168].

Finally, from the clinical point of view, a factor present in approximately 50% of cases is of limited use in managing a disease which has a poor outcome in no more than 10–15% of patients. Hence, a more detailed stratification is necessary [29, 71]. Indeed, implementing the *BRAF* mutation as a factor of poor prognosis in clinical practice would entail a large proportion of patients with stage I-II disease being moved from the low-risk to the high-risk group. There is very little evidence-based support for such strategy which would involve a significant risk of overtreatment [71]. Thus, it is particularly crucial to identify new molecular markers in order to recognize, among patients with *BRAF*-positive PTC, those with a truly poor prognosis [71].

3.3.2 Prognostic Impact of Other Genetic Alterations

Unlike *BRAF* mutation, the other above-mentioned genetic alterations have no clear impact on the prognosis of thyroid cancers. *Ras* mutations can be encountered in benign thyroid tumors, and their prognostic value in thyroid cancer remains unclear. Nevertheless, Garcia-Rostan et al. found that *Ras* mutations were associated with poorly differentiated tumors and worse prognosis in thyroid cancer patients [77]. In this study, 11 (55.0%) of 20 patients with differentiated thyroid carcinomas with mutated tumors died as a result of their disease as opposed to 9 (15.5%) of 58 patients with wild-type *RAS* tumors. Similarly, Volante et al. demonstrated that *RAS* mutations, essentially located at codon 61 of the *NRAS* gene, were the predominant mutations detected in PDTC and constituted a negative prognostic parameter [81].

The correlation between *RET/PTC* rearrangement and prognosis in PTC is still uncertain. *RET/PTC* rearrangement is the main mutation encountered in Hashimoto's thyroiditis-associated PTCs, which are generally negative for the *BRAF* mutation [38]. Interestingly, several studies have reported that a coexisting Hashimoto's thyroiditis was associated with an improved prognosis in patients with PTC [169, 170]. *RET/PTC* rearrangement has been found to be correlated to younger age and an increased frequency of lymph node metastases [59, 65]. In patients with PTC, young age is associated with more lymph node metastasis, but nevertheless, with a better outcome than in older patients [65, 71, 171]. Finally, the correlation between *RET/PTC* rearrangement and prognosis in patients with PTC is not yet well-defined [172].

The overexpression of the growth factor receptors of the HER family, and particularly of HER1 (EGFR), HER2 and HER3, have been reported in thyroid cancer and seems to be correlated with a worse outcome [173]. Interestingly, the activating mutations affecting the intra-cellular tyrosine kinase domain of EGFR, well described in lung cancer and correlated with the response to EGFR tyrosine kinase inhibitors, have also been found in thyroid cancer [174]. Mitogen-inducible gene-6 (MIG-6) is a tumor suppressor, downregulated in several human malignancies, which negatively regulates the EGFR signaling pathway. Interestingly, Ruan et al. demonstrated that a high level of MIG-6 expression was associated with favorable outcomes in a series of 106 patients with PTC [175]. Furthermore, the authors showed that MIG-6 overexpression was independently predictive of greater disease-free survival in *BRAF*(*V600E*)-positive PTC.

VEGF overexpression has been found in various human malignancies, including thyroid cancer. VEGF is one of the most potent proangiogenic factors and its overexpression is a marker of tumor hypoxia. Interestingly, Jo et al. demonstrated, in a study on 161 PTC patients, that VEGF expression levels were strongly positively correlated with tumor size, extrathyroidal invasion, and tumor stage [176].

E-cadherin is a calcium-dependent cell adhesion molecule which plays an important role in normal growth and development. Downregulation of E-cadherin has been found in PTC and correlated with poor prognosis [177, 178]. Mucin 1 (MUC1) is another glycoprotein playing an important role for cell adhesion. Preclinical data suggest that MUC1 overexpression promotes an aggressive phenotype in PTC,

which corroborates the fact that MUC1 overexpression has been frequently shown in tall-cell variant of PTC [179, 180]. If various studies seemed to find a correlation between MUC1 overexpression and poor outcomes in PTC patients, this issue, to date, remains controversial [71, 181]. The role of various other molecular factors, such as calvasculin, osteopontin, toll-like receptors 3 or 5 and chemokine receptors, in the prognosis of thyroid cancer, and particularly of PTC, has been investigated in several studies, but is not yet clearly established [71].

Recently, miRs have been reported to offer a promising diagnostic tool in the field of thyroid oncology, and several studies have demonstrated their utility as class identifiers, especially in the context of PTC, FTC and ATC [117]. Interestingly, Gao et al. showed that the miR expression profile varied with invasiveness in PTC cell lines and identified a set of 11 metastasis-related miRs differentially expressed between the invasive and the non-invasive cell line subpopulations [182]. Similarly, Chou et al. demonstrated, in a study of 100 PTC, that miR-146b, -221 and -222 expression was correlated to extrathyroidal tumor extension. Moreover, in this study, miR-146b and -221 expression levels were significantly higher in high risk than in low risk PTCs [126]. Larger studies and/or meta-analyses could further delineate the role of miRs in predicting cancer progression and prognosis, which is, to date, largely undetermined.

4 Molecular Biology and Thyroid Cancer: Implications for Targeted Therapies

With the improvement of our understanding of thyroid cancer molecular biology, several molecules have emerged as promising molecular targets for thyroid cancer therapy. The most frequent genetic alterations encountered in PTC result in the constitutive activation of the MAPK pathway. Consequently, the selective targeting of one of the components of this critical signaling pathway should offer a promising therapeutic approach [38].

4.1 RET Kinase and Multikinase Inhibitors

Carlomagno et al. have shown that RET kinase inhibitors such as pyrazolopirimidine derivatives are able to prevent the growth of two human PTC cell lines harboring the *RET/PTC* rearrangement [183]. In another study, they demonstrated that sorafenib (BAY 43-9006), a multikinase inhibitor, inhibited the growth of *RET/PTC*-positive thyroid cancer cell lines both in vitro and in vivo [184]. They also reported that sorafenib was active on cells harboring the mutations *RET*(*V804L*) and *RET*(*V804M*) of the RET kinase which are known to confer resistance to anilinoquinazolines and pyrazolopyrimidines.

In two recent phase II clinical trials on patients with metastatic and iodine-refractory thyroid carcinoma, sorafenib has shown promising clinical activity with an acceptable safety profile [185, 186]. In a study investigating the response to sorafenib at a low dose in patients with radioiodine-refractory pulmonary metastases from PTC, Chen et al. reported an objective partial response rate and a stable disease rate of 33 and 44% respectively [187]. Furthermore, the authors demonstrated a marked and rapid change in the serum thyroglobulin level after start of treatment, with a mean decrease of 60% within 12 weeks, consistent with radiographic findings. Several phase III clinical trials are currently ongoing to determine the benefit of sorafenib in this type of patient [38].

4.2 BRAF Kinase Inhibitors

Some specific BRAF kinase inhibitors have been recently developed and seem more effective than sorafenib on thyroid cell lines harboring the *BRAF*(*V600E*) mutation [38, 188]. Selective therapies against *BRAF*(*V600E*), like SB-590885, PLX4720 and PLX4032, are specific BRAF inhibitors, inhibiting preferentially tumor cells harboring the mutant BRAF allele [29]. SB-590885 inhibits BRAF kinase enzymatic activity 100-fold more potently than sorafenib [189]. Some specific BRAF kinase inhibitors have recently shown relative therapeutic efficacy in vitro or in xenograft animal models. In a recent study, Lee et al. found that a significant proportion of *BRAF*(*V600E*), but not wild-type *BRAF*, was detected in the mitochondrial fraction of thyroid cancer cells [190]. Interestingly, they also demonstrated that the mitochondrial localization and antiapoptotic activities of *BRAF*(*V600E*) were unaffected by sorafenib and U0126 suppression of MEK and ERK activities.

4.3 MEK Inhibitors

As MEK inhibitors are able to suppress MAPK pathway signaling, they are considered to be very promising therapeutic agents in several human malignancies, including thyroid cancers. Numerous preclinical studies have demonstrated that MEK inhibitors preferentially inhibit *BRAF* or *RAS* mutation-harboring cancer cells [191–193]. Although MEK inhibitors are able to inhibit ERK1/2 phosphorylation in both *RET/PTC* rearrangements and *BRAF* mutations-positive thyroid cancer cells, the effects of MEK inhibitors on cell proliferation and apoptosis are encountered essentially in PTC harboring *BRAF* mutations [193]. Indeed, Liu et al. showed that cell proliferation is potently inhibited by the MEK inhibitor CI-1040 in thyroid cancer cells harboring *BRAF* or *RAS* mutations but not in cells harboring *RET/PTC* rearrangement or wild-type alleles [192]. More recently, the same authors have reported

comparable results with the new-generation MEK inhibitor PD0325901 [194]. Interestingly, although there was no inhibition of the proliferation of *RET/PTC1*-harboring cells by PD0325901 itself, this inhibition could be induced or significantly potentiated by concurrent inhibition of the PI3K or the NF-kB pathway. This may be explained by the fact that *RET/PTC* rearrangements are coupled to these multiple signaling pathways which are important for proliferation of cancer cells. Consequently, it is not surprising that MEK inhibitor alone did not show significant inhibition on proliferation and some other cellular events of *RET/PTC1*-harboring thyroid cancer cells. Thus, inhibition of the different components of the MAPK signaling pathway in combination with the blockage of other critical signaling pathways for thyroid cancer development, survival regulation, apoptosis or inflammation appears to offer a promising therapeutic approach [38]. Thus, Jin et al. have recently investigated the effects of the association of a MEK inhibitor (AZD6244 or ARRY-142886) with the mTOR inhibitor rapamycin on 10 differentiated thyroid cancer and anaplastic thyroid cancer cell lines and in a xenograft model [195]. They found that all the cancer cell lines tested exhibited better than 60% growth inhibition with combined MEK and mTOR inhibition, including lines with *BRAF*, *RET/PTC*, *RAS*, and *PTEN* mutations. This dual-pathway inhibition in the *RET/PTC* mutant cell line TPC1 caused an intense G(1) arrest in cell culture and reversible cytostatic inhibition in a xenograft model without significant feedback up-regulation of Akt activation.

4.4 Potential Role of miRs for Thyroid Cancer Therapy

As a clear deregulation of miR expression has been found in several human malignancies including thyroid cancer, miRs represent a promising therapeutic target. Inhibition of miRs such as miR-221, -222, and -146, which have been reported to be overexpressed in PTC, is an attractive direction for therapy [117]. Indeed, inhibition of cell proliferation was obtained when thyroid carcinoma cell lines were treated with miR-221 and -222 antisense oligonucleotides [127]. Interestingly, Frezzetti et al. recently showed that miR-21, a miR overexpressed in ATC, was up-regulated both in vitro and in vivo by oncogenic RAS [196]. They also demonstrated that a LNA directed against miR-21 slowed down tumor growth in xenograft models.

Restoration of downregulated miRs could also provide a tool for improving patient care and response to treatment. Thus, Nasser et al. established that miR-1 was able to suppress the tumorigenic property of lung cancer cells and induced their sensitization to doxorubicin-related apoptosis [197]. This is of particular interest because miR-1 is down-regulated in PTC, and this down-regulation is implicated in the tropism of PTC cells to the local lymph nodes [117]. In this way, Xiong et al. demonstrated that miR-886-3p was down-regulated in sporadic PTC, and that the ectopic expression of miR-886-3p in thyroid cancer cell lines significantly inhibited cellular proliferation and migration [135].

5 Conclusion

Molecular biology is gradually playing a bigger role in the preoperative diagnosis of thyroid tumors, particularly in cases of indeterminate FNAB. The detection of a set of molecular biomarkers, including notably the *BRAF*(*V600E*) mutation, on cytological material has to be tested in large clinical series in order to determine the real clinical benefit for patients. Nevertheless, new thyroid biomarkers should be identified in order to more accurately distinguish malignant from benign thyroid lesions, and thus avoid unnecessary thyroid surgery.

These molecular markers should also improve the preoperative determination of the prognosis of thyroid tumors, and this would be particularly beneficial in adapting treatment to the potential aggressiveness of the tumor. There are of course predictable limitations and difficulties. Patients with WDTC, such as PTC and FTC, enjoy an excellent prognosis, and the future molecular biomarkers will have to be sensitive and specific enough to identify the very low proportion of patients who are at risk of recurrence, and therefore require therapeutic intensification. In contrast, the prognosis of ATC is so poor that all patients with ATC should be treated with the most intensive treatment regimen that they are able to tolerate.

Finally, various molecular targeted therapies are being developed with a view to personalizing treatment for every individual patient and to specific tumor mutations. These promising therapies will need to be evaluated in future clinical trials, particularly on patients with iodo-refractory thyroid cancer.

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Chapter 13
Sarcomas Genetics: From Point Mutation to Complex Karyotype, from Diagnosis to Therapies

Frédéric Chibon, Alain Aurias, and Jean-Michel Coindre

Abstract Sarcomas represent a heterogeneous group of rare tumours accounting for approximately 1% of adult cancers and with more than 50 histological subtypes. Almost half of all sarcomas bear a specific (or almost specific) relatively simple genetic lesion, i.e., recurrent chromosomal translocations, specific activating or rarely specific inactivating mutations and amplifications; the other half is composed of different histotypes characterized by a complex genetics. Even in sarcomas characterized by a single recurrent and specific genomic alteration, clinical outcome is associated to chromosomal instability. These different genetic markers, together with expression profiling are now daily helpful tools for diagnosis and prognosis but are still poorly useful regarding targeted therapies, with a few exceptions. Consequently, the next breakthrough toward a personalized medicine for sarcoma will be the identification of signature predicting drug response, the best being targetable oncogenic driver alteration.

1 Introduction

Sarcomas represent a heterogeneous group of rare tumours accounting for approximately 1% of adult cancers and with more than 50 histological subtypes [1]. They are diverse mesenchymal malignancies that arise in or from bone, cartilage or connective tissues, such as muscle, fat, peripheral nerves and fibrous or related tissues. Diagnosis of these tumours is often challenging and is currently based on

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histological evaluation supplemented with the use of immunohistochemistry and molecular techniques in selected cases. Identification of the first translocation in sarcomas [2] and its molecular characterisation [3] opened the field of molecular markers with the subsequent description of numerous specific simple genetic alterations which are now used as diagnostic tools in the daily practice [4]. Then, at the end of the 1990s, the development of the array technologies provides the medical community, several and sometimes contradictory data about genetics and biology of sarcomas. A "gene signature" can be defined as a single or a combined genetic alteration with specificity in terms of diagnosis, prognosis or prediction of therapeutic response. This specificity should have been validated in independent groups of tumors and, if possible, by different techniques and teams. According to this definition, this chapter will deal with almost exclusively validated "gene signatures" helpful for the daily practice in determining tumor diagnosis, prognosis and predictive response to treatment.

2 Gene Signature and Diagnosis

Almost half of all sarcomas bear a specific (or almost specific) relatively simple genetic lesion, i.e., recurrent chromosomal translocations, specific activating or rarely specific inactivating mutations and 12q13-15 amplifications which can be very useful for the diagnosis [4]. The other half is composed of different histotypes characterized by a complex genetics.

2.1 Sarcomas with Reciprocal Translocation

About 10–15% of all sarcomas bear a recurrent chromosomal translocation (Table 13.1). The most frequent sarcomas with such an abnormality are dermatofibrosarcoma protuberans (DFSP), myxoid liposarcoma, Ewing sarcoma and synovial sarcoma.

Depending on role and function of involved genes, a reciprocal translocation associated with sarcomas may give rise to at least three types of oncogenic factors: aberrant transcription factors; constitutively active receptor tyrosine kinases (RTKs) and constitutively active growth factors.

2.1.1 Fusion Generates Aberrant Transcription Factors

Fusion Genes Involving EWS Family Genes

Nearly half of the fusion genes participating to sarcoma oncogenesis contain a portion of TET gene family products, named after the initials of *TLS/FUS*, *EWSR1*, and *TAFII68*. This includes Ewing sarcoma, myxoid-round cell liposarcoma,

Table 13.1 Recurrent chromosomal translocations associated with soft tissue sarcomas

Sarcoma type	Translocation	Genes involved	Prevalence
Fusion generates aberrant transcription factors			
Ewing sarcoma/PNET	t(11;22) (q24;q12)	EWSR1-FLI1	85–95%
	t(21;22) (q22;q12)	EWSR1-ERG	5–10%
	t(7;22) (p22;q12)	EWSR1-ETV1	Rare (<1%)
	t(17;22) (q12;q12)	EWSR1-ETV4 (E1AF)	Rare
	t(2;22) (q33;q12)	EWSR1-FEV	Rare
	t(1;22) (p36;q12)	EWSR1-ZSG	Rare
	t(16;21) (p11;q22)	FUS-ERG	Rare
Myxoid liposarcoma	t(12;16) (q13;p11)	TLS(FUS)-DDIT3(CHOP)	95%
	t(12;22) (q13;q12)	EWSR1-DDIT3(CHOP)	Rare
Extraskeletal myxoid chondrosarcoma	t(9;22) (q22;q12)	EWSR1 - TEC(NR4A3/CHN/TEC)	75%
	t(9;17) (q22;q11)	TAF2N(RBP56)-TEC/CHN	25%
	t(9;15) (q22;q21)	TCF12-TEC(CHN)	Rare
Clear cell sarcoma	t(12;22) (q13;q12)	ATF1-EWSR1	>90%
	t(2;22) (q32;q12)	EWSR1-CREB1	Rare
Desmoplastic small round cell tumor	t(11;22) (p13;q12)	WT1-EWSR1	>90%
Angiomatoid fibrous histiocytoma	t(2 ;22)(q34;q12)	EWSR1-CREB1	90%
	t(12 ;22)(q13;q12)	EWSR1-ATF1	10%
Low-grade fibromyxoid sarcoma	t(7;16) (q32-34;p11)	TLS(FUS)-CREB3L2	90%
	t(11;16) (p11;p11)	TLS(FUS)-CREB3L1	10%
Alveolar rhabdomyosarcoma	t(2;13) (q35;q14)	PAX3-FOXO1A(FHKR)	60–80%
	t(1;13) (p36;q14)	PAX7-FOXO1A(FHKR)	10–20%
	t(X;2) (q13;q35)	PAX3-AFX	Rare
	t(2;2) (q35;p23)	PAX3-NCOA1	Rare
Alveolar soft part sarcoma	t(X;17) (p11.2;q25)	ASPL-TFE3	>90%
Fusion involves chromatin-remodeling genes			
Synovial sarcoma	t(X;18) (p11;q11)	SS18(SYT)-SSX1	65%
		SS18(SYT)-SXX2	35%
		SS18(SYT)-SSX4	Rare
Sarcoma stromal endometrial	t(7;17)(p15;q21)	JAZF1/JJAZ1	
	t(10;17)(q23;p13)	YWHAE-FAM22A/B	
Fusion involves genes encoding receptor tyrosine kinases (RTKs)			
Infantile fibrosarcoma (cell. mesoblastic nephroma)	t(12 ;15) (p13;q25)	ETV6(TEL)-NTRK3(TRKC)	80–90%
Inflammatory myofibroblastic tumor	t(2;19) (p23;p13.1)	TPM4-ALK	
	t(1;2) (q22-23;p23)	TPM3-ALK	
	t(2;17) (p23;q23)	CLTC-ALK	
	t(2;11) (p23;p15.5)	CARS-ALK	
	t(2;2) (p23;q13)	RANBP2-ALK	
	Other 2p23 rearrangements	ALK-other partners	
Fusion involves growth factors			
Dermatofibrosarcoma protuberans/giant cell fibroblastoma	t(17;22) (q22;q13)	COL1A1-PDGFB	>90%
	ring 17q, ring 22q, der(22)	COL1A1-PDGFB	75%

myxoid chondrosarcoma, clear cell sarcoma, desmoplastic round cell tumor and angiomatoid fibrous histyocytoma (Table 13.1). EWS family proteins contain a characteristic 87-aminoacid-RNA recognition motif that is implicated in protein-RNA binding and participate in transcription and RNA metabolism [5]. EWS family members may be interchangeable in the development of a subgroup of sarcomas. Thus, both EWSR1-DDIT3 and FUS-DDIT3 are found in myxoid liposarcoma with an indistinguishable phenotype; EWSR1-ERG as well as some FUS-ERG fusions are associated with Ewing Sarcoma Family Tumors, and both EWSR1 and TAFII68 fused to NR4A3 are found in myxoid chondrosarcoma [5]. The best representative of this category is the Ewing sarcoma into which translocation t(var;22) between EWSR1 and partners usually results in fusion of the N-terminal transcription activating domain of EWSR1 with removal of its RNA-binding domain and substitution with the C-terminal DNA binding of the fusion gene partner. The most frequent translocation is t(11;22) which fuse *EWSR1* to *FLI1* [3]. Among the genes that fuse with *EWSR1* are often members of the erythroblastosis virus-transforming sequence (avian ETS) transcription factor family: including FLI1, ERG, ETV1, ETV4, and FEV. The transformation effect of fusion protein is mediated by the abnormal activation of the target genes of the fusion partner contributing the DNA binding domain, i.e., ETS family member [6, 7].

Alveolar Rhabdomyosarcoma (ARMS)

Alveolar Rhabdomyosarcoma is the most common translocation-related soft tissue sarcoma in children and young adults and 70% of ARMS harbor the translocation t(2;13)(q35:q14), 10% of ARMS are associated with t(1;13)(p36;q14) leading to fusion of *PAX3* or *PAX7* to the *FOXO1A* (*FKFHR*) gene, respectively. The remaining 20% of ARMS do not have these fusion genes detectable by routine RT-PCR and comprise cases with a very low expression of a fusion gene, a rare variant fusion, or are true fusion negative cases. Both translocations fuse the 5′ end of *PAX3/7* with the 3′ end of the *FOXO1A* gene. *PAX3* and *PAX7* are transcription factors that initiate myogenesis in muscle stem cells, and the aberrant fusion of their DNA binding domain with the transactivation domain of FOXO1A creates a potent transcription factor that stimulates myogenesis and resists apoptosis [8, 9].

Alveolar Soft-Part Sarcoma (ASPS)

ASPS is a rare, malignant soft-tissue tumor that mostly occurs in adolescents and young adults and is usually located in the extremities. Cytogenetic studies have revealed an unbalanced, recurrent der(17)t(X;17)(p11;q25) translocation which leads to the fusion of alveolar soft-part sarcoma chromosome region candidate 1 (*ASPSCR1*; also known as *ASPL*) on the long arm of chromosome 17 to the transcription factor for immunoglobulin heavy-chain enhancer 3 (*TFE3*; located at Xp11) to form a chimeric protein that retains the TFE3 DNA binding domain [10–12].

2.1.2 Fusion Involves Chromatin-Remodeling Genes

Synovial Sarcoma (SS)

More than 95% of synovial sarcomas are characterized by t (X;18)(p11.2;q11.2), resulting in a fusion between the *SS18* (*SYT*) gene on chromosome 18 and one of the *SSX* genes on the X chromosome [13, 14]. The translocation creates a chimeric gene (*SS18-SSX1, SS18-SSX2* or *SS18-SSX4*) encoding a fusion protein that redirects the transcription factor function of SS18. Relevant downstream targets include cyclin D1 (CCND1) that enhances cell cycle progression. Recently, gene and tissue microarray studies have identified *TLE1*, which encodes a transcriptional co-repressor that is overexpressed in SS, as an excellent bio-marker for distinguishing SS from other soft tissue malignancies by using immunohistochemistry [15].

Endometrial Stromal Sarcoma (ESS)

ESS are rare uterine neoplasms including benign stromal nodules, low-grade ESS, and high-grade undifferentiated endometrial sarcomas (UES). Recently, a gene fusion has been discovered in these tumors and the molecular analysis of the translocation t(7;17) disclosed the fusion gene *JAZF1/JJAZ1* (juxtaposed with another zinc finger gene 1/joined to JAZF1) in approximately 50% of low-grade ESS. Although the JAZF1/JJAZ1 fusion is also common in benign endometrial stromal nodules, it is helpful for differentiating ESS from UES [16, 17]. A new translocation t(10;17) has been recently described and associated to UES. This translocation induces the nuclear localisation of chimeric protein coded by the fusion *FAM22-YWHAE* which keeps a functionally intact 14-3-3ε (YWHAE) protein binding domain.

2.1.3 Fusion Involves Genes Encoding Receptor Tyrosine Kinases (RTKs)

Infantil Fibrosarcomas (IFS) and Infantile Mesoblastic Nephromas (IMN)

IFS is a soft tissue sarcoma with low-grade behavior, principally arising in the extremities, generally in the first year of life and IMN is a renal tumor diagnosed generally within the first 3 months of life. These two unrelated uncommon paediatric tumors share exactly the same translocation t(12;15)(p13;q25) resulting in *ETV6-NTRK3* gene fusion. ETV6 is a transcription factor containing a basic helix-loop-helix (bHLH) dimerization domain. NTRK3 is the cell surface receptor for neurotropin 3 expressed primarily in the central nervous system. The ETV6–NTRK3 fusion protein, in which the ETV6 HLH domain is coupled with the NTRK3 tyrosine kinase residues, forms a homodimer or heterodimer with wild-type NTRK3, which displays receptor tyrosine kinase (RTK) activity and undergoes autophosphorylation at tyrosine domain [18, 19].

Inflammatory Myofibroblastic Tumor (IMT)

IMTs are mesenchymal solid tumors that occur preferentially in children and young adults. Cytogenetic studies showed abnormalities of chromosomal band 2p23 resulting in a rearrangement of the *ALK* gene, a tyrosine kinase oncogene initially found to be rearranged in anaplastic large cell lymphomas. The consistent involvement of ALK, together with the diversity of partner genes (more than six, Table 13.1), underlines the central role of chimeric fusion proteins in both *ALK* constitutive activation and homodimerization for IMT oncogenesis [20].

2.1.4 Fusion Involves Growth Factors

Dermatofibrosarcoma Protuberans (DFSP) and Giant Cell Fibroblastoma

DFSP, and its juvenile form the Giant Cell Fibroblastoma, is a rare skin tumor of low-grade malignant behavior that shows frequent local recurrence. Although histologically different these two diseases share the same genetics: a reciprocal translocation t(17;22) (q11;q13.1) resulting in the fusion of the *COL1A1* gene on chromosome 17 with the *PDGFB* gene on chromosome 22. Interestingly, this fusion doesn't generate a chimeric protein but the role of the *COL1A1* gene may be simply to up-regulate the expression of *PDGFR*, which acts as an auto- or paracrine growth factor [21].

2.2 Specific Activating or Inactivating Mutations

About 20% of sarcomas show a specific oncogenic mutation, which is the central event in GIST and in malignant rhabdoid tumors. Mutation of either *KIT* or *PDGFRA* in GIST leads to a constitutive activation KIT or PDGFRA tyrosine kinase receptors with subsequent activation of signal transduction cascades regulating proliferation, apoptosis and differentiation [22]. The most frequent sites of mutation are exon 11 of *KIT* (juxtamembrane domain), exon 18 of *PDGFRA* (activation loop) and exon 9 of *KIT (*extracellular domain). However, about 10% of GIST show no detectable mutation in either *KIT* or *PDGFRA*. A *BRAF* mutation has been reported in a small subset of these "wild type" GIST. Although *KIT* mutations are known in mast cell disease, seminoma, acute myeloid leukemia and sinonasal natural killer/T-cell lymphoma, the type of mutation encountered is clearly different from those found in GIST. The mutations demonstrated in GIST are nearly disease-specific but with two important exceptions: mutations of exons 11, 13 and 17 of *KIT* have been reported in acral and mucosal melanomas, as well as in melanomas arising on skin with chronic sun damage, and mutations of exons 12 and 18 of *PDGFRA* have been documented in inflammatory fibroid polyps. Mutated KIT and PDGFRA are the target of imatinib which is a KIT inhibitor used in GIST as a targeted therapie.

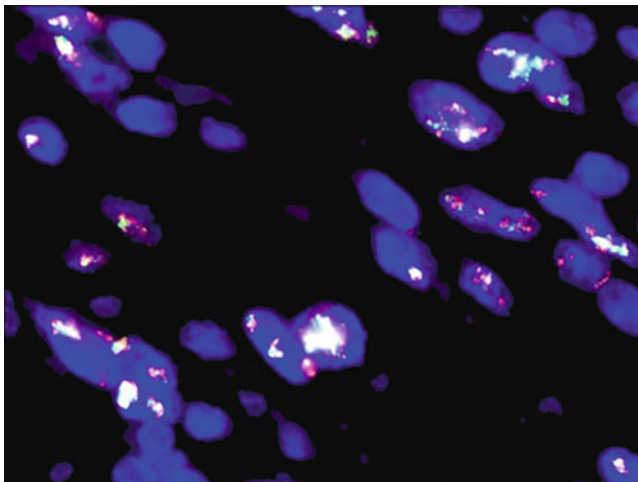

Fig. 13.1 High level amplification of *MDM2* (*green*) and *CDK4* (*red*) in a dedifferentiated liposarcoma. The clustered pattern (appearing as *yellow/white* large spots) is characteristic of high level amplification of more than 50 copies most frequently carried by a rod/ring marker

Malignant rhabdoid tumors are very rare sarcomas characterized by a specific biallelic inactivation of *SMARCB1* also known as *hSNF5* or *INI1* [23]. Biallelic inactivation of the *SMARCB1/INI1* tumor suppressor gene has been identified as the specific event of malignant rhabdoid tumors, whatever their location, but it has also been reported in some cases of proximal-type epithelioid sarcomas, suggesting a link between both tumor types.

2.3 Recurrent Genomic Profile with 12q14-15 Amplicon

Atypical lipomatous tumors/well differentiated liposarcomas (ALT/WDLPS) and dedifferentiated liposarcomas (DDLPS) as well as intimal sarcomas are characterized by this particular amplicon involving *MDM2* and often (90%) *CDK4* [24]. MDM2 binds and induce TP53 degradation; CK4 activates RB1 by phosphorylation. Thus, these two amplifications recapitulate fundamental steps of oncogenesis, i.e. proliferation and apoptosis inhibition. DDLPS is characterized by additional amplicons involving genes whose overexpression may explain dedifferentiation and aggressiveness. These tumors represent about 10–15% of all sarcomas. Although the presence of an *MDM2* amplification detected by FISH (Fig. 13.1) is highly suggestive of ALT/WDLPS or DDLPS, it is not absolutely specific, since such amplification has been reported in a few other malignant tumors including sarcomas such as rhabdomyosarcomas. In fact, the whole genomic profile as shown by array-CGH is much more specific and should be used in difficult cases.

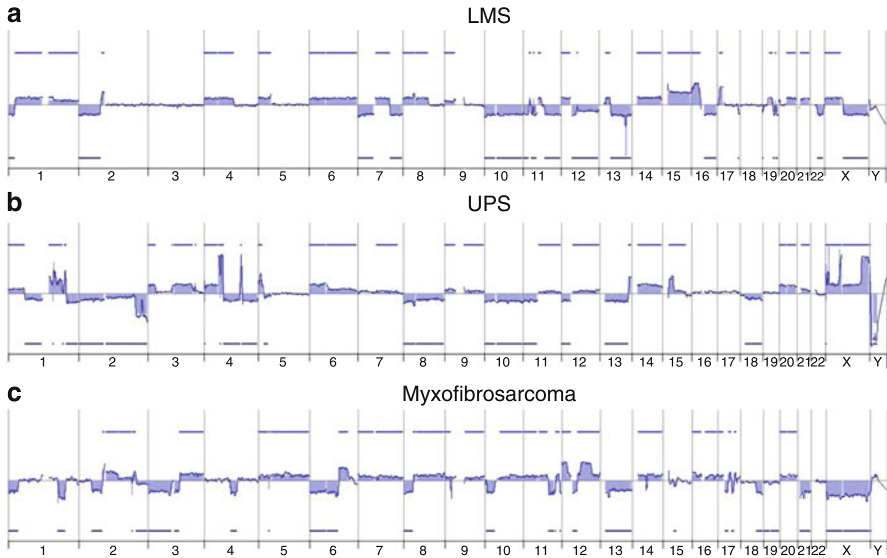

Fig. 13.2 Representative genomic profiles of LMS (**a**), undifferentiated pleomorphic sarcoma (**b**) and myxofibrosarcoma (**c**). Genomic alterations are presented and organized from chromosome 1 to 22 on the X axis and log ratio values are reported on the Y axis. Significant gains or losses are indicated by *blue lines* and *blue areas* above or below each profile, respectively. Expression values are log2 transformed

2.4 Sarcoma with a Complex Genetics

2.4.1 Leiomyosarcoma

Leiomyosarcoma (LMS) accounts for about 8–10% of adult soft tissue sarcomas [1, 25, 26]. These malignant neoplasms which show varying degree of smooth muscle differentiation can develop anywhere in the body, as well as in visceral organs (prostate, urinary bladder, uterus, etc.). Cytogenetics and molecular genetics LMS usually show complex karyotypic alterations [27, 28], and karyotypes differ generally from one tumor to another. Many LMS show many chromosomal imbalances aberrations in the form of gains, losses and amplifications (Fig. 13.2) [27–30]. Some gains and losses of chromosomal material, however, are more frequently observed and tend to correlate with poor outcome, large tumor size, and metastatic dissemination: loss of 1p12-pter, loss of 2p, loss of 13q14-q21 (targeting the Rb pathway) [31], loss of 10q (targeting *PTEN*) [32], and loss of 16q and gains of 17p, 8q, and 5p14 pter [31, 32]. LMS often show loss of *RB1*, correlating with the high frequency of LMS observed in hereditary retinoblastoma patients and the P53 pathway is systematically altered by inactivating either TP53 or p14 [33]. The 5p13-p15 region is often amplified in several pleomorphic STS including LMS. Residing in this amplicon are three candidates genes which are overexpressed:

TRIO, NKD2, and IRX2, of which TRIO seems to be particularly involved in tumor progression of LMS [34]. Activation of the PI3K–AKT pathway through different mechanisms (e.g., activation of IGFR, inactivation of PTEN, a negative regulator of the PI3K–AKT) also plays a crucial role in the development and maintenance of LMS [35]. Recently, it has been realized that the more differentiated retroperitoneal leiomyosarcomas tend to behave more aggressively and that this aggressiveness was mainly dependent upon myocardin amplification/overexpression [36]. Myocardin is a transcriptional cofactor of SRF regulating smooth muscle differentiation. Pérot et al. [36] showed that inactivation of the myocardin pathway resulted in a significant reduction of smooth muscle differentiation, cell proliferation, and cell migration and was associated with less differentiated histology. Leiomyosarcomas of the uterus tend to parallel LMS of soft tissue in terms of karyotypic abnormalities. Most frequent losses by CGH include 10q, 11q, 13q, and 2p, whereas the most common gains are Xp, 1q, 5p, 8q, and 17p [28, 37]. Aberrations involving 1q21 seem to be more common and aberrations of 1p13 and 10q22 less common in soft tissue leiomyosarcomas [27, 28]. Losses involving 1q and 3p are more frequent among soft tissue LMS whereas losses of chromosomes 14 and 15 and of 22q12-qter are more frequent in non-soft-tissue LMS [28]. TP53 mutations, MDM2 stabilization, and inactivation of p16INK4a are observed in both soft tissue and uterine LMS and seem to be associated with LMS progression.

2.4.2 Undifferentiated Spindle/Pleomorphic Sarcoma

Undifferentiated high-grade spindle/pleomorphic sarcoma (undifferentiated pleomorphic sarcoma (UPS)) is now the preferred term to designate high-grade soft tissue sarcomas which fail to show any specific line of differentiation using currently available ancillary techniques [1, 38, 39]. These neoplasms account for about 5–7% of adult sarcomas. Cytogenetics and molecular genetics UPS show complex cytogenetic rearrangements involving 30–35% of the genome, but no specific structural or numerical abnormalities have been proven, so far, to be useful for identification purposes [40]. UPS and other pleomorphic sarcomas share many of the aberrations observed in LMS [41–43], including losses of 1q32.1, 2p25.3, 2q36-q37, 8p23, 9p, 10q21-q23, 11q22, 13q14- q21, 16q11, and 16q23, gains of 1p36-p31, 1q21-q24, 2p, 4p16, 5p, 5q34, 6q, 7p15-p22, 7q21-qter, 17q, 9q, 14q, 16p13, 17q, 19p13, 19q13.11-q13.2, 20q, and 21q, and high-level amplifications of loci 1p33-p34, 12q13-q15, 17cen-p11.2, and 17p13-pter (Fig. 13.2). Both tumor types share also very similar protein expression patterns [44, 45]. Loss of chromosome 13q is the most frequent genomic imbalance in UPS [46], leading to inactivation of the RB pathway [47]. Alterations within the TP53/ARF/MDM2 pathway are also extremely frequent [33, 41]. A recent gene expression analysis of 64 spindle and pleomorphic sarcomas showed that, when performing hierarchical clustering analyses, dedifferentiated liposarcoma, myxofibrosarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, and adult-type fibrosarcoma formed their own clusters [48]. This study also showed

that many of the so-called UPS/MFH (Malignant Fibrous Histiocytoma) had heterogeneous profiles and could be reclassified into other histologic subcategories of pleomorphic sarcomas, especially in the myxofibrosarcoma category. Matrix-CGH studies showed that the gene *TRIO*, coding for a guanidine nucleotide exchange factor, is up-regulated in pleomorphic sarcomas, correlating with copy number gains and high-level amplifications of the short arm of chromosome 5 [49]. TRIO is implicated in the control of cytoskeleton organization, transcription regulation, cell cycle progression, apoptosis, vesicle trafficking, and cell-to- cell adhesion, through activation of the Rho GTPase mediated signalling pathway [34, 49]. As in LMS, TRIO seems to play a significant role in UPS progression [34]. Along with LMS, pleomorphic sarcomas also show *RASSF1A* hypermethylation [50] (albeit less than in LMS) and up regulation of several hypoxia-related genes (e.g., HIF1A and its targets) as well as of several genes involved in cell proliferation, adhesion, and motility, correlating with increased aggressiveness and/or increased metastatic potential [51, 52]. The cell of origin of STS is a matter of controversy. It has convincingly shown that mesenchymal stem cells are good progenitor candidates for Ewing [53] and myxoid liposarcoma [54] development. Mesenchymal stem cells seem also to be good progenitors for MFH development via inactivation of the Wnt pathway [55]. Recently, Matushansky et al. [55] nicely demonstrated that DKK1, a Wnt inhibitor and mediator of human mesenchymal stem cell proliferation is overexpressed in UPS/MFH and that human mesenchymal stem cells can be transformed via inhibition of the Wnt signaling to form UPS/MFH-like tumors in nude mice. This undifferentiation (or "dedifferentiation") can be reversed if the Wnt signaling pathway is appropriately reestablished.

2.4.3 Myxofibrosarcoma

Myxofibrosarcoma, previously called myxoid malignant fibrous histiocytoma, is a relatively common sarcoma of older patients (median age 60 years) [1, 26, 56–59]. Because myxofibrosarcoma has been, for a long time, included in the MFH category and because the separation between high-grade pleomorphic myxofibrosarcoma containing few (<10%) myxoid areas and undifferentiated pleomorphic sarcoma (UPS) is difficult and somewhat subjective [1, 56, 58], data on the cytogenetics and molecular genetics of myxofibrosarcoma are limited. Myxofibrosarcomas are generally associated with very complex karyotypes, sharing many of the aberrations observed in LMS and UPS [40, 48, 60] (Fig. 13.2). A CGH analysis of a series of 22 myxofibrosarcomas showed recurrent simple gains of the chromosome regions 19p and 19q, losses of chromosome 1q, 2q, 3p, 4q, 10q, 11q, and 13q, and high-level amplifications of the central regions of chromosomes 1, 5p, and 20q [60]. Loss of the 13q14-21 chromosomal region, encompassing the *RB1* locus, was observed in all 22 cases examined. Of interest, gain of 5p and loss of 4q are not observed in low-grade myxofibrosarcomas as opposed to myxofibrosarcomas of higher grades, suggesting that these aberrations are late events in the oncogenesis of myxofibrosarcoma [60]. In their comparative study, the authors also found similar chromosomal aberrations

in a series of nine pleomorphic liposarcomas, suggesting close relationship between myxofibrosarcoma and pleomorphic liposarcoma. A recent gene expression analysis of 64 spindle and pleomorphic sarcomas showed that, when performing hierarchical clustering analyses, myxofibrosarcoma could be separated from other spindle cell/pleomorphic sarcomas, namely dedifferentiated liposarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, and fibrosarcoma. This study also showed that many neoplasms which had initially been (mis)classified as UPS/MFH based on their morphology and immunoprofile could be reclassified as myxofibrosarcoma based on gene expression [48]. Two genes, *GPR64* and *TNXB*, were particularly expressed by myxofibrosarcomas but not by UPS/MFH, thus allowing distinction between the two histotypes [48]. Separating a low-grade myxofibrosarcoma from a cellular myxoma can be difficult. A recent study showed that this can be done based on the differences in genetic alterations and in the composition of extracellular matrix [61].

2.5 Indications for Molecular Analyses in Sarcomas

Since detection of specific translocations, activating or inactivating mutations and amplifications can be used reliably as histotype-specific markers, an increasing number of surgical pathologists and clinicians are now relying on molecular validation and this sort of validation will certainly be required in the near future for any sarcoma with a suspicion of specific molecular abnormality. This strategy definitively classifies tumors belonging to the group of sarcomas with a specific genetic abnormality. However, drawbacks such as high cost, low turnaround time and lack of quality assurance have limited the expansion of genetic analyses on a large scale. Nevertheless, given the value of genetic analyses for deciding treatment and the relatively low cost of these techniques as compared to the cost of treatment, it is highly recommended to use them whenever possible. A national network of laboratories specialized in molecular pathology of sarcomas would improve the turnaround time and help in the organization of quality assurance programs.

Most sarcomas with a suspicion of specific translocation should be tested molecularly, except perhaps for the obvious biphasic synovial sarcomas, myxoid/round cell liposarcomas and DFSP. For example, it is now acknowledged that the diagnosis of alveolar subtype of rhabdomyosarcoma requires the proof of the specific translocation, with important consequences in terms of prognosis and treatment [62]. A specific translocation should be suspected when faced with a sarcoma exclusively composed of monotonous round or spindle cells, with no cellular pleomorphism. These tumors most often arise in young patients but may be seen in older individuals.

In term of diagnosis, mutations of *KIT* and *PDGFRA* should be detected in any suspicion of GIST which is c-KIT and/or DOG1 negative.

MDM2 amplification is required for the diagnosis of DDLPS without any ALT/WDLPS component and for the diagnosis of ALT/WDLPS when located in the

extremities or trunk wall given the overdiagnosis of liposarcoma in these situations [63]. However, with the new therapeutic possibilities for these tumors, genetic characterization will probably be systematically required (see paragraph 4).

2.6 Recurrent Chromosomal Translocations, Point Mutations or Complex Karyotypes as Diagnosis Tools

Apart from a few exceptions, recurrent chromosomal translocations are specific of a tumor type. Although a few papers have questioned the specificity of t(X;18) (*SS18-SSX*) in synovial sarcomas, it has been demonstrated that this translocation is specific and sensitive for the diagnosis of synovial sarcoma. These papers described the RT-PCR detection of fusion transcripts in other tumors without being validated by other techniques or reproduced by others, and most likely correspond to PCR contamination [64]. A few fusion transcripts are common to different entities: *ETV6-NTRK3* is present in congenital fibrosarcoma/mesoblastic nephroma but also in secretary breast carcinoma and acute myelogenous leukemia; *ASLP-TFE3* is present in alveolar soft-part sarcoma and in some renal cell carcinomas arising preferentially in children and young adults; *TPM3-ALK* is present in both inflammatory myofibroblastic tumor and anaplastic lymphoma; both *EWSR1-ATF1* and *EWSR1-CREB1* are present in clear cell sarcoma and in angiomatoid fibrous histiocytomas. These data can be explained either by the occurrence of a second unknown tumor-specific molecular event or, more likely, by a divergent differentiation program of distinct precursor cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR) is the standard method for detecting specific fusion transcripts and should be used first when a translocation is suspected. It is more suitable with RNA extracted from frozen tissue but can also be used with RNA extracted from paraffin-embedded tissue, provided amplified fragments of less than 150 bp are used. It is highly recommended to use quantitative or real-time RT-PCR which is more adapted to daily diagnosis than conventional RT-PCR. This technique is highly specific but has several limitations when used in poorly experienced laboratories: variable success rate of RNA extraction from paraffin-embedded material, possible difficulty of primer design, and high risk of PCR contamination. However, when used in strictly controlled conditions, it is a specific and sensitive technique with about 90% of interpretable results on formalin paraffin-embedded tissues.

Fluorescence *in situ* hybridization (FISH) detects a specific DNA target in the nuclei of interphase cells and can be used to demonstrate a gene rearrangement such as a translocation [65]. Commercially available break apart probes are regularly used for demonstrating rearrangement of *EWSR1* (Fig. 13.3)*, SS18 (SYT), DDIT3 (CHOP), FOXO1A (FKHR), TLS (FUS), ETV6 and ALK*. Some probes can be used for several tumor types, such as *EWSR1* probes for Ewing sarcoma, clear cell sarcoma, desmoplastic small round cell tumor and some myxoid/round cell

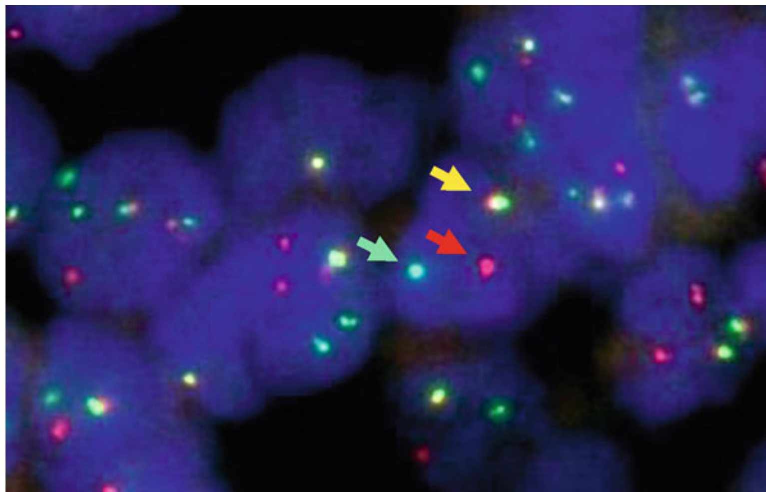

Fig. 13.3 FISH with break-apart probes for *EWSR1* in an Ewing sarcoma. Two probes flanking *EWSR1* gene are used, one centromeric (*red*) and one telomeric (*green*). Wild type allele is recognized by the fusion signal (*yellow arrow*) and gene rearrangement is shown by splitting of one pair of green and red signals (*green and red arrows*)

liposarcoma, extraskeletal myxoid chondrosarcoma, and angiomatoid fibrous histiocytoma, and *TLS (FUS)* for low grade fibromyxoid sarcoma and most myxoid/round cell liposarcoma. For DFSP, home-made fusion probes (*COL1A* and *PDGFB*) and break apart probes for *PDGFB* are used. FISH has several advantages: it can be used on fresh or fixed tissue, it can be performed fast (overnight procedure) and can be used on core needle biopsies or on touch preparations with a few cells. The main limitation is unsuccessful hybridization or detection in 10–20% on fixed tissue.

3 Gene Signature and Prognosis

The concept of histological grade was introduced by Broders in 1920 for squamous cell carcinoma of the lip and was based on the percentage of the well-differentiated component which was correlated with mortality. Since then, several grading systems have been proposed and used on many tumour types, such as breast, prostate, endometrium, ovary, bladder, kidney, colon/rectum, brain, lymphoma and sarcomas. The process of grading must be clearly distinguished from traditional staging and the more recently developed nomograms. Whereas grading is based only on the intrinsic quality of the primary tumour, staging also takes into account tumour extent, and nomograms assess multiple clinical and histological parameters to calculate the probability of recurrence for a given patient. In all cases, histological grade is based on the histological qualities of the primary tumour and is expected to predict tumour

aggressiveness. In fact, histological grade can be considered as a morphological translation of molecular events that determine tumour aggressiveness, and, therefore, it should be regarded as a transient practical method which should prompt research in order to establish a definitive system based on molecular parameters.

Prognosis in sarcoma is of major importance since therapeutic management will be essentially based on the evaluation of the local recurrence and metastatic risk. Since the middle of the 1980s, with the publication of the FNCLCC grading system [66], histopathologic grading has been considered as the "gold standard" in predicting patient outcome. Nevertheless it suffers from many limitations: it is an indirect evaluation of the underlying oncogenic changes in the tumor; its reproducibility from one pathologist to another is questionable; it is not applicable to all types of sarcomas and it is poorly informative for grade 2 (which represents about 40% of cases). Nowadays, the value of grading is also limited by the universal use of core needle biopsies. One can thus postulate that direct assessment of the genetic alterations underlying the tumor phenotype would provide a more precise estimate of tumor aggressiveness.

Molecular markers hold great promise also for refining our ability to establish early prognosis and to predict response to therapy. But legitimate excitement about the attractiveness of molecular technology and the promise of discovery-based research should not avoid adhering to rules of evidence, otherwise it could result in claims that are not reproducible and lead to disappointment. Among the most important criteria to make a molecular alteration a prognostic factor is, first to demonstrate its specific association to outcome, secondly its accuracy and reproducibility in an independent group of patients, and thirdly the independency of its prognostic value from other standard factors in a multivariate analysis. Considering these simple rules only few molecular markers could be recognized as prognostic in sarcomas.

Molecular history of prognostic markers in sarcomas started in the beginning of the 2000s, with the study reported by Wurl et al. [67]. The authors showed that expression of two genes *TERT* (telomerase reverse transcriptase) and *survivin* (also named *BIRC5*) is associated to poor outcome in a series of 89 sarcomas of different subtypes. This series was composed of sarcomas with a complex genetics such as leiomyosarcomas, pleomorphic rhabdomyosarcomas and liposarcomas, and in these tumors, co-expression of both genes was a significant prognostic factor ($P = 0.0004$; relative risk 20.1 95% CI 3.8–106.4). *Survivin* belongs to the family of genes which inhibit apoptosis and is also involved in chromosome segregation. *TERT* is involved in the immortal status of cells by maintaining telomere size. Even if this two-genes signature has never been validated in an independent group, it has a biological meaning which relies metastatic outcome and genes involved in chromosome structure and segregation.

With the development of microarray technology, approaches to identify signature changed and, as shown in Table 13.2, several studies reported molecular profiling analyses in sarcomas, but only a few has been validated on independent groups of patients. Lee and colleagues [89] reported one of the first expression profiling study

Table 13.2 Sarcomas profiling studies

First author	Year	Size	Sarcomas	Application	Validation
Lagarde [68]	2012	67	Gastrointestinal stromal tumor	Prognosis	YES
Chibon [69]	2010	310	Leimyosarcomas, US and dedifferentiated sarcomas	Prognosis	YES
Scotlandi [70]	2009	30	Ewing/PNET	Therapy	NO
Subramanian [71]	2009	80	Nerve sheath tumors	Biology	NA
Paoloni [72]	2009	80	Osteosarcoma	Biology	NA
Miller [73]	2009	77	Nerve sheath tumors	Prognosis	No
Beck [74]	2009	75	Leiomyosarcoma	Diagnosis	No
Carneiro [41]	2009	49	Leiomyosarcoma, other pleomorphic	Diag/prono	No
Cleton-Jansen [75]	2009	40	Osteosarcoma	Biology	NA
Yamaguchi [76]	2008	32	Gastrointestinal stromal tumor	Prognosis	YES
Skubitz [77]	2008	53	Malignant fibrous histiocytoma, 4 other	Diagnosis	No
Lee [78]	2008	51	Leiomyosarcoma (6 other)	Prognosis	No
Neale [79]	2008	199	Pediatric and xenografts	Biology	NA
Whiteford [80]	2007	163	Pediatric and xenografts	Biology	NA
Agaram [81]	2007	44	Gastrointestinal stromal tumor	Therapy/ Biology	NA
Francis [51]	2007	177	13 subtypes, high grade	Diagnostic/ Pronostic	No
Singer [82]	2007	129	Liposarcoma	Diagnostic/ Biology	No
Nakayama [48]	2007	105	Pleomorphic sarcomas	Diagnostic	No
Davicioni [83]	2006	186	Alveolar rhabdomyosarcoma	Prognosis	No
Baird [84]	2005	181	16 subtypes	Biology	NA
Henderson [85]	2005	96	19 subtypes	Biology	NA
Detwiller [86]	2005	54	8 subtypes	Biology	NA
Morgan [87]	2005	48	Giant cell tumors of bone, 4 other	Biology	NA
Segal [88]	2004	81	Clear cell, melanoma, other	Diagnostic	No
Lee [89]	2004	37	Leiomyosarcomas	Prognosis	No
Lee [90]	2003	27	9 Leiomyosarcomas, 9 US and 9 Synovialosarcomas	Diagnosis	No
Linn [91]	2003	53	Dermatofibrosarcoma protuberans, 7 other	Biology	NA
Segal [92]	2003	51	9 subtypes	Diagnosis	No
Nagayama [93]	2002	47	Synovialosarcomas, 5 other	Biology	NA
Nielsen [94]	2002	46	6 subtypes	Diagnostic	No

leading to a prognostic signature in sarcomas. Comparing expression profiles from both metastatic and non-metastatic leiomyosarcomas, they identified 335 genes differentially expressed between primary tumors (20 cases) and metastasis (7 cases). Although this signature has a significant prognostic value, the study suffered from two main limitations, the small size of the series and the non-validation of the results

compromising its clinical use. Francis and colleagues [51] analyzed 177 sarcomas and produced a meaningful expression portrait of different histotypes demonstrating that histological classification fits well to expression profiles of tumors. They also identified 220 genes associated to metastasis events in a subgroup of 89 pleomorphic sarcomas. Both studies developed supervised approaches with the metastatic event as end-point and, selected genes which, for most of them, have no biological link between them. Indeed, such an approach has proven to be inefficient for identifying genes or pathways implicated in the metastatic potential acquisition. This is the case in Gastrointestinal Stromal Tumors (GIST) for which Yamaguchi and colleagues [76] claim that the gene CD26 appears to be a very interesting and strong ($P < 10–5$, $n = 152$ gastric GISTs) prognostic marker but authors wrote "CD26 may not be the cause of malignant progression of gastric GISTs". Nevertheless, authors demonstrated that CD26 can be evaluated in the daily practice since they validated their data by immunohistochemistry, which makes this marker easily applicable as compared to array-based technologies. It is also interesting to note that in GIST, genomic alterations have been also identified to be associated to poor prognosis [68, 95–100]. Actually, different studies showed that 9p21 deletion is associated to metastatic outcome in patients with GIST, but the driver gene was not positively identified [99, 100]. Nevertheless Lagarde et al. recently showed that *CDKN2A* is the target of 9p deletions [68]. Genomic (and not expression) markers could be considered as the best form of molecular criteria because one can expect they drive the outcome and their identification in the daily practice is quite easy by using CGH-array, FISH or sequencing technologies which are widely used in pathology [68].

Recently, the French Sarcoma Group published the identification of a 67 genes-expression signature (CINSARC for Complexity INdex in SARComas) satisfying all the criteria mentioned above to be a prognostic molecular marker [69]. In a series of 310 primary untreated non-translocation related sarcomas with genomic and expression profiling associated to clinical data, expression profiles were compared according to histological grade and genome complexity to determine a prognosis gene-expression signature. Sixty seven genes were selected and validated this signature demonstrating in an independent group of sarcomas that it predicts metastatic outcome better than does current FNCLCC grading system. It is interesting to note that all these genes belong to pathways involved in mitosis control and chromosome integrity which means that these mechanisms are of primary importance in the development of metastasis. Such mechanism being activated across virtually all cancers, this signature could predict outcome in various cancer types and effectively CINSARC predicts metastatic outcome in all the tested public dataset and of particular interest, in GISTs [68]. Thus, one can expect that this signature could be applied to all sarcoma types.

Finally, this prognostic signature points out the same pathways than the first reported by Wurl and colleagues [67], chromosomes integrity, segregation and mitosis. This observation likely means that these mechanisms are among the most important to predict outcome in sarcomas.

4 Gene Signature and Prediction to Response to Treatment

After the diagnostic and prognostic steps, thanks to diagnosis and prognosis markers, the (molecular) pathologist should provide the clinician predictive markers in order to select the best therapy for a given tumor in a given patient. This new era of personal medicine is just beginning and will have to combine tumor-specific and patient-specific markers. Most of the latter will belong to the gene polymorphisms explaining, at least in part, the relative sensitivity or resistance to certain drugs. Regarding the tumor-specific factors, molecular markers can be used for predicting the response to either a non-specific chemotherapy or a targeted therapy. Of course, the best predictor of response is the identification of a target for therapy. In this setting, GIST is the paradigm of targeted therapy since a mutated *KIT* can be considered as the best marker of response to imatinib [101] even if response to therapy will be dependent on the mutation type [102].

Imatinib mesylate (gleevec®) is also an inhibitor of tyrosine kinase PDGFR, which together with other PDGFR inhibitors such as sunitinib and sorafenib is being tested in patients with metastatic DFSP [103].

Unfortunately, such situation does not exist in other sarcomas so far, but could arise rapidly. In the last 10 years, several studies have shown that most poorly differentiated sarcomas arising in the retroperitoneum are actually dedifferentiated liposarcomas showing a quite specific genomic profile with an amplification of *MDM2* and often *CDK4*. Thus, MDM2 and CDK4 are promising targets for therapy and drugs such as Nutlins (Roche) which are currently under investigation in clinical trial phase 0 and 1. Nutlin acts through inhibition of the MDM2- TP53 interaction, which leads in turn to TP53 induced apoptosis. The situation is quite more complex for most of other sarcomas for which instead of activated protein to target, activated pathways have been identified and drugs inhibiting the aberrant signaling have been developed: this is the case for HDAC inhibitors in sarcomas with a specific translocation such as synovial sarcomas and Ewing sarcomas [104–106], and anti-AKT – mTOR pathway in sarcoma histotypes which can show PTEN inactivation [35], PI3Kinase mutations or IGFR1 overexpression [46]. The last pathway seems to be very important in sarcomas as well as in other cancers, but there is no good predictive marker for predicting response to drugs targeting this pathway. This can be due to the fact that activation of the pathway is a secondary event and is not really responsible for oncogenesis or that tumor resists by activating the pathway downstream of protein targeted by the drug. Therefore, there is a huge need of supplementary markers to detect patients who will respond to therapy. In sarcomas such signatures do not exist with the exception of Ewing sarcomas where one study reported that over-expression of microsomal glutathione S-transferase (MGST1), a detoxification enzyme, likely leads to resistance to doxorubicin [70]. In this line, authors demonstrated that the use of MGST1 inhibitor (NBDHEX) in Ewing sarcoma cell lines confers sensitivity to doxorubicin, but this has still to be proven in a clinical context.

We can thus conclude that the next breakthrough to a real personalized medicine in sarcomas will be identification of markers (molecular or clinical) leading clinicians to be able to propose efficient treatment for each individual patient.

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Chapter 14
Novel Molecular Acquisitions in Leukemias

Sabina Chiaretti and Robin Foà

Abstract Over the past two decades, hematologic malignancies have been extensively evaluated also thanks to the introduction of powerful technologies, such as conventional karyotyping, FISH analysis, gene and microRNA expression profiling, array comparative genomic hybridization (a-CGH and SNP arrays and, very recently, whole exome sequencing (WES)).

These analyses have allowed to refine the mechanisms underlying the leukemic transformation in several onco-hematologic disorders and, more importantly, are permitting to define novel prognostic algorithms aimed at stratifying patients at the onset of disease and, consequently, at treating them in the most appropriate manner.

Furthermore, the identification of specific molecular markers is opening the way to targeted and personalized medicine.

This chapter will focus on the novel acquisitions obtained in the context of acute lymphoblastic leukemia (ALL) of both B- and T- lineage, chronic lymphocytic leukemia (CLL) and de novo acute myeloid leukemia (AML).

1 Acute Lymphobastic Leukemia (ALL)

1.1 Introduction

Acute lymphobastic leukemia (ALL) is a malignant disorder that originates from hemopoietic precursors, that can be of B-cell (80–85%) or T-cell (20–25%) derivation: the acquisition of a series of genetic aberrations leads to an impaired maturation, with an arrest in the differentiation process and an abnormal prolif-

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eration. As a consequence, the accumulation of leukemic cells occurs in both the bone marrow, where it suppresses the physiologic hemopoiesis, as well as in extra-medullary sites.

ALL is the most common neoplasm in childhood, with the highest peak of incidence in children with an age comprised between 2 and 5 years, whereas it is rather rare in adulthood. In fact, according to US-SEER, approximately 60.3% of ALL cases are diagnosed under the age of 20; 10.3% between 20 and 34, 5.9% between 35 and 44, 6.7% between 45 and 54, 6.1% between 55 and 64, 5.0% between 65 and 74, 4.0% between 75 and 84, 1.7% in patients older than 85 years.

Beyond the different incidence of the disease, outcome varies profoundly between children and adults; in fact, to date, the majority of children can be considered curable, while the prognosis of adults is still extremely poor, with only 40% of individuals that are long-term free of leukemia.

2 Historic Genetic Defects in B-Cell ALL

A set of genetic lesions, mostly represented by translocations and including *BCR/ABL ETV6/RUNX1*, *E2A/PBX1* and *MLL* rearrangements, have been well-recognized in B-lineage ALL and represent “historical” aberrations.

The *BCR/ABL* rearrangement, derived from the t(9;22)(q34;q11) translocation (Ph chromosome) [1], that represents the hallmark of chronic myeloid leukemia (CML) and can be also detected in ALL, induces a constitutive activation of the *ABL* kinase, that in turns activates mitogenic signaling pathways, induces altered cellular adhesion, inhibition of apoptosis and proteasomal degradation of physiologically important cellular proteins, ultimately contributing to tumor growth and proliferation [2, 3]. At least three different fusion proteins have been detected; the p210, prevalently found in CML, the p190, detected in roughly 50% of adult Ph+ ALL and in the majority of childhood Ph+ ALL [4], and the rare p230 protein, reported in chronic neutrophilic leukemia, and seldom observed in about 1% of classic Ph+ CML [5]. The frequency of *BCR/ABL* is very low in childhood ALL, while it starts to increase during adolescence and reaches more than 50% in the elderly. Prognosis of these cases was dismal until the introduction of the tyrosine kinase inhibitors (TKI), whose use in the clinical practice has profoundly changed the clinical management of these patients [6–11].

At variance, *ETV6/RUNX1* is more frequent in children and virtually disappears with age progression [12]. It originates from the t(12;21)(p13;q22) translocation, that creates a fusion gene including the 5′ portion of *ETV6*, a member of the *ETS* family of transcription factor genes, and almost the entire coding region of the transcription factor *RUNX1*, which encodes the α subunit of core binding factor, a master regulator of the formation of hematopoietic stem cells [13, 14]. As a result, the chimeric *ETV6/RUNX1* transcription factor retains an essential protein–protein interaction domain of ETV6 and the DNA-binding and transcriptional regulatory sequences of *RUNX1* [13, 14]. The most prominent effect of the *ETV6/RUNX1* fusion

protein is the inhibition of the transcriptional activity that is normally initiated when *RUNX1* binds to a DNA region termed the core enhanced sequence [15]. Clinically, children with *ETV6/RUNX1* rearrangement usually have an excellent outcome.

The *MLL* gene can be disrupted in leukemias of B- and T-cell origin, as well as in myeloid leukemias, and so far at least 300 partners have been recognized [16, 17]. The *MLL* fusion proteins have a dominant gain-of-function effect that enhances their transcriptional activity. These alterations mainly disrupt the normal pattern of expression of *HOX* genes, causing a change in the self-renewal and growth properties of hematopoietic stem cells and committed progenitors, thus eventually leading to leukemia [18, 19]. Among the *HOX* genes known to play a predominant function in these processes, *HOXA7* and *HOXA9* play a pivotal role [20]. *MLL* rearrangements represent the most frequent aberrations in infants (children <1 year), where they are detected in more than 90% of cases, whereas they are very rare in children and adults (2–5%); in all cases, *MLL* rearrangements are associated with a very unfavorable outcome.

Finally, the *E2A/PBX1* rearrangement arises from the t(1;19)(q23;p13) translocation, initially described in 1984 [21], is strongly associated with a pre-B immunophenotype as blasts usually express cytoplasmic immunoglobulins (cIg). Its frequency is similar in children and adults and is around 2–7%. This translocation juxtaposes the *E2A* gene on chromosome 19 with the *PBX1* gene on chromosome 1 to form the *E2A/PBX1* fusion gene [22]: the resulting protein induces cell differentiation arrest and tumor formation, most probably because of a reduction of the levels of wild type E2A [23], whose reduced levels eventually induce a deregulation of lymphoid cell maturation and proliferation; furthermore, *E2A/PBX1* itself induces transcription activation of target genes.

Overall, the identification of these molecular aberrations has been of pivotal importance in the establishment of a prognostic algorithm: *ETV6/RUNX1* is associated with a very favorable outcome, *MLL* rearrangements, as well as *BCR/ABL* aberrations, are associated with a poorer outcome [24], while *E2A/PBX1* is still controversial, although it is likely that patients harboring this aberration can benefit from intensive regimens [25, 26].

3 Novel Genetic Acquisitions in B-Cell ALL and Definition of High Risk Subsets

The introduction of powerful technologies, such as gene expression profiling (GEP) first and SNP array analysis later, have eventually allowed to better define the molecular scenario of B-ALL.

The first manuscript focusing on GEP revealed that the transcriptional profiling of ALL is different from that of acute myeloid leukemia (AML) [27]. However, the most important study, carried out by the St. Jude Children's Research Hospital group, highlighted specific gene expression signatures for all the known subgroups of pediatric ALL, namely T-ALL, hyperdyploid cases with more than 50 chromosomes, *E2A/PBX1*, *BCR/ABL*, *ETV6/RUNX1* and *MLL* rearrangements [28, 29];

similar results were also reported by us and others in adult cohorts [30–32], therefore indicating that at the transcription level there are no major differences between adult and pediatric cohorts, at least in the presence of specific aberrations. In adult B-ALL, GEP also showed that a set of cases without major molecular aberrations tend to cluster with BCR/ABL+ cases, hence identifying a subgroup that might be regarded as different and might benefit from aggressive treatment strategies [31, 32].

More recently, the introduction of the SNP array technology has further defined the spectrum of genetic lesions [33–37]; overall, this approach has shown that in ALL the number of CNA (copy number alterations) is rather high, with deletions always outnumbering gains. Lesions frequently affect fundamental pathways, such as B-cell differentiation, tumor suppression, cell cycle and apoptosis [34]. Interestingly, the number of CNA varies according to the molecular subgroups, with *MLL* rearrangements being characterized by very few additional lesions, while *BCR/ABL* and *ETV6/RUNX1* are associated with a mean number of 6 alterations. Taken together, these results further confirm that *MLL* itself is able to induce leukemic transformation [38], whereas *BCR/ABL* and *ETV6/RUNX1* may require supplementary hits.

Furthermore, this technique has allowed to reveal that *IKZF1*, that encodes for the transcription factor *Ikaros* and plays a pivotal role in lymphoid development [39], is frequently disrupted in ALL, particularly in BCR/ABL+ cases, where it is deleted, in both adult and pediatric cohorts, in roughly 80% of cases, thus making it the most frequent aberration associated with *BCR/ABL*. The deletion of *IKZF1* has functional consequences, since it impairs B-lymphoid maturation, pre-B cell receptor signaling and accelerates leukemogenesis in a BCR/ABL+ murine model [40–43]. Importantly, *IKZF1* deletions are important predictors of poor outcome in Ph+ ALL, regardless of age, and they currently represent the hallmark of high-risk leukemias [44–47]. In fact, in children, *IKZF1* can be deleted also in non-Ph+ ALL [35, 37], as well as in adult B-ALL without major molecular aberrations, and is correlated with poor prognosis. More importantly, its deletion recognizes a subgroup with inferior event-free survival (EFS) and disease-free survival (DFS) also in non-high risk childhood ALL, and it has been proposed as a useful marker for monitoring of minimal residual disease (MRD) [48].

Together with *IKZF1*, another lesion that has been recently recognized is represented by rearrangements involving *CRLF2*, a cytokine type I receptor, known to play a pivotal role in dendritic development, T-cell response, allergic inflammation and proliferation of normal and leukemic B cells [49, 50]. *CRLF2* is located on the pseudoautosomal region (PAR1) of chromosomes X and Y; rearrangements involving this transcript lead to its overexpression and can be of two types: either a rearrangement that involves *CRLF2* and the Ig heavy chain locus *(IGH@-CRLF2)* or an interstitial PAR1 deletion that juxtaposes intron 1 of *P2RY8* to the coding region of CRLF2 itself. Interestingly, the latter rearrangement is frequently detected in roughly 50% of Down syndrome ALL [51, 52]. In all cases, *CRFL2* alterations are coupled with the presence of *JAK* mutations (*JAK1* or *JAK2*), thus suggesting that these events together contribute to leukemogenesis. Moreover, they are frequently detected in *IKZF1* deleted Ph- ALL patients. Overall, a *CRLF2* impairment is detected in 5–10% of cases without molecular lesions, in both adult and pediatric

cohorts [53–56]. Its presence correlates with a poor outcome; more importantly, since it is correlated with activation of the *Jak-Stat* pathway, it might be of particular relevance in a therapeutic algorithm, since patients with such lesions might benefit of treatments contemplating the use of JAK inhibitors.

Finally, the integration of genome-wide technologies has allowed to better refine prognostic subgroups. In fact, as previously observed in adults, the "BCR/ABL-like" subgroup has also been identified in the pediatric setting: by combining a-CGH with GEP, the group of Den Boer et al. [57] elegantly showed the presence of a subgroup with a peculiar GEP that resembled that of BCR/ABL+ cases, poor outcome and genetic lesions similar to those observed in BCR/ABL+ patients, among which *IKZF1* deletions and *CRLF2* rearrangements.

Similarly, Harvey and colleagues performed a GEP analysis in a large set of high-risk ALL and showed the presence, by unsupervised analysis, of eight subgroups that differ from each other in terms of outcome and associated deletions: in line with Den Boer, cases with poorer outcome were characterized by *IKZF1* deletions [58].

Another lesion which has been recognized and analyzed in detail by SNPs array is represented by iAMP21 amplification [59–63]: it represents a rare aberration (2% of pediatric ALL) and was initially described as being characterized by multiple copies of the *RUNX1* gene, indeed located on chromosome 21. Initially, it was possible to define a common region of amplification (CRA) of 33.192 and 39.796 Mb on chromosome 21 (which includes *RUNX1*), that was later redefined to a 5.11 Mb region [63]. Survival analysis of patients harboring such lesion indicated that they had an increased risk of relapse in at least two studies [59, 62]. Subsequent SNP array analyses allowed to define recurrent abnormalities affecting genes in key pathways, such as *IKZF1*, *CDKN2A/B*, *PAX5*, *ETV6* and *RB1*. An analysis of clonal architecture indicated that these lesions, together with *P2RY8-CRLF2* aberrations, are secondary events to chromosome 21 rearrangements. Patients' outcome was reconfirmed to be poor if they are treated with standard therapy [63] thus rendering it another suitable marker of poor prognosis.

Thus, gene expression profiling and SNPs array analyses have allowed to identify lesions that define high-risk B-ALL also in cases that do not harbor historical molecular aberrations, traditionally associated with outcome.

4 Next-Generation Sequencing in B-ALL: What's Next?

By re-sequencing, Mullighan and colleagues [64], performing a paired diagnosis-relapse comparison, identified *CREBBP* mutations in about 18% of relapsed cases, whereas they are extremely rare in AML. *CREBBP* and its paralogue, *EP300* (p300), are transcriptional coactivators that are involved in haematopoiesis [65]. The functional consequences of *CREBBP* mutations included reduced acetylation and impaired expression of glucocorticoid-receptor-responsive genes. Overall, their presence in relapsed samples and their role in the regulation of glucocorticoid-responsive genes, suggests that these alterations may influence response to therapy

and relapse; they also suggest that therapeutic approaches directed towards acetylation may be useful in high-risk ALL.

The introduction of whole exome sequencing (WES) is further permitting to reveal novel lesions, in particular in high-risk B-ALL leukemias. Beyond the known *IGH@CRLF2* rearrangement, other rearrangements identified include *NUP214-ABL1,* in-frame fusions of *EBF1-PDGFRB*, *BCR-JAK2* or *STRN3-JAK2* and cryptic *IGH@-EPOR* rearrangements. Among the above mentioned lesions, *EBF1-PDGFRB* was reconfirmed also in an additional cohort of patients and has been shown to confer growth factor independence, induce constitutive activation of pSTAT5, pAkt and pERK1/2, and respond to imatinib, dasatinib and the specific PDGFRB/FGFR inhibitor dovitinib. Furthermore, it was possible to identify a recurrent in-frame activating insertion of *IL7R* in one case [66].

A summary of the findings and their relative incidence in children and adults described in Sect. 1 is summarized in Table 14.1. Figure 14.1 summarizes the changes in the knowledge of molecular lesions in B-ALL throughout the years.

5 Recurrent Lesions in T-Cell ALL

Up to a decade ago, little was known about the biology of T-cell ALL (T-ALL): well-recognized aberrations involved the T-cell receptor (TCR), that was juxtaposed with different fusion partners; in fact, aberrations involving the 14q11 (*TCRA/D*) and 7q34 (*TCRB*) regions can be detected in 35% of patients [67]. They juxtapose enhancer elements of the *TCR* genes with transcription factors involved in T-cell differentiation, such as *LMO1*, *LMO2*, *TAL1* and *TLX1* with consequent deregulation of hemopoiesis. Other rearrangements involve two transcription factors: they include *SIL-TAL1* in 10–25% of patients [68], *TLX3-BCL11B* in roughly 20% of patients, *PICALM-MLLT10* in 8% of patients, *NUP214-ABL1* fusion formed on episomes, deletions of *CDKN2A* and *CDKN2B* locus on chromosome 9p, *EML-ABL1* and *SET-NUP214* fusion, *MLL* gene rearrangements to numerous different translocation [69–72].

The improvement of cytogenetic assays, mutational analysis, GEP and the integration of these techniques has nowadays permitted to largely recapitulate the genomic complexity of T-ALL (Table 14.2).

By GEP, Ferrando and colleagues [73] showed the presence of several subgroups, each associated with the overexpression of known oncogenes. Among these clusters, it is interesting to notice that it was possible to define a novel subgroup, which clustered tightly to *TLX1 (HOX11)*, and was characterized by the overexpression of *TLX3*.

Furthermore, GEP allowed to show that several lesions, including *MLL* rearrangements, inv(7)(p15q34), t(10;11)(p13;q23), that results in the *CALM-AF10* rearrangement, and del(9)(q34.11q34.13), leading to *SET-NUP214,* all induce to the overexpression of HOXA genes thus indicating that different genetic lesions activate the same pathway [74–77].

Table 14.1 Overview of the most frequent and significant lesions occurring in B-lineage ALL

			Frequency		Clinical relevance
	Gene/s involved	Functional consequences	Children	Adults	
Translocations					
t(9;22)(q34;q11)	*BCR-ABL*	ABL constitutive activation, activation of mitogenic pathways, cellular adhesion deregulation	~5–10%	Up to 50%	Poor outcome
t(4;11)(q21;q23)	*MLL-AF4*	Disruption of HOX genes expression and of self-renewing properties of hemopoietic progenitors	3–5%; >90% infants	5–10%	Poor outcome
t(12;21)(p13;q22)	*ETV6-RUNX1*	Transcriptional activity inhibition	20–30%	<1%	Favorable outcome
t(1;19)(q23;p13)	*E2A-PBX1*	Cell differentiation deregulation	~5%	~5%	Not clearly established
	Gene involved and position	Functional consequences	Frequency: Children	Frequency: Adults	Clinical relevance
Other types of lesions					
Focal deletions; rarely mutations	*IKZF1*, 7p13-p11.1	Deregulation of lymphoid differentiation	15%; >80% *BCR-ABL* pos; ~30% HR *BCR-ABL* neg	7%; >80% *BCR-ABL* pos	Poor outcome
Rearrangements; interstitial Par1 deletion; mutations	*CRLF2*, Xp22.3; Yp11.3	Together with *JAK* mutations, constitutive JAK-STAT activation	5–10%; >50 DS-ALL	5–10%	Poor outcome

(continued)

Table 14.1 (continued)

	Gene/s involved	Functional consequences	Frequency		Clinical relevance
			Children	Adults	
Mutations	*JAK1*, 1p32.3-p31.3 *JAK2*, 9p24	Constitutive JAK-STAT activation	~10% HR-*BCR-ABL* pos; 18%–35% DS-ALL	–	Associated with *CRLF2*, *IKZF1*, poor outcome
Focal deletions; mutations	*CREBBP*, 16p13.3	Impaired histone acetylation and transcriptional regulation	18% of relapsed ALL		Increased incidence at relapse; association with gluco-corticoid resistance
Intrachromosomal amplification of chromosome 21	*RUNX1*, 21q22.3	Multiple copies of the *RUNX1* gene; possible secondary event	2%	–	Poor outcome

HR high-risk, *DS-ALL* Down syndrome ALL

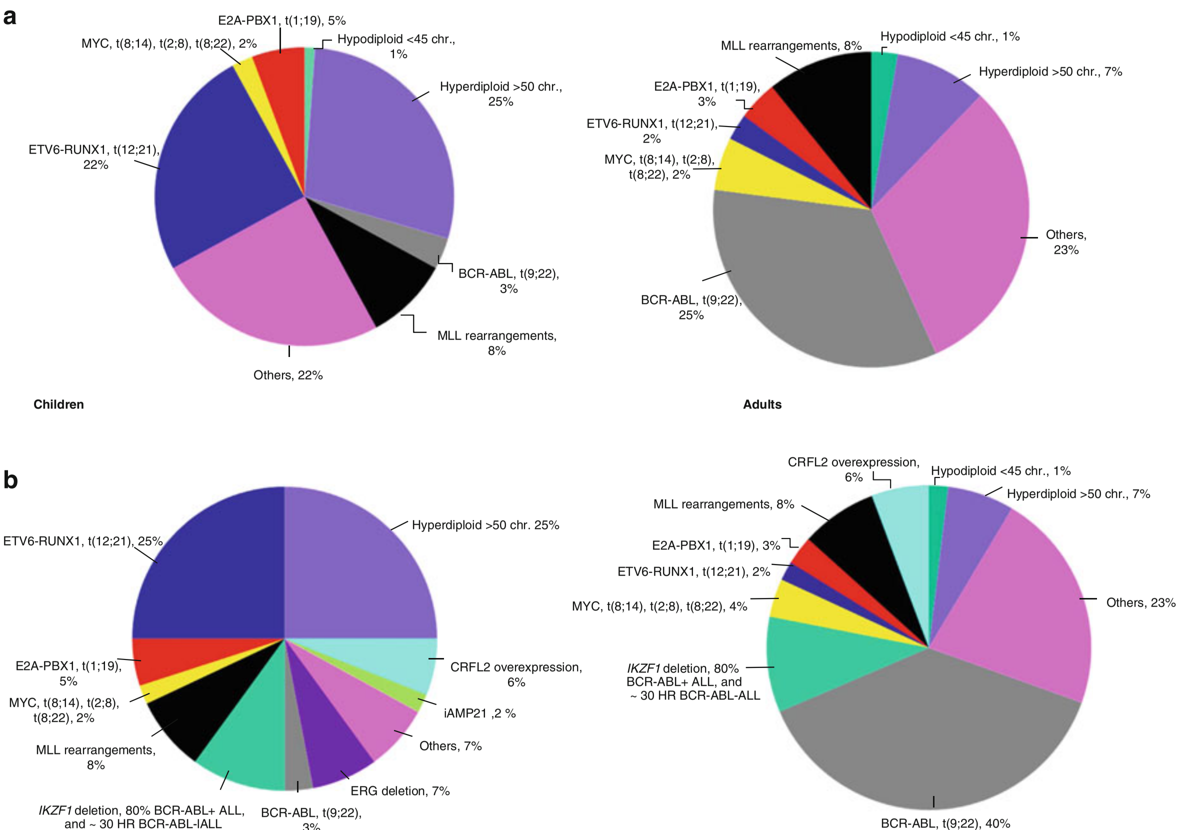

Fig. 14.1 Pie charts illustrating the incidence of known molecular-genetics of ALL in children and adults, respectively, in the first years of 2000 (**a**), and the current knowledge (**b**)

Table 14.2 Summary of recurrent genetic lesions in T-ALL

	Gene/s involved	Functional consequences	Frequency		Clinical relevance
			Children	Adults	
Translocation of TCR with various oncogenes	*LMO1*, *LMO2*, *TAL1*,*TLX1*, *TLX3*	Hemopoiesis deregulation, impairment of differentiation	~35%		No impact
Del(1)(p32)	*SIL-TAL1*	Impairment of differentiation	~10%	5–10%	Not clearly established
9p deletion	*CDKN2A* and *CDKN2B*	Loss of cell proliferation control	20–30%	<1%	No impact
11q23 rearrangements	*MLL* with various partners	Disruption of HOX genes expression and of self-renewing properties of hemopoietic progenitors	~5%		Poor outcome
t(9;9)(q34;q34)	*NUP214-ABL*	*ABL* constitutive activation	6%		No impact
t(9;14)(q34;q32)	*EML1-ABL*	*ABL* constitutive activation	1%		No impact

However, one of the most important and recent contribution of GEP, also in combination with immnunophenotyping analysis, is represented by the recognition of a subgroup, that accounts for about 10% of cases, defined either early T-cell precursor (ETP-ALL) [78] or myeloid-like [79], detected in both pediatric and adult cohorts (see below).

6 ETP/Myeloid-Like Leukemias

ETP/myeloid-like leukemias appear as a distinct subset of T-ALL and have been described in both pediatric and adult cohorts, with a similar incidence [78, 79]. In the pediatric setting, Coustan-Smith and colleagues [78] showed that, at the immunophenotypic level, these patients display an early T-cell phenotype and co-express at least one myeloid marker; at the transcriptional level these children have a stem-cell like profile. More importantly, ETP children are characterized by a very poor outcome. Our group [79], by performing an unsupervised analysis of 52 adult patients with T-ALL identified a subset of cases characterized by the overexpression of a large number of myeloid transcription factors, including *CEBPA*, *CEBPB*, *CEBPD*; furthermore, these cases also express miR223, a microRNA that is involved in the myeloid differentiation process [80]. Similarly to what observed in children, also in our cohort, myeloid-like patients appear to have an unfavorable outcome, since the majority was refractory to induction chemotherapy.

These cases have been recently further evaluated by sequencing and WES: Van Vlierberghe and colleagues [81] have in fact shown that ETP cases are indeed characterized by an immature transcriptional profiling and, more importantly, by a wide spectrum of gene mutations usually detected in AML, such as *IDH1*, *IDH2*, *DNMT3A*, *FLT3* and *NRAS*. Furthermore, a prominent role has been suggested for *ETV6*, since mutations of this gene were detected exclusively in such subgroup.

In line with these findings, Zhang et al. [82] reported that ETP cases are characterized by several intrachromosomal translocations, deletions and insertions. Among the mutations and/or translocations identified, it was possible to confirm the involvement of *ETV6*, *FLT3* and *NRAS*.

Thus, this subgroup clearly emerges as a grey zone between AML and T-ALL, is detected in all age cohorts, presents a peculiar phenotypic and transcriptional profiling, several additional lesions and, most importantly, defines subset of patients with a poor prognosis. In line with these findings, the use of myeloid-directed therapies appears an appealing approach.

7 Gene Mutations in T-ALL

An important contribution to the understanding of T-ALL has been provided by the mutational screening of a set of transcripts which might play a pivotal role in T-ALL leukemogenesis (Table 14.3). Beyond *NOTCH1* mutations that will be described in

Table 14.3 Gene mutations in T-ALL

Gene/s involved	Gene position	Functional consequences	Frequency		Clinical relevance
			Children	Adults	
NOTCH1	9q34.3	Impairment of differentiation of and proliferation	60–70%		Overall favorable outcome
FBW7	4q31.3	Arrest of differentiation, and aberrant self renewal activity	∼10%	∼10–20%	Usually evaluated in combination with *NOTCH1*
BCL11B	14q32.2	Loss of cell proliferation control	9%	–	Not defined
JAK1	1p32.3-p31.3	Cytokine growth independence, resistance to dexamethasone-induced apoptosis, JAK signaling activation	2%	7–18%	Unfavorable outcome
PTPN2	18p11.3-p11.2	Negative regulator of tyrosine kinases	6%	–	No impact
IL7R	5p13	Lymphoid development	6%	–	No impact
PHF6	Xq26.3	Putative tumor suppressor	5–16%	18–38%	No impact
ETV6	12p13	Various, including: signaling, developmental arrest, histone modification	Detected in ETP leukemia		Unfavorable, as per subgroup of the disease
IDH1	2q33.3				
IDH2	15q26.1				
DNMT3A	2q33.3				
FLT3	13q12				
NRAS	1p13.2				
JAK3	19p13.1				
IKZF1	7p13-p11.1				

detail, other recurrent lesions are represented by *FBW7*, *BCL11B*, *JAK1*, *PTPN2, IL7R* and *PHF6.*

The *FBW7* gene, located on chromosome 4q31.3, is a component of the ubiquitin ligase complex and is involved in the degradation of MYC, cyclin E and particularly *NOTCH1* [83, 84]. Mutations of this gene are detected in 8–16% of cases [84, 85]. In the presence of a mutation, the protein either fails to bind to its target proteins (*NOTCH1*) or binds its targets but fails to tag them for degradation (*MYC*): in both instances, this results in a prolongation of targets half-life.

FBW7 mutations have been widely investigated in clinical trials, usually in combination with *NOTCH1* mutations, since they concur to an elevated intracellular *NOTCH1* activity and overexpression of its downstream targets, ultimately resulting in deregulated cell cycle control and tumor development.

So far, their role in outcome prediction is still controversial: in fact, Asnafi et al. [86] analyzed adult patients, evaluated concomitantly *NOTCH1* and *FBW7* mutations, and reported an association with a favorable outcome; similar results were recently reported by the GRAAL study [87], that suggest *NOTCH1/FBW7* mutated cases have a particularly favorable outcome, especially when treated with intensified chemotherapeutic regimens. At variance, Park et al. [88] did not report any significant impact of *FBW7* in the pediatric ALL-97 protocol, in line with the results obtained in adult patients enrolled in the MRC UKALLXII/ECOG E2993 clinical trial [89]. Finally, Kox et al. [90], who analyzed separately the role of *FBW7* mutations, reported that *FBW7* mutations are indeed associated with an early MRD response that is lost at later time points.

BCL11B is a transcription factor that has an important role in normal T-cell development and is usually highly expressed almost at all stages of T-cell differentiation, with the exception of ETP cells. In murine thymocytes, *BCL11B* inactivation leads to the developmental arrest at a DN2-DN3 stage, acquisition of NK-like features and aberrant self-renewal activity [91]. *BCL11B* can be involved in leukemogenesis either through an inv (14) or a recurrent cryptic t(5;14)(q35;q32) translocation [92, 93]. More recent findings also indicate that *BCL11B* can be deregulated in T-ALL as a consequence of mutations, that are prevalent in 16% of *TLX1* overexpressing patients [94]; De Keersmaecker et al. [94] propose a mechanistic mouse model in which *TLX1* directly downregulates the expression of *CHEK1* together with additional mitotic control genes and induces loss of the mitotic checkpoint in non-transformed preleukemic thymocyte. This phenomenon, in turn, induces the accumulation of several mutations, including *BCL11B*. More recently, it has also been shown that mutations or deletions can occur in about 2–9% of T-ALL cases, not being limited to the presence of *TLX1* overexpression [95, 96].

The *JAK1* gene encodes a cytoplasmic tyrosine kinase and plays a role in lymphoid cell precursor proliferation, survival and differentiation. The first report focusing in this topic showed that *JAK1* mutations can be identified mostly in adult patients (18%), whereas they are rare in childhood T-ALL (2%). From a functional point of view, three mutations (A634D, R724H, and R879C) are able to promote JAK1 gain of function and confer interleukin (IL)-3-independent growth in Ba/F3 cells and/or IL-9-independent resistance to dexamethasone-induced apoptosis in the

T-cell lymphoma BW5147 cell line. In line with this, primary T-ALL cells harboring *JAK1* mutations display a gene expression signature characterized by transcriptional upregulation of genes positively controlled by JAK signaling. More importantly, the presence of *JAK1* mutations correlated with poor response to induction therapy and overall prognosis [97]. While similar results were reported by Jeong and colleagues [98], the French GRAAL group could not confirm both the relatively high incidence (3–7% in the French study) and the association with a poorer outcome [99], thus suggesting that other events might contribute to the impact of *JAK1* mutations on prognosis.

PTPN2 is a tyrosine phosphatase located on chromosome 18p11.3–11.2, engaged in a cell cycle dependent manner, and is considered a negative regulator of tyrosine kinases. *PTPN2* was recently found to be deleted in 6% of T-ALL patients, especially within subgroups overexpressing *TLX1* T-ALL, or in *NUP214-ABL*+ patients [100]. Recent evidences indicate that *PTPN2* can be lost also in *JAK1* mutated T-ALL, where it increased the transforming capability of *JAK1* mutations, thus overall inducing a more resistant phenotype when these cells are treated with a JAK inhibitor [101].

The *IL7R*, located on 5p13, encodes for the IL-7 receptor and is required for lymphoid development [102, 103]. This gene can be mutated in 9% of pediatric T-ALL [104, 105], as well as in 6% of B-ALL overexpressing *CRLF2* [104].

In T-ALL, mutations lead to a gain of function, with consequent constitutive JAK1 and JAK3 activation and enhancement of cell cycle progression. Overall, this mutation is more frequently detected in cases that, by GEP, fall in the HOXA cluster, is not associated with *JAK1* and *PTEN* mutations, and there is no significant difference in its distribution between *NOTCH1* mutated *vs.* wild-type cases. There is no association between the *IL7R* mutational status and clinical outcome [105].

PHF6 mutations have been recently described by Van Vlieberghe and colleagues [106]. This gene, previously known to be involved in the Börjeson-Forssman-Lehmann syndrome [107], is located on Xq26, encodes for a plant homeodomain factor (PHD), regulates gene expression, is phosphorylated in a cell cycle dependent manner and its expression is ubiquitous. Mutations of *PHF6* were initially reported to be almost exclusively found in males, associated with *TLX1* and *TLX3* overexpression, but not with *NOTCH1*, *FBW7* and *PTEN* [106], and were detected more frequently in adults than in children (38 *vs.* 16%). A more recent report [108] somehow showed contradicting results: in fact, mutations were found at a much lower frequency (18% of adults *vs.* 5.4% in children) and were not associated with a male gender, whereas there was a significant association with *NOTCH1* and *JAK1* mutations, as well as with the *SET-NUP214* rearrangement. Both studies showed that there was no significant correlation between the presence of *PHF6* mutations and outcome [106, 108].

Finally, a mutation in the *PTPRC* gene, located on chromosome 1q31-q32 and encoding for the protein tyrosine phosphatase CD45, has been described: it induces loss-of-function, are usually detected in combination with activating mutations of *IL7R*, *JAK1* or *LCK*, and are associated with downregulation of CD45 expression [109].

8 The Role of Notch1 Mutations in T-ALL

8.1 Notch Structure Function and Activation

Notch1 receptors (Notch1-4) play a pivotal role in tuning differentiation and proliferation in both physiological and leukemic T cells. Their activation is dependent from ligands sent from neighboring cells, that include Jagged 1–2 and Delta-like -1, -3, -4 (DLL-1,-3,-4) [110].

From a structural point of view, the mature form of Notch contains an intracellular and extracellular portion, which are associated by a non-covalent, extracellular and Ca^{++} dependent bond at the HD (heterodimerization) domain. The extracellular portion contains the EGF (epidermal growth factors)-like repeats, the LIN12/Notch1 cysteine rich repeats and the HD domain. The EGF-like repeats are required for the binding with Notch ligands; the LIN12/Notch1 cysteine rich repeats prevent receptor activation in the absence of the appropriate ligands, while the proteolyitic cleavage upon ligand binding occurs in the HD domain. The intracellular portion contains the RAM domain, involved in Notch dependent activation, and, at the C-terminal, the PEST domain; the latter is involved in the proteosomal degradation of Notch itself, since it has a sequence required for the polyubiquitination [111, 112].

Notch activation requires two cleavages, at both the extracellular and intracellular sites, operated by two distinct proteases, the ADAM/TACE metalloprotease and the γ-secretase, respectively. Upon cleavage, the Notch intracellular portion (ICN) migrates into the nucleus, displaces the transcriptional repressor complex (CSL), recruits several co-activators and eventually activates transcription of downstream targets, including proliferation (*MYC, CDKN1A, CDKN1B, CCND1*) and cytokine/cytokines receptors genes (*CCR4*, *CCR8*, *CXCR6*, *CCR6*, *CCR7 IL-6*, *IL-8*, *VEGFR*, and *IL7R*) [113–117].

8.2 Notch1 Involvement in T-ALL

The first evidence of *NOTCH1* involvement in T-ALL came from the description of a translocation t(7;9)(q34,q34.3), detected in about 1% of cases, that creates a truncated form of NOTCH1, by juxtaposing it to the *TCRB* [118]. Importantly, it was shown that retroviral-mediated expression of the resulting truncated form induced leukemia in irradiated mice, thus indicating that Notch1 aberrant forms might be causative of leukemia [119].

Besides the rare translocation mentioned above, *NOTCH1* mutations can be detected in about 60–70% of T-ALL patients [120, 121] and are mostly located in the HD or in the PEST domain, and in the extracellular juxtamembrane region (JME). HD domain mutations weaken the binding of the two subunits and favor ligand-independent activation, whereas PEST domain mutations are usually small deletions/insertions, that generate premature stop codons which, in turn, lead to

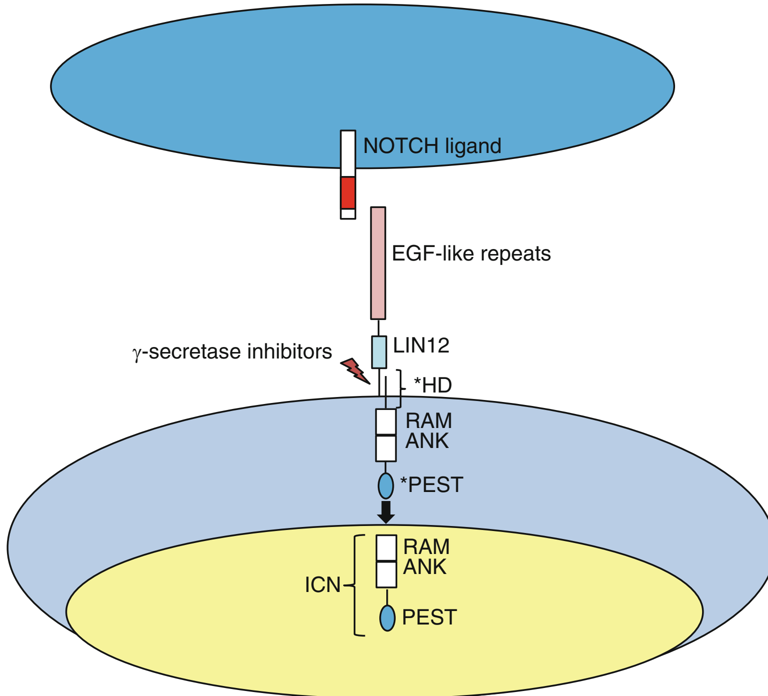

Fig. 14.2 Functional structure of mature NOTCH receptors and model for NOTCH1 activation. Structure: The N-terminus extracellular domain contains the EGF-like repeats, the LIN12 cysteinrich repeats (LIN12) and the heterodimerization domain (HD), which allows the interaction with the intracellular domain; the latter contains a RAM domain, followed by ankyrin repeats (ANK). The RAM-ANK region is required for the control of transcription. The C-terminus contains a polyglutamine and a proline, glutamic acid, serine and threonine rich region (PEST), which controls Notch ubiquitylation and proteasomal degradation. Activation: Heterodimerization (HD) and PEST domain mutations – indicated with an asterisk in the figure – lead to an increase of ICN levels, either by enhancing γ-secretase cleavage (HD mutations) or by increasing ICN half-life (PEST mutations). γ-secretase inhibitors might revert these effects by inhibiting NOTCH1 cleavage, and therefore its levels

a decreased proteosomal degradation and eventually to an increased half-life of NOTCH1 itself. In all cases, the final result is an augmented Notch1 pathway activity.

The structure and the impact of *NOTCH1* mutation in its signaling are summarized in Fig. 14.2.

8.3 Prognostic Role of Notch1 Activation in T-ALL

Given the high incidence of *NOTCH1* mutations and its key role in differentiation and proliferation, several studies have attempted to correlate their presence with

outcome, with non equivocal results. Overall, a number of studies showed that the presence of *NOTCH1* mutations, evaluated mostly in pediatric cohorts, was significantly correlated with a good prednisone response and favorable MRD levels, independently of gender, age, white blood cell count and T-cell immunophenotype, and was associated with an excellent prognosis [120, 121]. However, it must be kept in mind that the interaction with other lesions, such as *FBW7*, makes the scenario more complex and it is likely that the presence of either *NOTCH1* or *FBW* mutations, rather than *NOTCH1* only, may identify a subgroup with favorable prognosis, as suggested by several authors [86, 87, 89, 90], especially if intensive treatments are administered.

8.4 Notch1: A Therapeutic Target?

Since NOTCH1 activation is driven by the cleavage of the receptor, the blockage of this process would theoretically provide the molecular basis for therapeutic intervention. $\gamma-$secretase inhibitors (GSIs), which directly inhibit NOTCH1 cleavage, were initially developed for Alzheimer's disease [122] and have shown activity in T-ALL *in vitro* [120, 123]. However, their use presents some important limitations: first, since NOTCH1 is regulated and interacts with other intracellular mediators, particularly c-Myc and PTEN, their efficacy is somehow limited by the concomitant deregulations of these transcripts, which induce resistance to GSIs [124, 125]; second, the use these compound has been limited by an important gastrointestinal toxicity. More recently, it has been shown that the combined use of GSIs plus glucocorticoids can improve the anti-leukemic effects of GSIs and reduce gut toxicity in vivo, thus suggesting that their use is feasible in such a schedule [126].

The functional structure of mature Notch receptors and model for NOTCH1 activation are depicted in Fig. 14.2.

9 Chronic Lymphocytic Leukemia (CLL)

9.1 Introduction

Chronic lymphocytic leukemia (CLL) represents a clonal malignancy of mature $CD5^+$ B lymphocytes, is the most frequent leukemia in adults in the Western countries, where it accounts for about 30% of all leukemias [127, 128], and is usually diagnosed in elderly individuals, presenting a peak at the seventh decade of life. However, nowadays the improvement of diagnostic tools and routine blood test screenings has identified a growing number of patients who are diagnosed at a relatively young age [129, 130]. Form a clinical standpoint, CLL is characterized by a heterogeneous course, with some patients requiring early therapeutic intervention, whereas others have an indolent disease that does not require treatment for many

years. At present, the disease is curable only if treated with aggressive strategies, such as allogeneic stem cell transplantation; beyond this approach, the current standard chemotherapy regimens with or without the addition of monoclonal antibodies, have been shown to prolong survival. Given these premises, there has been great interest in identifying prognostic markers that can distinguish patients with an aggressive CLL from those who will not progress.

Among the factors that have shown prognostic significance, the IGHV mutational status, ZAP-70 and CD38 expression, and genetic aberrations have a pivotal importance and will be discussed in detail. Furthermore, since WES has provided striking results and is changing dramatically the current knowledge of CLL, a section will be dedicated to this topic.

10 IGHV Mutational Status, ZAP-70 and CD38 Expression

In physiologic conditions, immune diversity is the process that gives rise to a pool of mature B cells that express a Ig B-cell receptor and is achieved when variable (V), diversity and joining segments of the *Ig* genes are recombined. Upon encounter with an invading pathogen, B cells enter the germinal center, where somatic hypermutations of the V regions lead to selection of a B cell that produces an antibody with high affinity for its target antigen.

In CLL, patients can be segregated into two subgroups on the basis of their *Ig* heavy chain V region (IGHV) genomic sequence: if their sequence homology is ≥98% to the germline they will be defined IGHV unmutated, if less they will be defined IGHV mutated. The first evidence of the clinical impact of this difference came from two milestone studies [131, 132] that showed that the IGHV mutated status is associated with an early stage of disease and, more importantly, with a prolonged median survival [131–134]. While the significance of the IGHV mutational status as an independent prognostic factor is nowadays accepted, its role in predicting response to therapy is still not clearly defined [135]. Furthermore, it is worth noting that the usage of different IgV regions harbors prognostic significance: for example, *IGHV3-21* is associated with a poor outcome, although mutated [136–138], and similar findings have been reported for the *IGHV3-23* subset [139, 140] At variance, the *IGHV3-72* has been associated to a very favorable clinical scenario and *IGHV3-30* is more frequently detected in cases who undergo the exceptionally rare event of a CLL spontaneous regression [141].

In order to better understand the contribution of the IGHV mutational status in CLL pathogenesis, GEP studies specifically attempted to resolve this issue by comparing IGHV mutated *vs.* unmutated cases [142, 143]. Quite surprisingly, very few genes were differentially expressed between the two subsets, thus indicating that overall CLL is a unique disease; among the transcripts identified, the Zeta-associated protein 70 (ZAP-70) was more highly expressed in IGHV unmutated cases.

ZAP-70 is an intracellular protein normally expressed in T cells that transmits signals from the TCR to downstream pathways [144]. The increased expression of ZAP-70 in CLL and its association with an unmutated IGHV status has been widely reconfirmed [145]. Additional investigations demonstrated that ZAP-70 expression is relatively stable over time [146] and that this protein may be a better predictor of time to treatment initiation than CD38 expression and the IGHV mutation status [147, 148].

Finally, CD38 is a transmembrane glycoprotein normally expressed at high levels in B-cell precursors, germinal center B cells and plasma cells, with low expression on circulating B cells. In CLL, a high level of surface CD38 expression was initially found to correlate with an unmutated IGHV status [132]. The threshold for defining CD38 positivity has been controversial [149–151]. Nevertheless, CD38 expression is associated with a shorter time to first treatment, poor response to therapy and shorter PFS, although its prognostic role is not independent from other variables [135, 151–153].

11 Genomic Aberrations in CLL

CLL is characterized mostly by numerical aberrations, rather than chromosomal translocations. Approximately 80% of individuals have acquired chromosomal abnormalities; on a FISH-based classification basis, patients can be stratified into five prognostic groups: deletion 13q (median survival, 133 months), deletion 11q (median survival, 79 months), trisomy 12 (median survival, 114 months), normal cytogenetics (median survival, 111 months) and deletion 17p (median survival, 32 months) [154]. So far, reciprocal chromosome translocations are rare, although the introduction of CpG-oligodeoxynucleotides in conventional karyotyping analysis has enabled their identification in about a third of patients [155, 156]. A complex cytogenetic karyotype can be identified in ~16% of patients and is commonly associated with poor prognostic features, including CD38 expression and unmutated IGHV [157].

Among the most recurrent abnormalities, deletion 13q represent the most common, is found in ~55% of patients and has been associated with a favorable outcome [154]. Detailed analysis of the minimally deleted region (MDR) has highlighted the presence of miR-15a and miR-16-1 [158, 159] in the 13q14region. These two non-coding RNAs seem to a have a crucial role in the establishment of B-cell malignancies, since their deletion in mice causes clonal lymphoproliferative disorders and it has been postulated that they regulate the expression of genes important for proliferation (*CCND1*, *CCND3* and *CHK6*) and apoptosis (*BCL2*) [160].

Deletion 11q is identified in ~18% of CLL patients and is associated with several adverse prognostic factors, including lymphadenopathy, unmutated IGHV status, advanced disease at diagnosis, poor response to treatment and shorter PFS. The minimal region of deletion maps to 11q23 and involves the *ATM* gene, which can

be either mutated and/or deleted [154, 161–164]. It is likely that other genes, such as *BIRC3*, are deregulated by 11q deletions [165]. While in the past this patients' category had a poor outcome, the current use of intensive chemotherapy combined with the anti-CD20 monoclonal antibody has overcome the poor prognostic impact [166].

Trisomy 12 represents the third most frequent chromosomal aberration in CLL (15–20% of cases) and often (~60% of cases) occurs as the sole cytogenetic lesion [154]. The prognostic role of trisomy 12 has been debated, since it was shown that, when present as a single aberration, it conferred an intermediate prognostic risk, with a median time to progression of 33 months and a median OS of 114 months [154]. It is conceivable that the outcome of this subset of cases could be sustained by the concomitant presence of additional lesions. In line with this, it has been recently shown that *NOTCH1* mutations are detectable in roughly 25% of trisomy 12 cases, particularly in those harboring trisomy 12 as sole abnormality, are strikingly associated with unfavorable prognostic markers, as well as a shortening of survival [167, 168].

Finally, deletion 17p is found in a relatively small proportion (~3%) of CLL patients at diagnosis, but it significantly increases at the time of first-line treatment (~7%) and to a further extent in relapsed/refractory patients, where it can be found in 25–30% of cases [154, 169, 170] and is often associated with unmutated IGHV. More importantly, patients with such lesions display a very poor outcome, both in terms of disease progression, response to therapy and survival. The deletion always involves the locus of the *TP53* gene encoding the tumor suppressor p53. In addition, the majority of CLL patients with monoallelic deletions of 17p have point mutations in the remaining *TP53* allele, thus completely inactivating a critical component [171] of the DNA damage response pathway. Overall, CLL patients with p53 inactivation respond poorly to conventional fludarabine or alkylating agent-based regimens, possibly because both agents require p53-dependent pathways to induce cell death [171–174]. Thus, in an exhaustive diagnostic work-up, both deletions and mutations should be evaluated, given their prognostic impact.

12 Next-Generation Sequencing in CLL

In CLL, next-generation sequencing is providing important results and has led to the discovery of previously unknown mutations. The first study [175] aimed at comparing IGHV mutated *vs.* unmutated cases. Overall, this analysis showed that ~1,000 somatic mutations per sample can be detected, significantly less than in solid tumors. The most frequent mutations were represented by G > A/C > T transitions, frequently detected in CpG island context. Among the mutations identified in the training cohort, 46 were causative of a change in the protein coding sequence: recurrent lesions in an extended cohort included *NOTCH1*, *MYD88*, *XPO1* and *KLHL6*.

A similar approach has been undertaken by Wang and colleagues [176], who screened 88 CLL patients, including previously treated cases: recurrent mutations involved, as expected, *TP53* and *ATM*. Other recurrent mutations affected *NOTCH1*, *SF3B1*, *MYD88, FBW7, DDX3X, MAPK1* and *ZMYM3.*

As for *NOTCH1*, mutations were initially detected in a small cohort of patients and were regarded as an adverse prognostic factor in CLL [177]. Currently, *NOTCH1* mutations have been reported by several groups [167, 168, 175–179]: they affect the PEST domain, are usually represented by a 2 bp frameshift deletion (DCT7544-7545, P2515fs) and are more often associated with an unmutated IGHV status, advanced stage of the disease, shorter overall survival (OS) and treatment-free survival (TFS). Their frequency ranges from 4 to 12%.

At CLL diagnosis, they are almost mutually exclusive with *TP53* aberrations [179]; they are more frequently detected in trisomy 12 patients, mostly when this lesion occur as sole aberration, where it correlates with unfavorable prognostic markers and shorter OS, thus permitting a further dissection of this heterogeneous subgroup [167, 168, 179].

Furthermore, the incidence of *NOTCH1* mutations increases with disease progression, being 21% in cases with progressive/chemorefractory CLL and 30% in Richter' syndrome (RS) [179].

Thus, *NOTCH1* mutations represent a novel and relatively frequent lesion in CLL: these findings are particularly important, since their incidence increase during progression and also in light of the potential use of *NOTCH1* inhibitors in the context of this neoplasm.

SF3B1, a component of the SF3b complex involved in the spliceosome machinery, has been extensively evaluated by Rossi et al. [180] in CLL patients either at diagnosis, at fludarabine-refractoriness or at RS transformation, where they are detected in 5, 17 and 6% of cases, respectively. Mutations occur regardless of the IGHV mutational status, are almost mutually exclusive with *TP53* disruption and, at diagnosis, are associated with shorter OS and TFS survival. Subsequent studies [176, 181] reported a mutation frequency of 15 and 10%, and, as already shown by Rossi et al. [180], correlate with a poorer outcome. All the mutations affect the HEAT domain and have functional consequences, since the analysis of *SF3B1* target genes shows an impaired splicing capability.

Other mutations identified include *MYD88*, detected in 2–10% of cases [175, 176], *ZMYM3* [176], disrupted in 4% of cases and *XPO1* [175]*,* reported in 2.4% of cases.

MYD88, a critical adaptor molecule of the interleukin-1 receptor–toll-like receptor (TLR) signaling pathway, also affecting the NF-κB pathway, is more frequently disrupted in IGHV mutated cases and seems to have no evident impact on OS [175, 176].

ZMYM3 is a component of multiprotein complexes containing histone deacetylase, is involved in gene silencing and has been found more frequently mutated in IGHV unmutated patients [176].

XPO1, implicated in the nuclear export of proteins and mRNAs in yeast, was found mutated only in IGHV unmutated patients. In all cases, the mutations affected the same residue, indicating a functional effect in XPO1 activity [175].

Mutations of *BIRC3,* a negative regulator of the non-canonical NF-κB pathway, have been first identified in splenic marginal-zone lymphoma and in CLL, whilst they are absent in other lymphoproliferative disorders [182]. Subsequently, *BIRC3* mutations and deletions have been identified in 4% of CLL at diagnosis and in 24% of chemorefractory CLL with wild-type *TP53*. They introduce a stop codon that causes the truncation of the C-terminal RING domain, whose ubiquitin-ligase activity is essential for MAP3K14 degradation by the proteasome, the principal activator of the non-canonical NF-κB pathway [165]. Beyond *TP53*, *BIRC3* mutations appear to be mutually exclusive with *NOTCH1* and *SF3B1,* and their presence correlate with shortening of OS.

Finally, mutations identified at low frequency included *KLHL6*, *CCDN2*, *MAPK1*, *DDX3X* and *POT1* [175, 176]. The most important findings obtained by WES are summarized in Table 14.4.

13 *De Novo* Acute Myeloid Leukemia (AML)

13.1 Introduction

AML represents one the most frequent leukemia in adults (25%), with a median age at diagnosis of 67 years [183, 184]. While the majority of patients aged less than 60 years achieve a complete remission (CR), the overall long-term survival rates continue to be poor, ranging around 30–40% [185, 186]. The prognosis is even poorer for those patients with high-risk AML, where the CR rate is less than 40% and survival rates are below 10% [186].

From a biological point of view, it is represented by a clonal hematopoietic disorder resulting from genetic alterations in hematopoietic stem cells. These alterations disrupt normal differentiation and/or cause excessive proliferation of abnormal immature leukemic blasts. As the disease progresses, blast cells accumulate in the bone marrow, blood and organs, and interfere with the production of normal blood cells.

In recent years, technologic progress has allowed to better define the lesions underlying the malignant transformation and has permitted to more precisely elucidate the heterogeneity of the disease. As a proof of principle, the WHO classification now includes several subsets of AML, indeed defined on the basis of their genomic characteristics and characterized by specific morphologic and prognostic features [187]. In this section, the most important findings that have arisen from cytogenetic analyses and SNP arrays, gene and miRNA profiling, gene mutation and WES sequencing will be described.

Table 14.4 Gene mutations detected by WES in CLL

Gene involved	Gene position	Functional consequences	Frequency	Clinical relevance
NOTCH1	9q34.3	Impairment of differentiation of and proliferation	4–12%	Reduced OS and TFS
			Incidence increases at disease progression, refractoriness and RS transformation.	Significantly associated to an IGHV unmutated status and trisomy 12
				Almost mutually exclusive with *TP53* and *SF3B1* disruption
SF3B1	2q33.1	Impaired splicesome machinery	5–15%	Reduced OS and TFS
			Incidence increases at refractoriness	Preferentially detected in IGHV unmutated patients
				No clear association with specific FISH cytogenetic subgroups
				Almost mutually exclusive with *TP53* and *NOTCH1* disruption
MYD88	3p22	Toll-like receptor (TLR) pathway	2–9%	Significantly associated to an IGHV mutated status and 13q deletion
				Survival analysis to be assessed
FBW7	4q31.3	Arrest of differentiation, and aberrant self renewal activity	4%	Not assessed
POT1	7q31.33	Telomere maintenance	~5%	Significantly associated to an IGHV unmutated status
				Survival analysis to be assessed
ZMYM3	Xq13.1	Histone modification	4%	Survival analysis to be assessed
XPO1	2p15	Protein nuclear export	~2%	Significantly associated to an IGHV unmutated status
				Survival analysis to be assessed

(continued)

Table 14.4 (continued)

Gene involved	Gene position	Functional consequences	Frequency	Clinical relevance
BIRC3	11q22	Constitutive non-canonical NF-κB activation	4% at diagnosis, 24% at refractoriness	Mutually exclusive with *TP53*, *NOTCH1* and *SF3B1* Reduced OS
KLHL6	3q27.3	BCR signal transduction and formation	~1%	Not assessed
MAPK1	22q11.21	Toll-like receptor (TLR) pathway	3%	Not assessed
DDX3X	Xp11.3-p11.23	RNA helicase	3%	Not assessed
CCND2	12p13	Cell cycle	0.6	Not assessed

14 Cytogenetic Aberrations

Cytogenetic abnormalities can be detected in approximately 50–60% of newly diagnosed AML patients and are usually represented by non-random chromosomal translocations that often result in gene rearrangements [187], monosomies or deletions of part or all of chromosomes 5 or 7 (−5/−7 AML) and trisomy 8 [188]. Chromosomal abnormalities also include balanced translocations between chromosomes 15 and 17 (t(15;17)), chromosomes 8 and 21 (t(8;21)) and inversions, such as inv(16) and lesions involving the long arm of chromosome 11 (11q) [189].

The t(15;17) translocation is always associated with acute promyleocytic leukemia (APL) and leads to the expression of the *PML-RARα* oncofusion gene in hematopoietic myeloid cells. The PML-RARα protein acts as a transcriptional repressor that interferes with gene expression programs involved in differentiation, apoptosis and self-renewal [190].

Following the understanding of the underlying molecular mechanisms, patients have been treated with all-trans retinoic acid (ATRA) as part of the remission induction. This has had a profound clinical impact both in adults and in children [191–195], since ATRA alters corepressor activity, bypasses the differentiation block of the leukemic cells and eventually induces differentiation of the myeloid lineage [196].

The t(8;21) translocation results in the *AML1-ETO* oncofusion protein. *AML1* (*RUNX1*), a DNA-binding transcription factor, is a master regulator of the formation of hematopoietic stem cells (13, 14), while *ETO* encodes for a protein harboring transcriptional repressor activities [197]. The fusion protein AML1-ETO is suggested to function as a transcriptional repressor that blocks AML1-dependent transactivation. This aberration is associated to a relatively favorable outcome [198].

Inv(16) leads to a *CBFβ-MYH11* rearrangement that is found in approximately 8% of AML cases. It fuses the first 165 amino acids of core binding factor β (CBFβ) to the C-terminal coiled-coil region of a smooth muscle myosin heavy chain (MYH11). The CBFβ-MYH11 fusion protein is suggested to cooperate with AML1 to repress transcription [199].

Finally, *MLL* (11q23) rearrangements are implicated in different types of acute leukemias and so far at least 300 partners have been recognized [16, 17]. The MLL fusion proteins have a dominant gain-of-function effect that enhances transcriptional activity. In general, prognosis of patients with MLL translocations is poor [200].

Beyond these recurrent translocations, a recent study that evaluated more than 100 AML patients using the SNP array technology showed that the rate of acquired chromosomal copy number changes and LOH (loss of heterozygosity) is variable among cases. More importantly, using multivariate analysis, the authors found that the presence of ≥2 genomic lesions doubles the risk of death when controlling for age- and karyotype-based risk, thus confirming the impact of genomic complexity on outcome. Finally, as expected, the negative prognostic impact of *TP53* mutations, or *TP53* mutations plus 17p-loss of heterozygosity, was confirmed [201].

15 Gene Mutations in AML and the Contribution of Next-Generation Sequencing

Mutations of important genes have now been recognized in AML and can be categorized in at least two groups: class I mutations, which activate signal transduction, and class II mutations that impair differentiation. The identification of such mutations has been particularly useful in the prognostic stratification of AML cases with normal karyotype (CN-AML) (Fig. 14.3).

Among class I mutations, *KIT* mutations are mostly frequently reported in inv(16) and t(8;21), are very rare in other AML subtypes [183] and are usually associated with an unfavorable outcome.

FLT3 mutations can be of two types: internal tandem duplication (ITD) and tyrosine kinase domain mutations (TKD). *FLT3-ITD* are found in approximately 20% of unselected cases of AML and mainly cluster in the juxtamembrane domain, whereas *TKD* are represented by point mutations, small insertions, or deletions, mainly at codons 835 and 836, in 5–10% of AML cases. *FLT3-ITD* in CN-AML are usually associated with a dismal outcome [202–204]; clinical trials based on the use of FLT3 inhibitors are ongoing.

Similarly, among class II mutations, *CEBPA* mutations are predominantly found in CN-AML [205] and can be of two types. Nonsense mutations affecting the

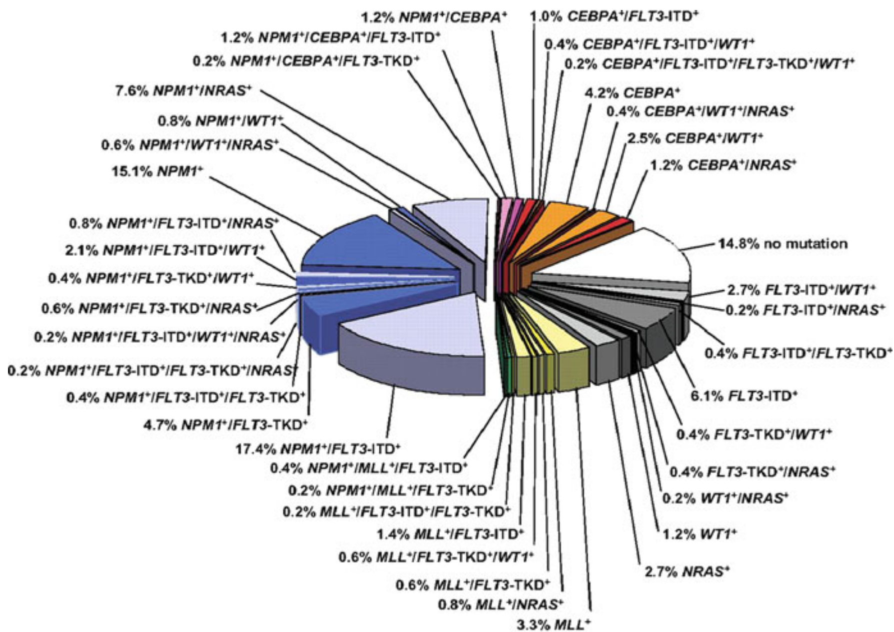

Fig. 14.3 Pie chart illustrating the molecular heterogeneity of cytogenetically normal AML based on mutations in the *NPM1*, *CEBPA*, *MLL*, *FLT3* (ITD and TKD mutations at codons D835 and I836), *NRAS*, and *WT1* genes. From [213]

N-terminal region result in a truncated CEBPA isoform with dominant-negative properties, while in-frame mutations in the C-terminal basic result in CEBPA proteins with decreased DNA binding or dimerization activity. Such mutations can be biallelic. From a prognostic point of view, they have been associated with a relatively favorable outcome [203–205]; however, recent findings indicate that only double *CEBPA* mutations predict for this favorable outcome, as also corroborated by a discrete gene-expression signature of double mutated cases [206].

NPM1 mutations, located in exon 12, induce an abnormal cytoplasmic localization of the NPM1 protein [207]. They are usually found in one third of adult cases of AML, making it the most frequent mutation in AML [208] and can be associated with other recurrent genetic changes, secondary chromosome abnormalities, such as trisomy 8, trisomy 4 and del(9q), and additional gene mutations, most frequently in *FLT3* and *IDH1* genes [209–212].

Within CN-AML, *NPM1* mutations have an important prognostic impact, mostly when they are not concomitant with *FLT3-ITD*: in fact, they are associated with achievement of CR and favorable outcome [210, 211]. For this reason, AML with mutated *NPM1* without *FLT3-ITD* has recently been allocated to the genetic favorable-risk category of AML [213]. Furthermore, given the presence of specific gene and microRNA expression signatures [214–216], they have been incorporated as provisional entities in the 2008 WHO classification of AML [187].

The introduction of WES has made it possible to identify a potential third class of mutations in AML that induces an impairment of epigenetic regulation [217].

The first of such genes identified by WES is represented by *IDH1/IDH2* [218]. This gene encodes for a protein that has a significant role in cytoplasmic NADPH production: since mutations have been reported also in gliomas [219, 220], it has been postulated that *IDH1* might function as tumor suppressor gene. *IDH1/IDH2* mutations were firstly reported in more than 15% of adult CN-AML patients and this frequency was later confirmed in other studies [212, 218, 221]. Similar results were also observed in pediatric cohorts [222]. Furthermore, they are more frequently detected in patients without *FLT3-ITD* and with NPM1 mutations. In this subset of patients, at least two independent studies have shown an impact on DFS and OS [212, 222], whereas a third study, carried out by Wagner and colleagues highlighted a role for *IDH1* SNP rs11554137 polymorphism [223]. Finally, from a functional point of view, both *IDH1* and *IDH2* mutations appear to induce hypermethylation [224].

Another mutated gene in AML is *DNMT3A,* a transcript with methyltransferase activity that catalyzes the methylation of cytosine residues of CpG dinucleotides. *DNMT3A* mutations are represented mostly by nonsense, frameshift and missense mutations throughout the open-reading frame, with the most recurrent hotspot being a missense mutation at amino acid R882. They are found in approximately 20–22% of adults with *de novo* AML [225] and are associated with intermediate-risk AML, where they also correlate with an inferior outcome [225, 226]. At variance, *DNMT3A* mutations appear to be rare in children [227, 228].

BCOR (BCL6 corepressor) mutations have been initially identified by WES in a CN-AML patient lacking *NPM1*, *CEBPA*, *FLT3-ITD*, *IDH1* and *MLL-PTD*

mutations. Extended analysis on a large cohort of patients (>500 cases) showed that *BCOR* mutations are relatively recurrent (17.1%) in CN-AML patients without *NPM1*, *CEBPA*, *FLT3*-ITD, *IDH1* mutations and *MLL-PTD* whereas they are rarely detected in unselected CN-AML (3.8%) and virtually absent in the other subgroups [229]. The mutations identified are similar to those reported in the oculo-facio-cardio-dental genetic syndrome [230], are scattered across the whole coding sequence and associated with decreased *BCOR* mRNA and absence of the protein. Furthermore, they are frequently associated with *DNMT3A* mutations and tend to be associated with a reduced OS.

Finally, *TET2* mutations, which play a pivotal role in DNA demethylation, are detected mostly in myelodisplastic syndromes, myeloproliferative disorders and are highly recurrent in chronic myelomonocytic leukemias, in which they have been found to be associated with significant monocytosis and poor outcomes [231–234]. While the impact on prognosis is controversial, it is interesting to highlight that *TET2* mutations appear to be mutually exclusive with *IDH1* mutations. This is in line with the role of such mutations, both shown to induce an hypermethylated state [224] and thus acting on the same pathway.

16 Gene and miRNA Expression Profiling

Similarly to what observed in ALL, GEP was initially used to discriminate known chromosomal translocations. This approach clearly showed that AML with t(8;21)/*RUNX1-RUNX1T1* and inv(16)/*CBFB-MYH11* could be easily discriminated from other cytogenetic subgroups [235–237]. Similarly, it was shown that cases with a complex karyotype had a peculiar profile characterized by the upregulation of genes with a role in DNA repair such as *RAD21* [238, 239].

GEP studies have been also applied to identify transcriptional profiles associated with emerging mutations.

NPM1-mutated AMLs were found to be associated with overexpression of distinct *HOX* cluster genes and genes involved in apoptosis [214, 215].

Similarly, *CEBPA* mutations have been associated with distinct GEPs, that include downregulation of *HOXA* and *HOXB* cluster genes and upregulation of erythroid-specific genes, including *GATA1* and *EPOR* [240]. Wouters et al. [241] revealed the presence of a subset of AML that did not harbor *CEBPA* mutations, but had a similar transcriptional profile. These cases were characterized by *CEBPA* silencing through promoter hypermethylation and, phenotypically, showed the aberrant expression of T-cell genes, among which CD7. Furthermore, these cases harbored *NOTCH1* mutations, suggesting the presence of mixed myeloid-lymphoid commitment. Subsequently, the same group showed the presence of a distinct signature for *CEBPA* double-mutated cases; this signature was not evident in *CEBPA* (single-mut) and is associated with a favorable outcome [206].

As for *FLT3*, Neben and colleagues [242] showed that GEP is able to discriminate *FLT3-ITD* from *FLT3-TKD* mutations. The discriminating set included genes involved in cell cycle control, gene transcription and signal transduction.

Gene expression signatures have also been linked to the high expression of specific genes. Langer et al. [243] identified a high *BAALC* signature consisting of overexpression of genes involved in drug resistance and stem cell markers. The same group [244] showed high expression levels of *BAALC*, *CD200* and *ABCB1* in patients overexpressing *MN1*.

Finally, GEP has been used to define prognostic classifiers, mostly in normal karyotype patients (CN-AML). Bullinger et al. and Valk and colleagues identified clusters associated with a different outcome [236, 237]. In particular, one cluster was characterized by the overexpression of several transcriptional regulators such as *GATA2*, and the second one was characterized by involvement of genes playing a role in leukocyte differentiation and immune response. These results, validated by Radmacher et al. [245], confirm the prognostic value of this approach.

MicroRNAs (miRs) have been extensively evaluated in AML: the first study showed the ability of distinguishing AML from ALL on the basis of 21 miRs, of which 4 – *let-7b*, *miR-128a*, *miR-128b* and *miR-223* – were the most discriminative [246]. Furthermore, miR expression profiling has been shown to be able to distinguish different cytogenetic subtypes of AML: *MLL* rearrangements are characterized by the high expression of the *miR-17-92* polycistronic microRNA cluster, as well as of *miR-196b*, the latter located between the homeobox (*HOX*) *A9* and *HOXA10* genes at 7p15 [247]. APL displays high expression levels of miRs localized at chromosome band 14q32, while the downregulation of *miR-133a* in patients with t(8;21) has been described [248, 249].

Distinctive miR profiles were correlated to several mutations: *NPM1* mutations display upregulation of *miR-10a*, *miR-10b* and *miR-196a*, and downregulation of *miR-204* and *miR-128a*, predicted to target the HOX genes [216]. *FLT3*-ITD mutations have been reported to be associated with *miR-155* upregulation which, in turn, interferes with SHP1, ultimately leading to leukemic expansion [250], while *CEBPA* mutations are characterized by the upregulation of members of the *miR-181* family in CN-AML [204].

Finally, miR expression was correlated with outcome: low expression levels of *let7b* and *miR-9* were detected in patients classified in the favorable risk group [249], whereas among the miRs likely to be associated with unfavorable outcome it is worth mentioning the overexpression of *miR-20a, miR-25*, *miR-191*, *miR-199a* and *miR-199b* [251]. A more recent study identified, within CN-AML belonging to the molecular high-risk group (i.e. *FLT3*-ITD mutations and wild-type *NPM1*), a set of miRs discriminative of outcome. Among these, an increased expression of *miR-181a* and *miR-181b* was again associated with outcome [252].

17 Concluding Remarks

Overall, the genomic era has permitted to re-define the molecular bases of leukemic transformation.

In B-lineage ALL, beyond the well-recognized molecular rearrangements, i.e. *BCR/ABL*, *ETV6/RUNX1*, *E2A/PBX1* and *MLL* rearrangements, it was to possible to identify a "BCR/ABL-like" subset, lesions of *IKZF1*, *CRLF2* and of genes involved in lymphocyte development and differentiation.

Similarly, the constellation of lesions in T-ALL now includes the presence of several mutations, such as *NOTCH1*, *FBW7*, *BCL11B, JAK1, PTPN2, IL7R* and *PHF6,* and the recognition of a grey zone between T-ALL and AML.

In CLL, WES analysis has revealed a handful of mutations in genes previously not known to be related to this disease, and which include *NOTCH1*, *SF3B1, BIRC3*, *MYD88*, *XPO1* and *KLHL6*.

Finally, in AML a major contribution has been provided by gene and microRNA expression profiling, as well as by mutational screening of several transcripts: the integration of these approaches has allowed to subdivide patients in prognostic subgroups, to redefine the current WHO classification and has proven particularly useful in the dissection of cases with a normal karyotype.

Last, but not less important, is the fact that the use of all these powerful technologies has highlighted that some genes are recurrently deregulated in more than one onco-hematologic disorder (i.e. *NOTCH1* in both T-ALL and CLL, *IDH1*, *IDH2*, *FT3* and *RAS* members in at least a T-ALL subset, etc.). These results indicate that in the forthcoming future a more refined patients' stratification and possibly the development of personalized treatments, based on the use of targeted therapies, will prove feasible. Moreover, these results suggest that few targeted inhibitors directed toward recurrent lesions may be applied to different therapeutic approaches, in order to reduce the toxicity and maximize efficacy.

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Chapter 15
Where Do We Stand in the Genomics of Lymphomas?

Francesco Bertoni, Zhi-Ming Li, and Emanuele Zucca

Abstract Malignant lymphomas comprise over 60 different neoplastic disorders that originate from lymphoid cells. The different lymphoma subtypes can be distinguished based on a combination of histological, immunophenotypic, genetic and clinical features. Malignant lymphomas are among the malignancies with the highest success rate of cure, although there are still large differences among the different subtypes and the need for therapeutic improvements. A series of recurrent chromosomal translocations, DNA losses and gains, and somatic mutations are now known, and in this chapter, we will summarize the current knowledge on the genomics of the most common lymphomas, with particular emphasis on the recent findings.

Malignant lymphomas are a group of neoplastic disorders that originate from lymphoid cells [1]. Their incidence has steadily increased worldwide, and they represent between the 5th and 6th most common tumor in adults [2–4]. According to

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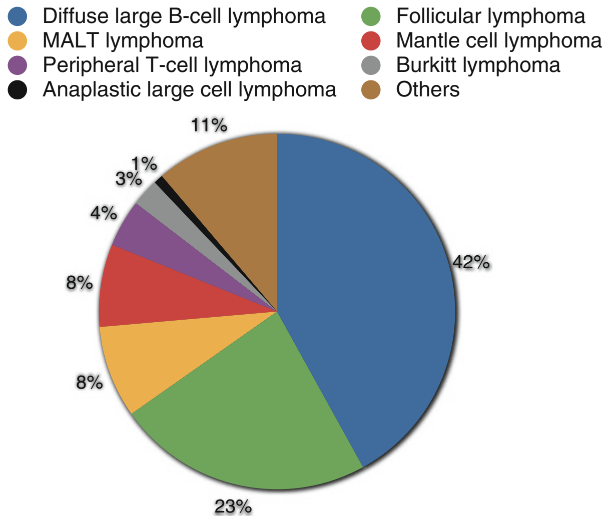

Fig. 15.1 Distribution of the most common lymphoma subtypes at the IOSI Oncology Institute of Southern Switzerland in the period 1980–2008. The database comprises a total of 1,555 patients; chronic lymphocytic leukemia patients have been excluded from the analysis

the last 2008 World Health Organization (WHO) classification, over 60 lymphoma entities or provisional entities can be distinguished based on a combination of histological, immunophenotypic, genetic and clinical features. Figure 15.1 shows, as an example, the distribution of the most common lymphoma subtypes at our Institute. Malignant lymphomas are among the malignancies with the highest success rate of cure, although there are still large differences among the different subtypes and the need for therapeutic improvements.

Most lymphoma subtypes are characterized by non-random chromosomal translocations (Table 15.1), but only a few of them can taken as pathognomonic. Genomic gains or losses and somatic mutations can also be either restricted to specific subtypes or, more usually, shared by different lymphoma entities. Lymphomas are derived from the only cells in the human body which, physiologically, undergo profound DNA rearrangements to express the functional B- and T-cell receptors (BCRs, TCRs), which determine the specificity of the immune response. This process involves multiple DNA double-strand breaks, which directly contribute to the pathogenesis by increasing the risk of genomic lesions. Notably, the rearrangements occurring at BCR and TCR loci also provide fingerprints reflecting the different lymphocytes development stages, thus allowing the identification of the normal cell counterparts.

In this chapter, we will summarize the current knowledge on the genomics of the most common lymphomas, with particular emphasis on the recent findings.

Table 15.1 Main clinical and genetic features of the most common lymphomas

Lymphoma type	Main recurrent chromosomal translocations	Main recurrent somatic mutations	Main recurrent unbalanced genomic lesions
Diffuse large B-cell lymphoma	t(14;18)(q32;q21) *IGHV@-BCL2*, 20–45%[a];	Chromatin remodeling[a] (*EZH2*, *MLL2*, *MEF2B*, *EP300*, *CREBBP*), BCR signaling and NFKB pathway (*TNFAIP3*, *CARD11*, *CD79B*, *MYD88*, *TRAF2*, *TRAF3*, *TRAF5*, *MAP3K7*, *TNFRSF11A*, *ITPKB*)[b], *PRDM1*[b], *TP53*, *BCL6* regulatory region and other ASHM targets	+1q, +2p16 (*REL*)[a], trisomy 3/+3q[b], +7q, +12q (*MDM2*)[a], +13q31 (*MIRHG1*)[a], +18q21 (*BCL2*, *NFATC1*)[b], +19q13 (SPIB)[b], −1p36 (*TNFRSF14*)[a], −6q21 (*PRDM1*)[b], −6q23 (*TNFAIP3*)[b], −9p21 (*CDKN2A*)[b], −10q23 (*PTEN*)[a], −13q34 (*ING1*)[a], −17p (*TP53*)
	3q27 rearrangements involving *BCL6*, 25%;		
	8q24 rearrangements involving *MYC*, 20%[a]		
Primary mediastinal large B-cell lymphoma	3q27 rearrangements involving *BCL6*, 30%;	*SOCS1*, *TNFAIP3*, *BCL6* regulatory region and other ASHM targets	+2p16 (*REL*), +9p21 (*JAK2*, *JMJD2C*), −6q23 (*TNFAIP3*), −16p13 (*SOCS1*)
	16p13 rearrangements disrupting *CIITA* and SOCS1, 40–45%		
Follicular lymphoma	t(14;18)(q32;q21) *IGHV@-BCL2*, 90%	Chromatin remodeling (*EZH2*, *MLL2*, *MEF2B*, *EP300*, *CREBBP*), *TNFRSF14*, *TNFAIP3*, *TP53*	+1q, +2p16 (*REL*), +7, +12q (*MDM2*), +X, +18q21 (*BCL2*) −1p36 (*TNFRSF14*), −4q, −6q, −9p21 (*CDKN2A*), −17p (*TP53*)
Mantle cell lymphoma	t(11;14)(q13;q32) *IGHV@-CCND1*, 90%	*CCND1*, *ATM*, *TP53*, *TNFAIP3*, *NOTCH1*	+3q, +7p, +8q, +15q22−q26, +18q, −1p, −6q (*TNFAIP3*), −8p, −9p (*CDKN2A*), −11q21−q23 (*ATM*), −17p (*TP53*)

(continued)

Table 15.1 (continued)

Lymphoma type	Main recurrent chromosomal translocations	Main recurrent somatic mutations	Main recurrent unbalanced genomic lesions
MALT lymphoma	t(11;18)(q21;q21), *BIRC3-MALT1*, 15–40%;	*TNFAIP3*, MYD88, *BCL6* regulatory region and other ASHM targets	trisomy 3/+3q, trisomy 18/+18q, −6q23 (*TNFAIP3*)
	t(14;18)(q32;q21) *IGHV@-MALT1*, 20%;		
	t(1;14)(p22;q32) *IGHV@-BCL10*, <5%;		
	t(3;14)(p14.1:q32) *IGHV@-FOXP1*, <5%		
Splenic MZL		NFKB and BCR signaling pathway (*BIRC3*, *TNFAIP3*, *TRAF3*, *IKBKB*, *MAP3K14, MYD88, CARD11, CD79A*), NOTCH pathway (NOTCH2, SPEN, NOTCH1), Chromatin remodeling (*MLL2*, TBL1XR1, SIN3A, *EP300*, *ARID1A)*	trisomy 3/+3q, trisomy 18/+18q, −6q23 (*TNFAIP3*), −7q31−q32, −8p, −17p (*TP53*)
Burkitt lymphoma	t(8;14)(q24;q32): *IGHV@-MYC*	*TP53, TCF3, ID3, CCND3*	+1q, +7q, +12q, +13q (*MIR17HG*), −6q, −17p (*TP53*)
	t(2;8)(p12;q24): *IGK@-MYC*		
	t(8;22)(q24;q11): *IGL@-MYC*		
Peripheral T-cell lymphoma, not otherwise specified	t(5;9)(q33;q22) *ITK-SYK*, ?		+1q32-qter, +2p15-p16 (*REL*), +7q22-ter (*CDK6*), +8q24, +9q33-qter, +11q13, +17q12-q21, −6q21−q22, −9p21 (*CDKN2A*), −13q21−q22, −14q12-q21 (*NFKBIA*), −16q11-q21 (*CYLD*), −17p13 (*TP53*)
	t(6;14)(p25;q11.2) *IRF4-TCRA*, <5%		
	Other 6p25 rearrangements involving *IRF4*, <5%		

Anaplastic large cell lymphoma, ALK-positive	t(2;5)(p23;q35) *ALK-NPM*, 80–85%		+7p, +17p11-pter, +17q, −4q13−q28, −6q13-q22, −11q14-q23, −13q
	t(1;2)(q25;p23) *TPM3-ALK*, 10–15%		
	Other 2p23 rearrangement involving *ALK*, <15%		
Anaplastic large cell lymphoma, ALK-negative	t(6;7)(p25;q32) *DUSP2-FRA7H*, 18% (29% in cALCL, ALK-)		+1q, +7q, +8q, +12q, +17q, −4q, −6q21 (*PRDM1*),−11q, −13q,−17p13 (*TP53*)
	Other 6p25 rearrangements involving *IRF4*, <5% (50% in cALCL, ALK-)		
Hodgkin lymphoma	16p13 rearrangements disrupting *CIITA* and SOCS1, 15%	SOCS1, NFKB pathway (*BIRC3*, *TNFAIP3*, *TRAF3*, *IKBKB*, *MAP3K14*, *CYLD*, *NFKBIA*, *NFKBIE*)	+2p16 (*REL*), +9p21 (*JAK2*, *JMJD2C*), +13q (*MIR17HG*), −6q23 (*TNFAIP3*), −16p13 (*SOCS1*), −17p (*TP53*)
	Translocations involving *BCL6* with IGHV, IKAROS, and ABR in NLPHL;		

ASHM Aberrant somatic hypermutation
[a]More common in GCB-DLBCL than in ABC-DLBCL
[b]More common in ABC-DLBCL than in GCB-DLBCL

1 Diffuse Large B-Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) accounts for 30–40% of all lymphomas, and represents the most common lymphoma subtype [1, 5–8]. It is a clinically aggressive lymphoma (as indicated by median survival of untreated patients that is less than 1 year), and it can present at both nodal or at extra-nodal sites. Most DLBCL patients can nowadays be cured with chemo-immunotherapy regimens, such as R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone). However, approximately 30–40% of patients will present with a refractory disease or will experience a relapse, indicating the need of further therapeutic improvement. Clinical prognostic models, such as the International Prognostic Index (IPI) [9], can identify groups of patients with different outcome, but more precise, possibly biologically-based, prognostic factors are warranted to improve the management of patients.

Microarray-based techniques, namely gene expression profiling (GEP) and array-based comparative genomic hybridization (array-CGH), and, more recently, the “next-generation” deep sequencing have allowed the recognition of genetic lesions underlying DLBCL, highlighting that this lymphoma is an indeed heterogeneous group of relatively distinct disorders [7, 8, 10–26].

At least two main biologically different DLBCL subtypes, with different clinical outcome, have been identified by GEP studies resembling the gene expression profile of two types of normal B-cells, likely to represent the lymphoma cells of origin (COO): germinal center (GC) B-cell like (GCB) subtype and activated B cell-like (ABC) subtype [8, 10, 11, 14, 15, 24, 26].

These supposed COOs are supported also by the study of the immunoglobulin heavy chain (*IGHV*) genes, which, whilst somatically hypermutated in both subtypes, show the presence of on-going mutations only in the GCB-type, indicating that this derives from centroblasts, which in the dark area of GC, undergo rapid proliferation and the process of somatic mutation of the *IGHV@* genes to improve the affinity to antigens.

The ABC-type DLBCL has a worse outcome than GCB-type when patients are treated with the chemotherapy regimen CHOP, also when combined with rituximab [8, 15]. Due to technical issues, GEP is still not feasible in the daily clinical practice. Different algorithms based on the detection of a limited number of surrogate markers for the GCB-GEP signature (CD10, BCL6, GCET1), or ABC-markers (MUM1, FOXP1) by immunohistochemistry have been implemented, but a total overlap with GEP signature has not been reached yet, causing important differences in the outcome prediction [27–30].

Without any overlap with the GCB/ABC classification, a GEP approach identified three types of DLBCL characterized by genes expressed by the immune microenvironment (“host response” cluster), genes involved in oxidative phosphorylation, mitochondrial function and electron transport chain (“oxidative phosphorylation” cluster), and genes involved in cell-cycle regulation, DNA repair and BCR

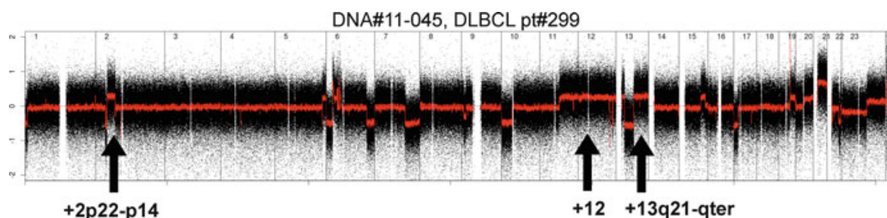

Fig. 15.2 Genomic profile of one case of DLBCL bearing, among others, gains at 2p (REL), at 13q (*MIRHG1*) and of chromosome 12, which are some of the recurrent lesions associated with the GCB-type. Profile obtained using the Affymetrix Genome-Wide Human SNP Array Version 6.0 *Black*, raw copy number values; *red*, smoothed copy number values. X-axis, genomic mapping; Y-axis, log2 copy-number values

signaling ("BCR/proliferation" cluster) [13]. These groups, despite biologically distinct, did not appear to identify patients with different clinical outcome [13].

To highlight that also the outcome of patients affected by aggressive lymphomas may depend not only on the intrinsic features of the tumor cell but also on its relationship with the tissue microenvironment, two novel gene expression signatures associated with the immune microenvironment have been reported to carry a prognostic significance in DLBCL patients treated with R-CHOP [15]. Both signatures are composed by genes expressed by non-tumoral cells: stromal-1 signature reflects extracellular-matrix deposition and histiocytic infiltration and has a favorable prognostic significance, while stromal-2 signature, reflecting tumor blood-vessel density, bears a negative prognostic significance and suggests the evaluation of antiangiogenic drugs in DLBCL [15] (for example, bevacizumab in the NCT00788606 trial).

The GCB-type of DLBCL present high expression of genes characteristic of normal GC B-cells, whilst the ABC-group is characterized by genes up-regulated in peripheral blood B-cells activated by *in vitro* mitogenic stimuli. The presence of the t(14;18) chromosomal translocation, deregulating the *BCL2* oncogene, DNA gains affecting regions on the short arm of chromosome 2 and on the long arms of 7, 12 and 13 (*MIRHG1*), DNA losses at 1p (*TNFRSF14*), 10q (*PTEN*), 13q (*ING1*), as well as somatic mutations of genes coding proteins involved in the chromatin remodeling (*EZH2*, *MLL2*, *MEF2B*, *EP300*, *CREBBP*) are all more common in GCB-type DLBCL [8, 14, 22–24, 26, 31] (Fig. 15.2). On the converse, chromosomal rearrangements involving *BCL6* (3q27), DNA gains at 3q (*FOXP1*, *NFKBIZ*), 18q (*BCL2*), 19q (*SPIB*) and losses at 6q (*PRDM1*, *TNFAIP3*) and 9p (*CDKN2A*) and mutations of genes determining a constitutive activation of the B-cell receptor (BCR) signaling ("tonic signaling") and of the NFκB pathway (*MYD88*, *TNFAIP3*, *CARD11*, *TRAF2*, *TRAF5*, *MAP3K7*, *TNFRSF11A*, *CD79A*, *CD79B*) are observed more commonly in the ABC subtype [10, 14, 16–18, 20, 22, 24, 26, 32–38] (Fig. 15.3).

The activation of the NFKB pathway in the ABC-subtype of DLBCL has driven the testing of the proteasome inhibitor bortezomib [39]. In combination

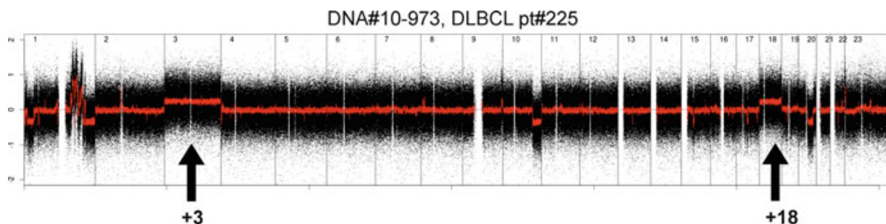

Fig. 15.3 Genomic profile of one case of DLBCL bearing, among others, gains of chromosomes 3 and 18, some of the recurrent lesions associated with the ABC-type. Profile obtained using the Affymetrix Genome-Wide Human SNP Array Version 6.0 *Black*, raw copy number values; *red*, smoothed copy number values. X-axis, genomic mapping; Y-axis, log2 copy-number values

with standard CHOP chemotherapy or with the infusional dose-adjusted EPOCH regimen (etoposide, prednisone, vincristine, cyclophosphamide and doxorubicin), bortezomib has shown a significantly better activity in the ABC compared with GCB subtype [39, 40]. A phase 3 trial evaluating the addition of bortezomib to the R-CHOP regimen is currently on-going (REMoDL-B, NCT01324596). Also lenalidomide, an analog of thalidomide acting as immunomodulant but also with a direct anti-lymphoma effect, has been evaluated in the relapsed/refractory disease setting and showed higher response rates in the ABC-subtype [41]. The preferential activity in one specific subtype would be justified since, in DLBCL, lenalidomide down-regulates IRF4 and SPIB, both factors important in ABC- but not in GCB-DLBCL [38].

By comparing the GEP of DLBCL samples from patients cured or not cured with CHOP, possible therapeutic targets have been identified, such as PDE4B and PKCB [12, 42]. A PKCB inhibitor, enzastaurin [43], has shown ability to induce disease stability as single agent and improved clinical activity when combined with R-CHOP [44]; this has led to additional trials evaluating its combination with R-CHOP (NCT00332202).

The tonic BCR signaling observed in both the GCB- and ABC-type [45] provided the rationale for the clinical development of several signal transduction inhibitors. Small molecules undergoing clinical evaluation comprise the SYK inhibitor fostamatinib disodium (NCT01499303 trial), the BTK inhibitor PCI-32765 (NCT01325701 trial), the PKC inhibitor AEB071 (NCT01402440 trial).

The COO seems to have an effect in predicting the response to salvage therapies as well: patients with GCB-DLBCL have a significant better outcome when high-dose therapy and autologous bone marrow transplant is preceded by the R-DHAP regimen (rituximab, dexamethasone, high-dose cytarabine, cisplatin) than by the R-ICE scheme (rituximab, ifosfamide, carboplatin and etoposide) [46].

Genetic aberrations have been reported associated with poor outcome in DLBCL patients. These lesions include BCL2 translocation (and protein expression) and TP53 inactivation (both apparently restricted to the GCB subtype), MYC translocations, gains at 3q, losses at 8p and 9p21 (CDKN2A) [14, 46–60]. A particularly poor prognosis seems associated with the concomitant involvement of MYC, BCL2 and

BCL6 or CCND1 (Cyclin D1) in the so called “double” and “triple-hit” lymphomas [49, 54, 60–66]. However, up to now, only the search by fluorescence *in situ* hybridization (FISH) for chromosomal translocations involving the oncogene MYC, present in 5–15% of DLBCL cases, seems worth of being incorporated in the diagnostic panel of newly diagnosed DLBCL patients, since it identifies patients who have a very poor outcome with the current treatment approaches, including high-dose therapies.

Trying to implement the results obtained with GEP studies in the diagnostic workflow, some investigators have developed methods using real-time polymerase chain reaction assays (PCR), which can be performed on formalin-fixed paraffin-embedded (FFPE) tissues [67, 68]. A very recent publication has suggested a score combining the IPI with the expression level of only two genes, one expressed by DLBCL cells (LMO2), and one expressed by cells of the immune microenvironment (TNFRSF9) [68].

Other recent studies have identified a series of alterations that may contribute to the lymphoma immune-escape in both DLBCL subtypes: affected genes are *B2M*, *CD58*, *TNFSF9*, *PDL1* and *PDL2* [21, 22, 69] but their prognostic relevance is still unknown.

2 Primary Mediastinal Large B-Cell Lymphoma

GEP studies have contributed to the definitely separation of the uncommon but not rare primary mediastinal large B-cell lymphoma (PMLBCL) from the other DLBCLs [70, 71]. PMLBCL indeed, is nowadays regarded as an distinct clinico-pathologic entity [1, 6, 7]. PMLBCL has a peculiar immunohistochemical profile [72] and unique histopathological characteristics and clinical features, partially overlapping with classical Hodgkin lymphoma (cHL) [73, 74]. As a whole, the outcome for PMLBCL patients appears better than DLBCL, possibly also as a result of their younger age and earlier stage at presentation [1, 6, 7].

GEP studies have shown that PMLBCL is different from DLBCL, and it is more similar to cHL [70, 71]. PMLBCL and cHL present the same unique profile of low expression levels of genes participating to the BCR signaling pathway, high levels of interleukin-13 receptor and its downstream effectors JAK2 and STAT1, as well as frequent activation of NF-κB pathway [70–72, 75–77]. PMLBCL and cHL also share common genetic aberrations [14, 78–81]. Twenty percent of PMLBCLs and up to one half of cHL cells have gains of chromosome 2p, which are associated with amplification of the REL locus, with increased NFκB activity and tumor cell resistance to apoptosis. Seventy-five percent of PMLBCLs and a quarter of cHLs have gains/amplifications of chromosome 9p24. An elegant recent publication has demonstrated that this lesion co-deregulates two co-operating oncogenes, *JAK2* and *JMJD2C* [77]. More recently, chromosomal rearrangements disrupting *CIITA* have been reported in 15% of cHL and in 38% of PMLBCL [78]. The rearrangements determine the downregulation of the major histocompatibility complex (MHC) class

II molecules and over-expression of ligands of the receptor molecule programmed cell death 1 (CD274/PDL1 and CD273/PDL2), and, in PMLBCL, seem to be associated with poorer outcome [78]. Other lesions which are frequent in PMLBCL are the inactivation of the NFKB negative regulator TNFAIP3 and of SOCS1, the latter participating to the activation of JAK2 [82, 83].

PMLBCL and nodular sclerosis cHL may represent related tumors on either ends of a continuum, whose interface may include tumors that have a transitional morphology and phenotype, combining clinical and pathologic features of both PMLBCL and cHL [84]. These intermediate forms of B-cell lymphoma, the so-called “mediastinal gray zone lymphomas” (MGZL), cannot be discriminated from neither PMLBCL nor cHL with nodular sclerosis and represent an important diagnostic issue due to the therapeutic implications of the final diagnosis which might lead to quite different therapeutic choices [1, 64, 85].

3 Follicular Lymphoma

Follicular lymphoma (FL) is the most common indolent subtype of NHL, and the second most common NHL [1, 6, 7]. FL is composed by a mixture of two cell populations, centrocytes (small to medium-sized cells) and centroblasts (large cells), which reflect the cell populations observed in normal GCs. The current WHO classification recognizes four grades of FL based on the proportion of large cells: grade 1, 2, 3A and 3B. The clinical management of FL grade 3B is that of patients with DLBCL. As a whole, FL is a usually incurable slow-growing disease characterized by its responsiveness to initial therapy with disease-free intervals alternating with progression/relapses. The clinical course of FL is highly variable: the median survival is approximately 10 years in historical series, but a significant improvement over time was observed in the last decade by several groups [86]. Most patients are today expected to live more than 15 years after diagnosis, but some show a rapidly fatal disease course often associated with disease transformation to a more aggressive DLBCL.

Follicular Lymphoma International Prognostic Index (FLIPI) and Follicular Lymphoma International Prognostic Index 2 (FLIPI2) containing clinical baseline parameters are used in the clinics as the best predictor of clinical outcome in patients with FL [87–90]. Due to the diverse clinical course of FL patients, treatment options range from a ‘watch and wait’ approach to rituximab alone to chemo-immunotherapy [6, 90].

FL cells always present somatically mutated *IGHV@* genes and the presence of on-going/intra-clonal mutations, in accordance with the origin from GC-cells [91–93]. Importantly, the mutation load on *IGHV@* can be very high, determining a relevant risk of false negativity when using PCR targeting the *IGHV@* [94].

Up to 90% of FL cases have the genomic hallmark of t(14;18)(q32;q21) translocation, which relocates *BCL2* on the *IGHV@* locus resulting in the overexpression and accumulation of BCL2 [95–97] (Table 15.1). FISH is the best technique able

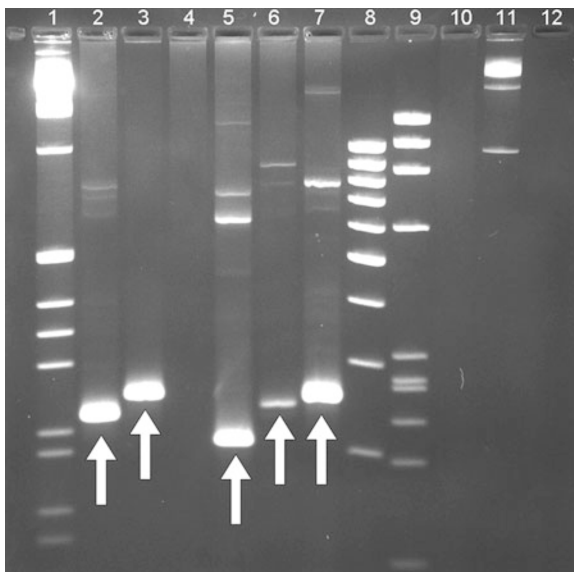

Fig. 15.4 Example of PCR assay detecting the presence of the t(14;18)(q32;q21) translocation in lymphoma DNA samples. The photo represents a 2% agarose gel, and the *white arrow* highlights the positive PCR-products. The lanes contain: (*1*) 1Kb DNA marker; (*2*) DNA of the t(14;18)-positive DoHH2 cell line; (*3*) DNA of the t(14;18)-positive SU-DHL-6 cell line; (*4*) DNA of a t(14;18)-negative follicular lymphoma; (*5*) DNA of the t(14;18)-negative Karpas-422 cell line; (*6*) DNA of a t(14;18)-positive follicular lymphoma; (*7*) positive control; (*8*) 100 bp DNA marker; (*9*) OX174/HaeIII DNA marker; (*10*) t(14;18)-negative sample DNA (negative control); (*11*) 1Kb DNA marker; (*12*) no DNA control (negative control)

to demonstrate virtually all translocated cases [98]. PCR approach using primers directed toward different breakpoint regions is also a feasible approach, especially useful for the detection of minimal residual disease in the context of clinical trials [99], although it misses part of the breakpoints [98] (Fig. 15.4). Approximately 10% of FL present without *BCL2* rearrangements [100–102], and these cases seem to present a few genetic and phenotypic differences possibly indicating an origin from a B-cell in a later GC stage. Due to the small number of cases reported, it is not clear whether t(14;18)-negative FL have a different outcome than the t(14;18)-positive [100].

The t(14;18) is also present at low frequency in the peripheral blood of healthy individuals and can be considered an early "driver" event of FL pathogenesis [103]. In patients with overt FL, nearly never t(14;18) is the only lesion, and various genomic gains and losses are usually also present. The most common unbalanced genomic lesions are gains on chromosome 7, 12, 18q (*BCL2*), and X, losses at 1p36 (*TNFRSF14*), 6q13-q26 (*TNFAIP3*), 9p21 (*CDKN2A*) and 17p (*TP53*), and copy neutral LOH (cnLOH) at 1p36, 6p21, 12q21-q24, and 16p13 [100, 104–108]. A recent paper has reported a new tumor suppressor gene, *EPHA7*, coding for the ephrin receptor A7, which is mapped at 6q16 and appears to be inactivated by

heterozygous losses and promoter methylation in up to 70% of FL [109]. Deep sequencing studies have shown the presence of somatic mutations in genes involved in transcription regulation and chromatin remodeling, and also affected in DLBCL (mainly GCB-subytpe), such as *MLL2*, *MEF2B*, *CREBBP*, *EP300* and *EZH2* [19, 21–23]. Although independent series will be necessary to confirm the real incidence, the first data indicate in *MLL2* and *CREBBP* the most commonly affected genes with frequencies of inactivation of 89% (31/35 cases) [21] and 33% (15/46) [23], respectively.

The presence of additional lesions can bear a prognostic significance [105, 107, 108, 110, 111]. *TNFRSF14*, *TP53* or *CDKN2A* inactivation, 16p cnLOH, 6q losses and 17q gains have been associated with poorer outcome; 1p cnLOH/loss, 6q loss, 16p cnLOH with higher risk of transformation to DLBCL.

Compared with the other grades of FL, grade 3B presents differences at both immunophenotypic and genetic level [102, 112–114]. In particular, FL3B present the t(14;18)(q32;q21) much less commonly and 3q27 rearrangements involving *BCL6* more frequently.

GEP studies have also been performed on FL [101, 115–120]. The largest study has very clearly highlighted the importance of the micro-environment in determining the outcome of FL patients. Dave et al. [115] has identified two signature, "immune-response 1" and "immune-response 2", both composed of transcripts expressed by the non-neoplastic infiltrating cells and not by FL cells. Expression of immune response-1 signature and immune response-2 signature was related to long survival and short survival of FL patients, respectively. The immune response-1 signature includes genes encoding well-known T-cell markers, T-cell signaling proteins and macrophages markers, such as CD7, CD8B1, IL-7 receptor, STAT4, LEF, ITK, ACTN1 and TNFSF13B (BLYS/BAFF). The immune response-2 signature includes genes expressed in monocytes and/or dendritic cells, such as SEPT10, LGMN, TLR5, C3AR1 and FCGR1A. These data suggest that the manipulation of the microenvironment might be a therapeutic approach (for examples, in the NCT00670358 and NCT01476787 trials). However, they have to be interpreted with caution, since the prognostic impact of a particular component of the microenvironment might change based on the given treatment [121–123].

4 Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) is characterized by the incurability with conventional chemotherapy also seen in indolent lymphomas and an aggressive clinical course typical of aggressive lymphomas [1, 6, 7]. There is no established standard of care [6, 7, 124]. The median survival in MCL is in the range of 3–5 years, but some patients with very aggressive disease, such as the blastoid variant, could face a survival of approximately 1 year. On the converse, other patients have a relatively indolent disease and some of them might expect more than 10 years of life expectancy.

MCL shows unmutated *IGHV@* genes in the majority of the cases, but one quarter of the patients present with mutated *IGHV@* genes. At least a subset of MCL might derive from marginal zone or peripheral blood memory B-cells which have undergone an extra-follicular T-independent antigen response or which bear antigen-independent mutated *IGHV@* [125], and not from pre-germinal center B-cells of the mantle zone, as previously believed. A highly restricted immunoglobulin gene repertoire with stereotyped *IGHV@* complementarity-determining regions 3 (CDR3s) has been very recently reported, strongly implying a role for antigen-driven selection for at least a subset of MCL cases [126]. Differently from what observed in chronic lymphocytic leukemia (CLL) [127, 128], the *IGHV@* mutational status is not a striking prognostic factor in MCL, although cases with mutated *IGHV@* seem to have a better outcome, especially in the presence of non-nodal leukemic disease [129–131].

The main genetic feature of MCL is the t(11;14)(q13;q32) chromosomal translocation with the deregulated ectopic expression of *CCND1,* coding for the cyclin D1, due to juxtaposition to *IGHV@* region [96, 97, 132–136] (Table 15.1; Fig. 15.5). The demonstration of the t(11;14) can have diagnostic relevance in the differential diagnosis of CD5+ small cell lymphomas. Fluorescence *in situ* hybridization (FISH) is the technique of choice to demonstrate the presence of the translocation. Immunohistochemistry success in determining cyclin D1 over-expression could be hampered by the quality of available material. PCR with primers directed to the breakpoint regions on 11q13 and 14q32 has a high false negative rate (40–60%), although, when positive, it represents an excellent marker for molecular follow-up studies. Besides chromosomal translocations, CCND1 gene is also target of mutations and deletions determining a higher expression of cyclin D1 [137]. Approximately, 10% of MCL lack the translocation, despite presenting a disorder phenotypically and clinically compatible with MCL [138–140]. The t(11;14) is present also in 15–20% of multiple myelomas, in which, differently from MCL, the 14q32 breakpoints more commonly target the switch and not the VDJ region [96].

An "indolent MCL" signature has been reported consisting in the lack or low levels of SOX11, a transcriptional factor usually highly specific in both t(11;14)-positive and t(11;14)-negative MCL [141], stable genomes and no inactivation of *TP53*, *CDKN2A* and *ATM* genes[140]. A GEP-based molecular predictor of survival based on the average expression of proliferation signature genes has been shown capable to stratify patients into groups with different prognosis [142], but GEP is still not feasible in the diagnostic practice. A PCR-based prognostic model evaluating only five genes (RAN, MYC, TNFRSF10B, POLE2, and SLC29A2) and working on both frozen and archival material, has been proposed [143], but not yet widely applied and validated.

Secondary genomic aberrations contribute to the MCL pathogenesis [139, 144–146] and some of them, such as gains of 3q and 18q (*BCL2*) and losses of 8p, 9p (*CDKN2A*) and 17p (*TP53*), would confer a worse outcome [139, 145, 147], but their detection is not part of the routine diagnostic practice. Also, recent whole exome sequencing has identified *NOTCH1* somatic mutations in 12% of MCL patients [148], similar to what observed in chronic lymphocytic

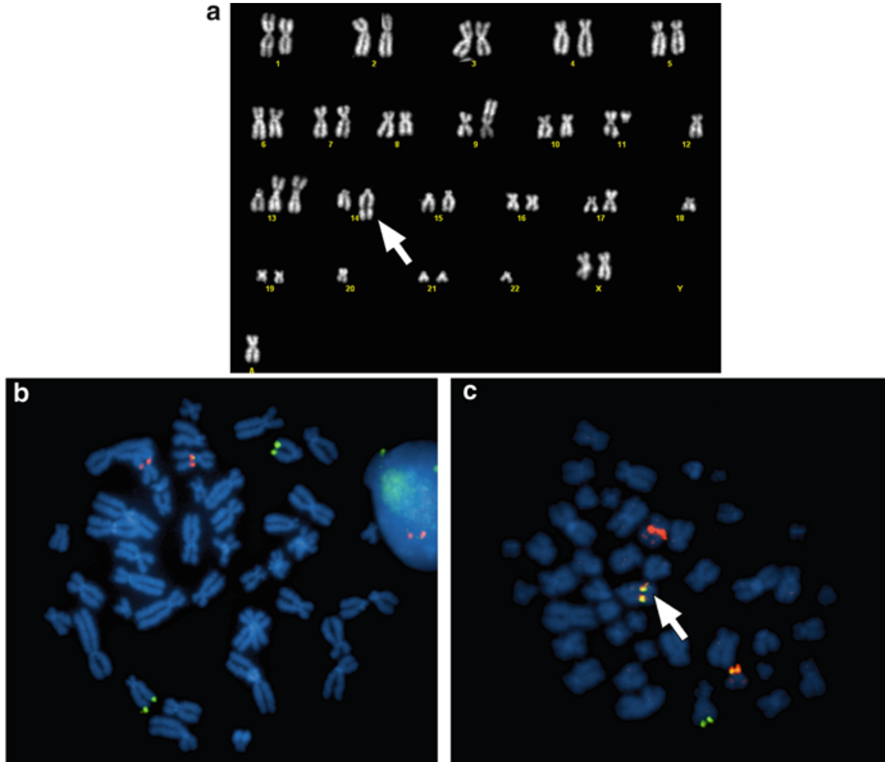

Fig. 15.5 Examples of t(11;14) chromosomal translocation in mantle cell lymphoma. The *white arrow* indicates the pathogenetic derivative chromosome. (**a**) Karyotype analysis of Granta-519 cell line. (**b**) Metaphases of a t(11;14)-negative case bearing two normal copies of the *IGHV@* locus (*in red*) and two normal copies of the CCND1 gene (*in green*), as detected by FISH. (**c**) Metaphases of a t(11;14)-positive case bearing two fusion signals (*in yellow*) highlighting the reciprocal translocation with juxtaposition of the CCND1 and *IGHV@* loci, and only one normal copy of the *IGHV@* locus (*in green*) and also of the CCND1 gene (*in red*)

leukemia (CLL) [149, 150]. Both MCL and CLL patients bearing *NOTCH1* mutations bear a poorer outcome than patients without *NOTCH1* mutations [148–150].

5 Extranodal Marginal Zone Lymphoma

The extranodal marginal zone lymphoma (MZL) of the mucosa-associated lymphoid tissue (MALT), currently named MALT lymphoma, is a peculiar, indolent lymphoma, which can occur in any extranodal anatomical sites (stomach, thyroid, salivary glands, lung, orbital adnexal, skin and others) [1, 6, 7]. It is highly

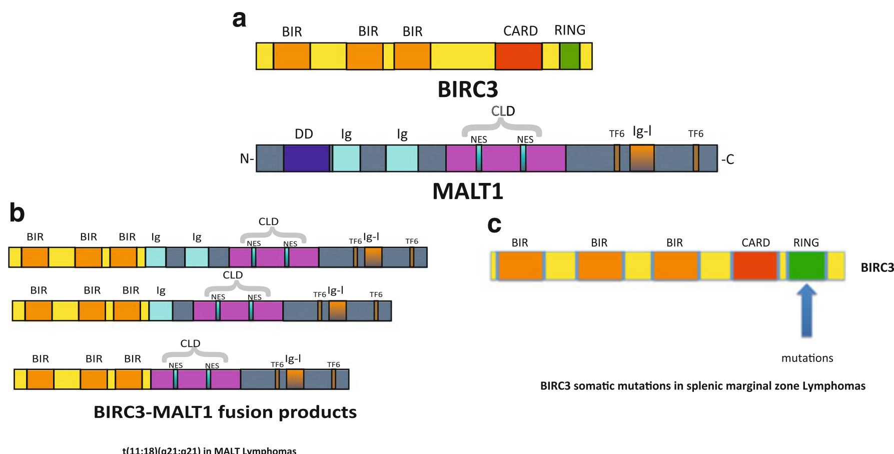

Fig. 15.6 Alterations of the *BIRC3* gene in marginal zone lymphomas. (**a**) Structure of normal *BIRC3* and *MALT1* genes; (**b**) Possible fusion BIRC3-MALT1 fusion transcripts observed in MALT lymphomas; (**c**) structure of the normal *BIRC3* gene with highlighted the region which is frequent target of somatic mutations in splenic MZL. *BIR* baculovirus IAP repeat, *CARD* Caspase recruitment domain, *RING* really interesting new gene, *DD* death domain, *Ig* immunoglobulin-like, *CLD* Caspase-like domain, *NES* nuclear export signal, *TF6* TRAF6 binding domain, *Ig-l* immunoglobulin-like domain, *N-* N-terminal, *-C* C-terminal

associated with chronic infections (such as *Helicobacter pylori* in the stomach) or autoimmune disorders (such as Hashimoto's thyroiditis or Sjögren's syndrome) which induce an acquired lymphoid tissue (absent in normal conditions) that could then give origin to the MALT lymphoma [151–154]. Virtually all MALT lymphomas present somatically mutated *IGHV@* with on-going mutations, suggestive of a continuous antigen-driven process [155–161].

5.1 MALT Lymphoma

Recurrent chromosomal translocations and unbalanced genomic aberrations have been identified in MALT lymphomas [162–174] (Table 15.1).

The most common translocation is the t(11;18)(q21;q21), fusing *BIRC3* (*cIAP2*) on 11q21 with *MALT1* on 18q21 [162, 174, 175] (Fig. 15.6). MALT lymphoma cases bearing the t(11;18) have a low probability of response to antibiotics, present with a more advanced disease and, if with a primary gastric localization, are usually *H. pylori* negative [176–181]. On the other hand, they seem to have a lower risk of transformation to DLBCL [182], and to bear less additional genomic aberrations [181, 183]. The translocation might also predict the resistance to chlorambucil or thalidomide as single agent [184], but apparently not to rituximab [185] or other therapeutic approaches [186–189].

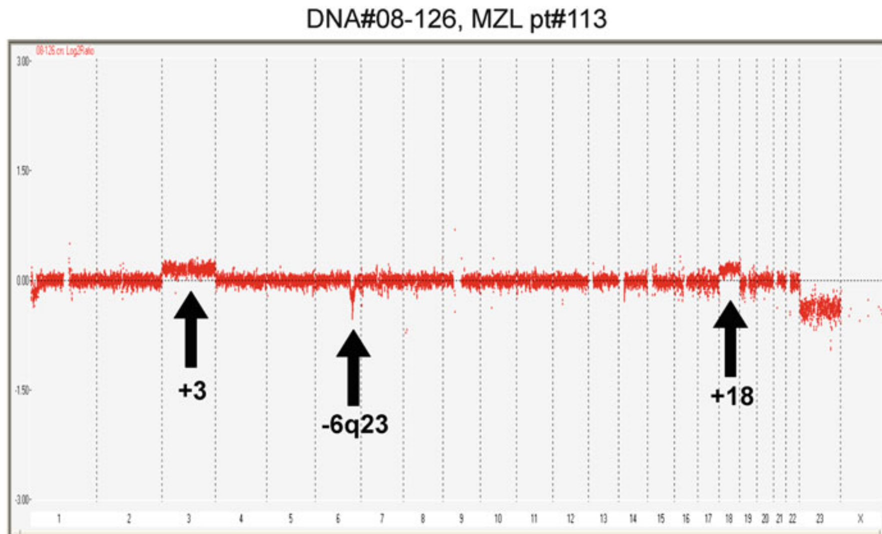

Fig. 15.7 Genomic profile of one case of MALT lymphoma bearing trisomies of chromosomes 3 and 18 and a loss at 6q23, the typical recurrent lesions of this lymphoma subtype. Profile obtained using the Affymetrix Genome-Wide Human 250k NspI SNP Array. *Red*, smoothed copy number values. X-axis, genomic mapping; Y-axis, log2 copy-number values

The t(14;18)(q32;q21) translocation is cytogenetically virtually identical to the one involving *BCL2* in FL or DLBCL, but in MALT lymphomas it brings *MALT1* under the control of the promoter region of the *IGHV@* genes with subsequent deregulation of *MALT1* expression [164].

The t(1;14)(p22;q32) translocation determines high level of *BCL10* expression due to its juxtaposition to the *IGHV@* promoter region [163]. MALT lymphomas carrying this translocation have a high BCL10 nuclear expression, observed also in t(11;18)-positive cases and in other patients [179, 180, 190, 191]. The presence of the translocation and the strong BCL10 nuclear staining are both associated with resistance to lymphoma eradication with antibiotics [180, 192, 193].

The t(3;14)(p13;q32) juxtaposes *FOXP1*, coding for a transcription factor, next to the enhancer region of the *IGHV@* genes [165, 172]. The translocation is not limited to MALT lymphomas, detected also in DLBCL. A high expression of FOXP1 has been associated with a poor outcome in both DLBCL and in MALT lymphomas, in which it could be associated with a higher risk of transformation to an aggressive lymphoma [194, 195].

Similarly to splenic MZL, MALT lymphomas present gains of the whole chromosomes 3 and 18 or of their long arms at a frequency higher than other B-cell tumors [167, 168, 170, 196]. Both gains at 3q and 18q have been associated with a poorer outcome and/or a more advanced disease [168, 197–199]. Also, a recurrent 6q23.3 deletion has been described, which, together with somatic mutations, inactivates *TNFAIP3 (A20)* [168, 170, 200–202]. Figure 15.7 shows a

genomic profile of a case bearing trisomies 3 and 18 and *TNFAIP3* gene loss. The high prevalence of gains affecting chromosomes 3 and 18 with the lack of other lesions such as deletions at 7q31 (common in splenic MZL), at 13q14.3 (common in CLL) or at 11q22 (common in CLL or MCL) can help in the differential diagnosis of MALT lymphomas from other indolent lymphomas.

Importantly, at least four of the recurrent lesions observed in MALT lymphomas (*TNFAIP3* inactivation, *BIRC3-MALT1*, *IGHV-BCL10*, *IGHV-MALT1*) determine the activation of the nuclear factor kappa B (NFKB) pathway, which can represent a therapeutic target [203]. The chromosomal translocations are mutually exclusive, and, differently from 3/3q and 18/18q gains and 6q23 losses, they present differences in their anatomical distribution [166, 170, 171].

Although the recurrent genomic lesions do affect the expression of genes mapped on them, deregulating pathways such apoptosis or ubiquitin proteasome pathways [168], not very much is known about the transcriptome of MALT lymphomas. One study showed an over-expression of NFKB target genes involving biological processes or molecular pathways such as immunoreceptors, chemokine receptor, and molecules involved in G-protein receptor signaling in MALT lymphomas with MALT1-translocations, while a strong inflammatory gene signature for cases without translocations [204]. *RARA*, the gene coding for the retinoic acid receptor alpha, has been suggested as possibly deregulated in primary pulmonary MALT lymphomas [205].

Recently, somatic mutations of *MYD88* gene, similar to those observed in ABC-DLBCL [20], have been reported in less than 10% of MALT lymphomas [20, 206–208]. It is worth of mentioning that MYD88 mutations can been seen in almost all cases of IgM-secreting lymphoplasmacytic lymphoma (Waldenstrom's macroglobulinemia) [208].

6 Splenic Marginal Zone Lymphoma

Splenic MZL is an indolent lymphoma [1, 6, 7, 209]. Differently from MALT lymphoma, but similarly to nodal MZL, splenic MZL can present with both mutated and unmutated *IGHV*, in accordance with the different normal B-cell populations resident within the marginal zone, that comprise both naïve and post-germinal centre B cells [210–217]. Differently from what observed in chronic lymphocytic leukemia, the *IGHV@* mutational status is not a strong prognostic factor, although cases with unmutated *IGHV@* seem to behave worse and to be associated with poorer biologic and clinical features [168, 214, 215, 218].

Splenic MZL is characterized by the presence of deletions affecting 7q31-q32 in up to half of the cases [168, 215, 219, 220] (Fig. 15.8), possibly affecting IRF5 [221], but additional data are still required. Other lesions are losses at 8p and at 17p (*TP53*), which, when concomitant, would determine a poor outcome [168]. Recent papers have undiscovered important pathogenetic lesions underlying splenic MZL [222–224]. First, recurrent somatic mutations of genes involved in the NFKB

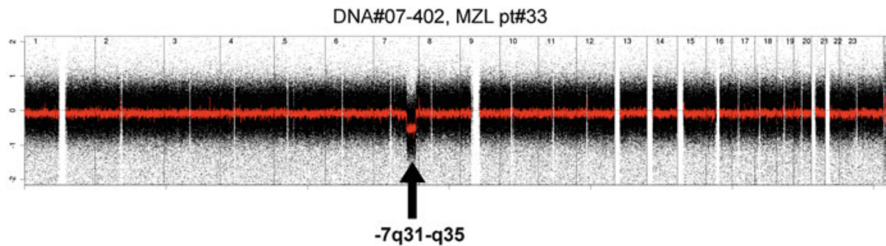

Fig. 15.8 Genomic profile of one case of MALT lymphoma bearing a loss affecting the long arm of chromosome 7, the typical recurrent lesion of this lymphoma subtype. Profile obtained using the Affymetrix Genome-Wide Human SNP Array Version 6.0. *Black*, raw copy number values; *red*, smoothed copy number values. X-axis, genomic mapping; Y-axis, log2 copy-number values

pathway have been reported in 36% (36/101) of splenic MZL cases; the mutations affect both canonical (TNFAIP3 and IKBKB) and non-canonical (BIRC3, TRAF3, MAP3K14) NFKB pathways [222]. Interestingly, the observed mutations in *BIRC3* disrupt the same RING domain that is removed by the t(11;18) in MALT lymphomas (Fig. 15.6), in which BIRC3 mutations have not been observed [222]. More recently, two concomitant papers have reported data derived by sequencing the whole coding exome of splenic MZL, confirming the relevance of the deregulation of the NFKB pathway, but, importantly, also identifying recurrent mutations of genes coding for members of the NOTCH pathway [223, 224]. In particular, the *NOTCH2* gene is mutated in 20–25% of the cases, with possible future therapeutic implications [223, 224]. Further studies are needed to understand the prognostic significance of the presence of NOTCH2 gene mutations.

GEP studies have reported an up-regulation of genes coding for proteins involved in apoptosis, cell cycle, BCR and TCR, TNF and NF-κB signaling [218], intracellular signaling or transcription [225], apoptosis or ubiquitin proteasome pathways [168].

7 Burkitt Lymphoma

Burkitt lymphoma (BL) was first described as a clinical entity in equatorial Africa children by Irish surgeon Denis Burkitt in 1958 [1, 6, 7, 226–228]. BL is a highly aggressive B-cell lymphoma that accounts for 30–50% of pediatric lymphomas, but only 1–2% of adult cases. According to WHO classification in 2008, BL is classified into three clinical variants: endemic BL, sporadic BL and immunodeficiency-associated BL [1]. The endemic BL is the most common form in African children, and almost all of the endemic BL cases are related with Epstein-Barr virus (EBV) infection. In Western Countries, sporadic BL is the most common variant, and only 20–30% of the cases are EBV-positive. Immunodeficiency-associated BL mainly occurs in patients whose immune system is severely compromised, such

as patients infected with human immunodeficiency virus (HIV), patients after organ transplantation or patients with congenital immunodeficiency.

Similarly to FL, BL cells have somatically mutated *IGHV@* with intra-clonal heterogeneity, consistently with an origin from GC B-cells [229–231].

BL is characterized by the t(8;14)(q24;q32) chromosomal translocation, which juxtaposes the MYC oncogene into the immunoglobulin heavy-chain locus [1, 6, 7, 96, 232, 233]. Rare variants include the t(2;8)(p12;q24) involving immunoglobulin kappa locus and t(8;22)(q24;q11) involving immunoglobulin lambda locus.

In adults, the differential diagnosis between BL and other aggressive lymphomas mimicking the BL has important clinical consequences, due to the specific regimes used for BL [1, 6, 7, 234, 235]. GEP studies have shown that BL presents a peculiar gene expression profile, different from other aggressive lymphomas [236–238]. GEP reflects the origin from GC B-cells, but with a deregulation of genes involved in cell proliferation, immune response, and signal transduction.

Especially when compared with DLBCL, BL presents a relatively stable genome [237, 239]. The most frequent lesions additional to the t(14;18) are extracopies of 1q, trisomy 7/7q, trisomy 12/+12q and 13q (*MIR17HG*) and losses at 6q and 17p. The latter regions contains TP53 which is also frequently mutated [239–244].

Very recently, highly recurrent mutations have been reported in the genes coding for TCF3 (11%), its negative regulator ID3 (58%), and for CCND3 (38%) [245]. The first two mutations would cause the activation of the PI3K pathway, in part by augmenting tonic B-cell receptor signalling, while the third lesion would determine a stabilization of CCND3 protein that contributing to the cell cycle deregulation of BL [245]. While mutations in *TCF3* and *ID3* genes are observed in both sporadic and endemic BL, those affecting *CCND3* appear rare in the endemic cases of BL [245]. All of them are more common in BL than in other lymphoma subtypes [245].

8 Peripheral T-Cell Lymphoma, Not Otherwise Specified

Peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) is an aggressive and predominantly nodal lymphoma [6, 7, 246–248].

Although representing the majority of T-NHL, the lesions underlying PTCL, NOS are poorly recognized [249–251]. PTCL, NOS frequently shows a complex karyotype [96, 252–261]. The most frequent gains occur at 1q32-qter, 2p15-p16 (*REL*), 7q21-ter (*CDK6*), 8q24, 9q33-qter, and 17cen-q21. Recurrent losses mainly affect 6q21, 9p21 (*CDK2NA*), 10q23-q24, 14q12-q21, 16q11-q21, 17p13 (*TP53*).

Differently from B-NHL, chromosomal translocations deregulating oncogenes by juxtaposing them to the TCR genes are present in only up to 1% [96, 261–263]. A t(5;9)(q33;q22) chromosomal translocation determining the fusion of the tyrosine kinase domain of *SYK* to the N-terminal pleckstrin homology domain and proline-rich region of *ITK* [264–267] has been described and its real incidence has still to be assessed. The over-expression of *SYK*, important in proliferation and

pro-survival signaling, is present in the majority of PTCL, even without *SYK/ITK* translocations [268], providing a rationale for evaluating SYK inhibitors, such fostamatinib disodium.

GEP studies have indicated a general heterogeneity alongside a deregulation of genes participating to T-cell activation, apoptosis regulation, and to NFKB and IFN/JAK/STAT pathways [249, 251, 269–274]. PDGFRA has been highlighted as possible therapeutic target [273]. A subgroup of PTCL-NOS with features of cytotoxic T-cells and with an apparently poorer outcome has been maybe identified [274].

9 Anaplastic Large Cell Lymphoma, ALK-Positive

Anaplastic large cell lymphoma (ALCL), ALK-positive (ALK+ALCL) is characterized by chromosomal translocations involving the *ALK* gene on chromosome 2, coding for a tyrosine kinase [6, 275–277] (Table 15.1). The most common translocation is the t(2;5)(p23;q35) generating the nucleophosmin (NPM)-ALK fusion protein with transforming properties [276, 278, 279]. NPM-ALK expression leads to the activation of several downstream signal transduction events, providing positive survival and proliferation signals. Alternative translocations with *ALK* fused to a variety of other partners are found in about 20% of cases, and the type of translocation does not affect the outcome [276, 280]. ALK represents a therapeutic target under evaluation with both kinase inhibitors, such as crizotinib (PF-02341066; NCT00939770 and NCT01121588 trials), or LDK378 (NCT01283516) or AP26113 (NCT01449461) but also for vaccine interventions [281–284].

ALK+ ALCL also carries frequent secondary chromosomal imbalances including losses of at 4q13-q28, 6q13-q22, 11q14-q23 and 13q, and gains on chromosomes 7 and 17 [255, 285–288]. Interestingly, unique among lymphomas, ALK+ ALCL is virtually devoid of losses affecting *TP53* gene.

10 Anaplastic Large Cell Lymphoma, ALK-Negative

In the WHO classification, ALK-negative ALCL (ALK-ALCL) is a provisional entity, and is defined as an CD30+ T-cell lymphoma, morphologically overlapping with ALK+ALCL, but that lacks ALK expression or *ALK* translocations. Importantly, ALK-ALCL has a different clinical presentation involving predominantly adults, with advanced age, and a more aggressive clinical course [277, 289].

Genetic lesions observed in ALK-ALCL differs from what observed in ALK+ALCL, but do not fully overlap with the aberrations reported PTCL, NOS or cutaneous ALCL [255, 286, 288, 290, 291] (Table 15.1). The most frequent lesions are gains of 1q41-qter, 5q, 6p, 7p 8q, 12q and 17q12-q21, and losses at 6q21-q22

and 13q21-q22. The 6q21 gene seems to be the already above-mentioned *PRDM1*, coding the BLIMP1 transcription factor [288], also shown to be deregulated in other B- and T-cell lymphomas [34, 292, 293]. Importantly, these chromosomal imbalances differ from those identified in ALK+ALCL, supporting the concept that they are different biological entities. The abnormalities of ALK-ALCL do not fully overlap with whose reported for other T-cell neoplasms, but can be shared with primary cutaneous ALCL.

Two translocations have been recently reported in ALK-ALCL, a t(6;7)(p25.3; q32.3), involving the *DUSP22* gene and the FRA7H fragile site [294], and one involving *IRF4*, mapped less than 50 Kb telomeric to the *DUSP22* [295, 296]. The biologic and clinical meaning of these lesions are still to be elucidated.

GEP studies have mainly tried to define markers able to discriminate between ALK-negative and ALK-positive ALCL and between ALCL and PTCL-NOS [274, 297–300]. ALK+ and ALK- ALCL appear relatively in terms of gene expression, although differentially expressed genes can be identified. Both ALK+ and ALK-ALCL present a gene expression signature suggesting an activation of STAT3 signaling, possibly representing a rational therapeutic target.

A three-gene model has been recently published to improve the differential diagnosis of ALK-negative ALCL from PTCL-NOS [300]. Based on the analysis by real-time PCR of the expression level of TNFRSF8, BATF3, TMOD1 RNA levels, the method has been optimized for both frozen and formalin-fixed paraffin embedded biopsies [300].

11 Hodgkin Lymphoma

Hodgkin lymphoma (HL) is one of the most curable types of malignant diseases, being curable in 90–95% of patients with limited disease and up to 70% of those with advanced disease [6, 7, 301, 302]. HL is divided in two main entities:nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), representing 5% of all HL, and classical Hodgkin lymphoma (cHL), accounting for over 95% of the cases [1, 6, 7, 123, 303]. The neoplastic cells, which are usually only 0.1–10% of the cellular population, are the Hodgkin and Reed-Sternberg (HRS) cells in cHL and the so-called LP (lymphocyte predominant) cells in NLPHL. Both cell types are derived from GC B-cells: LP cells from antigen-selected GC B-cells, and HRS cells from GC B-cells bearing unfavorable *IGHV@* mutations which have escaped apoptosis [303]. Importantly, the GEP of both cell types is very similar, characterized by a strong NFKB signature, and is reminiscent of B-cells at the transition from GC to memory B-cells, thus, more similar to non-GCB DLBCL than to FL [303, 304].

A series of genetic, viral and/or microenvironment-mediated stimuli largely contribute the main biologic features of HD. Despite being derived from B-cells, most B-cell lineage markers are down-regulated [303, 305–307]. There is an activation of NFKB, JAK/STAT and NOTCH1 signaling, as well of tyrosine kinase down-stream cascade [77, 79, 123, 303, 308–319]. Up to 40% of HRS cells are EBV-positive,

and the virus is recognized to play an important role in the pathogenesis of the disease, for example, by mimicking both CD40 and the BCR via its own LMP1 and LMP2, respectively, and rescuing signal to the cells which would otherwise undergo apoptosis due to the acquisition of the unfavorable *IGHV@* mutations [303, 320]. The main genetic lesions identified so far target genes coding for members of the NFKB pathway: inactivation of *TNFAIP3* gene (30–40%) [79], *REL* amplification (30% of the cases) [310, 311], and inactivation *of TRAF3* [321], *NFKBIA* [308, 317], *NFKBIE* (*IKBE*) [312] and *CYLD* [317] and gains affecting *MAP3K14* [321]. Interestingly, TNFAIP3 inactivation appears highly enriched in EBV-negative cHL cases, indicating the two events, TNFAIP3 loss or viral infection, might play a similar role in the HD pathogenesis. Lesions such as SOCS1 inactivation and JAK2 amplification contribute to the JAK/STAT pathway constitutive activation. Also, the cross-talk between HD cells and the normal cells present in the tumor microenvironment contribute with the genetic or EBV effects. The relevance of the microenvironment has been highlighted by a GEP study on 130 cHL cases, in which a gene expression signature of tumor-associated macrophages was significantly associated with treatment failure [322]. Very recently two molecular subgroups of cHL have been identified, with one of them apparently driven by high expression of MYC, IRF4 and NOTCH1 [319].

As discussed above regarding PMBCL, rearrangements disrupting CIITA, causing down-regulation of MHCII and up-regulation of CD273 and CD274, in approximately 15% of cHL [78].

12 Conclusions

In conclusion, data obtained in these last years, also thanks to the application of technologies able to study the genome in an unbiased way, have allowed important steps forward in clarifying the molecular mechanisms and key genetic elements causing biologic behaviors and clinical features of malignant lymphomas. Further genetic and functional studies will help to obtain the accurate diagnosis, to estimate the outcome, and, of uttermost importance, to define the appropriate individualized therapeutic plan for each individual lymphoma patient.

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Chapter 16
The Genomics of Multiple Myeloma and Its Relevance in the Molecular Classification and Risk Stratification of the Disease

Antonino Neri and Luca Agnelli

Abstract Multiple myeloma, an incurable disease characterized by uncontrolled proliferation of Ig-secreting plasma cells, is the second most frequent hematologic malignancies. By virtue of its clinical, biological, and molecular heterogeneity, it represents a distinctive challenge for the application of new high-throughput technologies, with the aims of a better comprehension of the molecular basis of the disease, a fine stratification and early identification of high-risk patients, and to gain insights towards targeted therapy. Particularly, over the last decade, global gene and microRNA expression, and genome-wide DNA profilings have been widely used to investigate the genomic alterations underlying the bio-clinical heterogeneity in multiple myeloma. Each approach led to promising results, either per se or when the data have been analyzed in an integrated fashion. Herein, we describe some of the most referenced or peculiar "–omic" approaches that had significantly improved the knowledge of multiple myeloma disease.

Multiple myeloma (MM) is an incurable disease characterized by heterogeneous molecular features, presentation and outcome, and accounts for up to 10% of haematological malignancies, with an incidence in Western countries of about 3–5 per 100,000. The average age of onset is 70 years [1–3]. MM affects antibody-secreting bone marrow (BM) plasma cells (PCs) and shows a wide clinical presentation ranging from the presumed pre-malignant condition of monoclonal gammopathy of undetermined significance (MGUS) to smoldering MM, truly overt and symptomatic MM, and extra-medullary myeloma or plasma cell leukaemia (PCL). To date, MM diagnosis is based on the criteria established by the International

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Myeloma Working Group in 2003 [4], subsequently updated in 2009 [5]. MM is defined as symptomatic upon the simultaneous occurrence of clonal plasma cells >10% in bone marrow biopsy, monoclonal serum (or urinary) paraprotein and related organ/tissue impairment. Patients are staged according to Durie and Salmon criteria [6] or, now widely increasingly and more effectively, to the International Staging System (ISS) [7] that consider serum β2-microglobulin and albumin levels. Indeed, ISS could be considered a prognostic index rather than a true staging system, as it is a valid measure of risk of progression and short outcome for patients with MM presentation, and is not strictly a measure of tumor burden or extent unlike staging systems used in other cancers.

MM is characterized by a remarkable genomic instability that encompasses ploidy and structural rearrangements [8]. Approximately half of MM tumors are hyperdiploid with non-random trisomies of odd chromosomes 3, 5, 7, 9, 11, 15, 19 and 21, together with a low prevalence of chromosomal translocations involving the immunoglobulin heavy chain locus (IGH) on chromosome 14q32.4. The remaining tumors are referred as non-hyperdiploid and are frequently associated with the constitutive activation of *CCND1* (11q13), *CCND3* (6p21), *MAF* (16q23), *MAFB* (20q11), or *FGFR3/MMSET* (4p16.3) genes as a result of *IGH@* translocations. The mechanisms underlying this dichotomic pattern have not been elucidated but hyperdiploid patients have a generally better prognosis, whereas the t(4;14) and the t(14;16) are associated with a poor prognosis. Other genetic abnormalities (*Ras* mutations, inactivation of *p53* and/or del(17)(p13), *Myc* deregulation) characterize a fraction of MM cases, and are specifically associated with advanced stages of the disease [8]. Therefore, the understanding of the molecular bases of the disease represents an important step to dissect the clinical heterogeneity of the disease and the variety of clinical presentation.

Noteworthy, the treatment of MM has so far been independent of any of these molecular or genetic features, and is related to other factors (e.g. the age of onset, being considered younger patients those with MM presentation before 65 years). In fact, although the conventional long-lasting anti-myeloma therapy (alkylators and corticosteroids, with a median post-treatment overall survival [OS] of 3–4 years), first implemented by high-dose therapy followed by autologous stem cell transplantation (ASCT, median OS 5–7 years) and then, over the last few years, by the new-generation treatments (the immuno-modulatory drugs thalidomide and lenalidomide, and the proteasome inhibitor bortezomib) [9], there is still a lack of effective therapies targeting the deregulated biological/molecular pathways specifically associated with subsets of the disease. Only the recent updates in myeloma treatment guidelines have partially introduced risk stratification and molecular features as factors for choosing therapy. For instance, the Mayo Clinic has developed a risk-stratification model termed Mayo Stratification for Myeloma and Risk-adapted Therapy (mSMART), which dissects patients into high-risk (bortezomib-based treatments) and standard-risk categories (lenalidomide and dexamethasone for patients ineligible for ASCT). Patients with deletion of chromosome 13 or hypodiploidy by conventional cytogenetics, t(4;14), t(14;16) or 17p- by molecular genetic studies, or with a high plasma cell labeling index (3% or more) are considered as high-risk myeloma [10]. The unfavorable prognostic

value of t(4;14) and deletion of 17p is in line with other studies that introduced serum β2-microglobulin as discriminant and independent variable in association with genomic alterations [11].

Thus, in this context, MM poses a unique challenge for genotyping and for gene and microRNA expression profiling by virtue of its inherent heterogeneity, with the aim of a fine stratification and early identification of which patients are at risk of progression or relapse, or ultimately to provide the most effective therapeutic regimen to individual patients. Nevertheless, the complexity of the disease represents a serious obstacle to reaching these aims, which is further issued by intrinsic limitations and variability of high-throughput technologies.

Over the last few years, the high-throughput microarray technologies, particularly global gene expression (GEP) and genome-wide DNA profiling (GWP), have been widely used to investigate the genomic instability underlying the bio-clinical heterogeneity of the disease. Such approach led to promising results, either *per se* or when analyzed in an integrated fashion. Herein, we therefore describe some of the most referenced or peculiar approaches applied to significantly improve MM stratification.

1 The Definition of High-Risk Transcriptional Signatures Based on Gene Expression Profiling Data

So far, GEP has been undoubtedly the most largely used microarray application since early 1990s. The study of MM transcriptional profiles has taken advantage of the high incidence among hematologic malignancies, which granted the availability of large datasets, and of the facility to obtain highly purified and enriched tumor specimens for microarray analysis purpose. Preliminary investigations of CD138-enriched plasma cells of healthy subjects, MGUS, MM an PCL cases described either peculiar tumor-associated profiles, which are able to discriminate between normal and tumor phenotypes, or fingerprints characterizing MM molecular subtypes [12–18].

Later on, several investigations were aimed at associating peculiar transcriptional profiles with clinical outcome, in order to provide further molecular elements to define high-risk tumors. Although the efforts in prognostic stratification using microarray data have been basically aimed at the definition of reduced and powerful gene expression signatures, the approaches used display substantial differences.

Hideshima et al. in 2004 [19], proposed a model for the molecular pathogenesis of multiple myeloma, known as TC (Translocation/Cyclin) classification. Their model stemmed from the notion that the deregulation of at least one of the *Cyclin D* genes (*CCND1*, *CCND2*, *CCND3*) is a unifying oncogenic event in MM. This aspect prompted from the evidences that (i) *CCND1* is not expressed in normal lymphocytes; (ii) t(11;14) or t(6;14) translocations that dysregulate respectively *CCND1* and *CCND3* occurred in 20% of MM tumors; (iii) *CCND1* is expressed in nearly 40% of tumors lacking a t(11;14) translocation; (iv) *CCND2* is

expressed in most if not all the remaining tumors. These observations led to the definition of five groups, namely corresponding to: TC1, expressing high levels of either cyclin D1 or cyclin D3 as a consequence of *IGH@* translocation; TC2, ectopically expressing low to moderate levels of *CCND1* but lacking t(11;14) translocation; TC3, which collects tumors that do not fall into any of the other groups, mostly expressing *CCND2*; TC4, expressing high levels of *CCND2* and harboring t(4;14) translocation; and TC5, expressing the highest levels of *CCND2* as a direct target deregulated by *MAF* or *MAFB* transcription factors (ectopically expressed as a consequence of t(14;16) or t(14;20) translocations, respectively). The Authors underlined that the TC groups are correlated with different outcome, based on previous evidences indicating the discriminating prognostic role of the *IGH@* translocations: particularly, t(4;14) cases, namely TC4, were reported with substantially shortened survival either with standard or high-dose therapy (median OS, 26 and 33 months, respectively); t(14;16) and t(14;20) patients (TC5) showed an even worse prognosis (median OS, 16 months under conventional therapy); whereas t(11;14) patients (TC1) were associated with a slightly better OS under conventional therapy (50 months) and with a better survival under high-dose therapy (predicted 88% OS at 80 months) [19–21]. The perspective presented in the Review by Hideshima et al. has been subsequently investigated by our group, which analyzed a subset of 50 MM samples on Affymetrix GeneChip® HG-U133A arrays [22]. In our classification analysis, we identified 112 probe sets, specific for 89 genes that distinguished TC1, TC2, TC4 and TC5 groups. TC1, TC4 and TC5 groups are easily detectable by microarray analysis: the *IGH@* translocations cause normally silent genes to be juxtaposed with powerful enhancer elements. Our analysis identified a significant fraction of transcripts that are putatively (if not yet demonstrated) modulated as a consequence of the marked deregulation of *CCND1*, *CCND3*, *MMSET* or *MAF/MAFB*. In addition, our results indicated that TC2 tumors represent a well distinct entity with peculiar overexpression of the genes involved in the translational machinery. Conversely, TC3 samples showed heterogeneous phenotypes and we could not identify a peculiar transcriptional signature characterizing the group. These findings were validated in two large publicly available datasets, both profiled at the University of Arkansas Medical Science (UAMS), Myeloma Institute for Research and Therapy, and demonstrated the robustness of the identified signature, neither affected by cohort-specific nor lab-dependent biases (prediction accuracy of 85.71% and 90.27%) [22]. It is worth underlying that a meta-analysis, where possible, is desirable if not mandatory in order to validate the results obtained in proprietary dataset, especially given the high number of expected false positives when such large-scale data were analyzed. Specifically, as regards this aspect, it is worth mention that a commonly accepted recommendation for tumor diagnostic/prognostic biomarker studies indicates the use of independent cohorts to validate the obtained results [23].

The TC classification has been revised 1 year later by Bergsagel et al. who defined up to eight groups associated with discriminant Cyclin D expression and/or *IGH@* translocation events, undoubtedly supported by a larger number of samples in their dataset. Particularly, the original five TC groups were dissected

to derive as follows: the "6p21", the "11q13", the "4p16", and the "maf" groups, the former three exactly matching the occurrence of *IGH@* translocations on the locus described by the name itself, and the latter encompassing patients t(14;16) and t(14;20) that deregulated *MAF*-family transcription factors; the "D1" and the "D2" groups, overexpressing *CCND1* and *CCND2* respectively; the "D1 + D2" group, representing 8% of their samples, that showed increased levels of both genes; and the "none" group, including about 2% of tumors, that neither expressed increased levels of any of the three Cyclin genes compared with normal bone marrow PCs nor harbored any primary *IGH@* translocation. The eight groups differed by the expression of 576 genes, the large part of which characterized 4p16, D1 and, above all, maf groups. As observed in our previous report [22], the D1 group, actually corresponding to TC2, represents a well distinct transcriptional entity. Furthermore, the Authors take advantage of microarray data to define an expression-based proliferation index (PI), calculated using the median value of 12 genes associated with proliferation (*TYMS*, *TK1*, *CCNB1*, *MKI67*, *KIAA101*, *KIAA0186*, *CKS1B*, *TOP2A*, *UBE2C*, *ZWINT*, *TRIP1*3, *KIF11*). They demonstrated that PI showed a good correlation (r = 0.73) with Plasma Cell Labeling Index (PCLI) in an independent dataset, and is capable to discriminate highly-proliferating plasmablasts or human myeloma cell lines (HMCLs) from normal plasma cells or the majority of MM tumors. However, in MM tumors, the PI actually did not correlate with Cyclin D expression.

Zahn et al. analyzed the expression profiles of purified PCs from 414 samples included in UARK 98–026 and UARK 03–033 clinical trials, treated with Total Therapy 2 (TT2) and Total therapy 3 (TT3) regimen, respectively, and incorporating tandem autologous stem cells transplantation after high-dose therapy [24, 25]. Largely consistent with the TC classification model, they proposed that MM could be stratified into at least seven groups showing peculiar transcriptional signatures and associated with significantly different outcome [26]. The Authors firstly defined the seven classes by means of a two-dimensional unsupervised hierarchical clustering (HC) of the 1,559 most variable genes in 256 newly diagnosed MM cases included in TT2 trial. Then, they applied a classification model (Prediction Analysis of Microarray) [27] to the training set TT2 and correctly classified almost all of the samples (98%), based on the classes previously assigned by HC. The Authors listed 700 classifying genes, namely the top-ranked 50 genes both up- and down-regulated in each of the seven groups of the training set; they also verified that a similar group distribution existed in the TT3 test set based on the expression of the 700 genes. Four classes (named by the Authors as "CD-1" and "CD-2", "MS", and "MF") exactly correspond to TC1, TC4 and TC5 group, and may represent up to 40% of MM. The "HY" is characterized by moderate expression of *CCND1* and is associated with hyperdiploid karyotype in the large fraction of cases (90%), although hyperdiploidy patients were not restricted to this group. In HY group is reported the overexpression of *DKK1*, which was demonstrated by the same Authors to be associated with bone disease in primary MM tumors [14], and the overexpression of other transcripts, such as *TRAIL* or *FRZB*, characterizing hyperdiploid signature. Due to these characteristics, it is

conceivable that HY group encompasses the large majority of TC2 cases. The "PR" group is characterized by the overexpression of proliferation-associated and cancer–testis antigen genes. This class might share molecular features with the other classes, e.g. the occurrence of *IGH@* translocation, although these will be in all likelihood masked by the expression fingerprint of proliferation genes. Finally, "LB" patients are characterized by a low incidence of magnetic resonance imaging (MRI)-defined bone lesions, and *CCND2* overexpression. The seven subgroups show different outcome: particularly, the LB group had 84% of 3-year favorable probabilities of event-free survival (EFS); the HY group had 72%; and the CD-1 and CD-2, 82% and 86%, respectively. These four groups are those defined by the Authors as "low-risk". Conversely, "high-risk" groups were PR, MS, and MF, being the 3-year EFS probabilities 44%, 39%, and 50%, respectively. Similarly, as regards OS, the 3-year probabilities were 55% for PR, 69% for MS, 71% for MF, 81% for CD1, 84% for HY, 87% for LB, and 88% in CD2.

The approach used to define the seven groups was undoubtedly helpful to elucidate both their transcriptional *milieu* and the specific outcome associated with each phenotype; however, a 700-gene classifier is impractical for general prognostic purposes. To this aim, the UAMS group investigated the expression profile of myeloma cells in 532 newly diagnosed MM patients, included in the above-mentioned UARK 98–026 and UARK 03–033 clinical trials [28]. The PCs were profiled on Affymetrix GeneChip® HG-U133Plus arrays. Shaughnessy et al. applied a test for univariate association with OS to each of the probe sets represented on the array: specifically, they performed a log-rank test on samples divided according to expression quartiles in order to identify under- and overexpressed prognostic genes, respectively, and then corrected for false discovery rate. This procedure yielded 19 underexpressed and 51 overexpressed probe sets in their training set ($n = 351$), namely a 70-gene signature (30% of which mapped to 1q chromosome), able to predict prognosis (both EFS and OS) in the training as well as in the test set ($n = 181$). Specifically, to estimate the proportion of high-risk tumors, the Authors applied an unsupervised K-means clustering to the $\log_2$ ratio of mean expression of up- versus down-regulated genes, which lead to the threshold set at 13.4%. The 70-gene model is an independent predictor of outcome endpoints in multivariate analysis that included the ISS and high-risk translocations. The Authors additionally showed that the expression profile of the 70 genes in low-risk myeloma samples resembles that of MGUS patients and normal donors, whereas HMCLs had a pattern similar to that of high-risk MMs. Moreover, Shaughnessy et al. demonstrated that, in the prediction of high-risk MMs, the 70-gene model could be reduced to a 17-gene model without suffering the decrease of the number of genes (Table 16.1). This simplified signature was reached applying a multiple linear discriminant analysis model, which allowed selecting a minimal subset of the 70 genes capable of differentiating high-risk and low-risk MM. The 70-gene model only partially fitted the previous seven group model. In fact, despite the high overlap between high-risk signature and PR group, many other high-risk patients fall into different groups, mostly but non-exclusively MS and MF, thus suggesting that other factors than proliferation or adverse translocations are likely to contribute

Table 16.1 Gene-risk models and cohorts used to derive and/or validate them

Gene-risk model	No. genes	Genes	Cohorts	References
UAMS-70	70	*FABP5, PDHA1, TRIP13, AIM2, SELI, SLCI19A1, LARS2, OPN3, ASPM, CCT2, UBE2I, STK6, FLJ13052, LAS1L, BIRC5, RFC4, CKS1B, CKAP1, MGC57827, DKFZp779O175, PFN1, ILF3, IFI16, TBRG4, PAPD1, EIF2C2, MGC4308, ENO1, DSG2, C6orf173, EXOSC4, TAGLN2, RUVBL1, ALDOA, CPSF3, CHMR3, MGC15606, LGALS1, RAD18, SNX5, PSMD4, RAN, KIF14, CBX3, TMPO, DKFZP586L0724, WEE1, ROBO1, TCOF1, YWHAZ, MPHOSPH1, GNG10, TAF13*[a], *PNPLA4, KIAA1754, AHCYL1, MCLC, EVI5, AD-020, PARG1, CTBS, UBE2R2, FUCA1, RFP2, FLJ20489, LTBP1, TRIM33, NA (3)*	GSE2658	[28]
UAMS-17	17	*KIF14, SLC19A1, CKS1B, YWHAZ, MPHOSPH1, TMPO, NADK, LARS2, TBRG4, AIM2, CHMR3*[a], *ASPM, AHCYL1, CTBS, MCLC, LTBP1, NA (1)*	GSE2658	[28]
GPI-HM	50	*ASPM, AURKA, AURKB, BIRC5, BRCA1, BUB1, BUB1B, CCNA2, CCNB1, CCNB2, CDC2, CDC20, CDC25C, CDC6, CDCA8, CDKN3, CEP55, CHEK1, CKS1B, CKS2, DLG7, ESPL1, GINS1, GTSE1, KIAA1794, KIF11, KIF15, KIF20A, KIF2C, KNTC2, MAD2L1, MCM10, MCM6, MKI67, NCAPD3, NCAPG, NCAPG2, NEK2, NPM1, PCNA, PGAM1, PLK4, PTTG1, RACGAP1, SMC2, SPAG5, STIL, TPX2, UBE2C, ZWINT*	E-MTAB-316, E-MTAB-317, GSE2658, GSE4581	[33]
IFM-99	15	*FAM49A, ATF4, CTSF, ALDH2, CNDP2, STMN1, AFG3L2, STK38, PARP1, CPSF6, LOC151162, TOX2, FRY, FLJ21438, MGST1*	GSE7039, GSE2658, GSE6477, GSE9782	[34]
A-CG[b]	4	*FAM53B, KIF21, TMPO, WHSC1*	GSE755, GSE2658, GSE6477, GSE9782, GSE13591, GSE15695, GSE19784	[37]
A-NG[c]	2	*CSGALNACT1, SLC7A7*	GSE755, GSE2658, GSE6477, GSE9782, GSE13591, GSE15695, GSE19784	[37]

(continued)

Table 16.1 (continued)

Gene-risk model	No. genes	Genes	Cohorts	References
UK-97	97	*TNFRSF14, UBE4B, HDAC1, MARCKSL1, RRAGC, UTP11L, FAF1, DEDD, NIT1, SLAMF7, EIF2AK2, FBXO11, MSH6, RTN4, TIA1, STAMBP, ALS2CR12, CASP8, HSPE1, FASTKD2, NDUFS1, BARD1, IL8RB, CUL3, AXUD1, GPX1, RHOA, RYBP, RNF7, PDCD10, BCL6, ANXA5, FASTKD3, PRKAA1, XRCC4, RASA1, HDAC3, CYFIP2, RNF130, TNFRSF21, FOXO3, TPD52L1, CYCS, FIS1, ZC3HC1, CASP2, CUL1, DNAJB6, BAG4, STK3, RAD21, SETX, CDC2, PTEN, PTENP1, TCTN3, HTATIP2, API5, BAD, BIRC3, DNM1L, B3GNT4, DIABLO, RNF34, TPT1, ITM2B, ACIN1, ARF6, GPR65, BUB1B, DNAJA3, CLN3, APPBP1, P2RX1, TNFSF12, TNFSF13, TP53, PSMC5, SMARCD2, BIRC5, SOCS3, PIK3R2, PLEKHF1, DEDD2, NUP62, CD40, SERINC3, CSE1L, DONSON, SON, BID, DGCR6, DGCR6L, PRODH, BIK, TSC22D3, ARHGEF6*	GSE2658, GSE9782, GSE15695	[59]
UK-6	6	*BUB1B/HDAC3*, *CDC2/FIS1*, and *RAD21/ITM2B* ratios	GSE2658, GSE9782, GSE15695	[59]

[a]Reported as "NA" in the original publication; re-annotated using NetAffx Annotation Update 32 (July, 2011)
[b]A-CG: ARACNe-derived Critical-Gene model
[c]A-NG: ARACNe-derived Neighbor-Gene model

to define worst outcome. The UAMS model highlighted the key role of genes mapped to chromosome 1 in MM prognosis: as stated by the Authors, a main hypothesis of what presented in their paper was that the unbalanced expression of a specific subset of genes correlating with survival might be representative of the effects of DNA copy number changes in myeloma disease progression. In line with this, the 70-gene high-risk signature is consistent with the identification of a class of disease, defined by high-resolution aCGH profiling, characterized by high-level amplification of 1q21 and deletion of 1p13 [29]. The transcriptional profiles of MM tumors characterized by 1q gain/amplification, as assessed by FISH, were specifically investigated by our group, which led to the identification of a marked gene dosage effect, robustly validated on UAMS dataset [30].

Afterwards, the seven-group model has been reconsidered and extended by Broyl et al. which analyzed purified PCs of 320 newly diagnosed MM patients included in the HOVON-65/GMMG-HD4 trial. The Authors identified the seven groups of UAMS dataset, and described three additional expression patterns, which thus led to a global ten-group classification: first, a subgroup of patients (11.6%) characterized by overexpression of genes involved in the NFkB pattern; the patients in the second novel cluster (<3%) showed upregulation of protein tyrosine phosphatases *PRL3*, and shared as common features the occurrence of bone lesion, the lack of 17p loss, and the ISS staging I. Finally, the third subgroup of patients (less than 7%) was characterized by normal expression of proliferation genes and overexpression of cancer testis antigens, which have been also previously described using microarray analysis and whose overexpression has been associated with poor outcome [18, 31]. This latter group shared with the PR group the overexpression of Aurora kinase A (*AURKA*), reported to be associated with a greater proliferation rate and poor outcome [32]. However, the Authors did not provide any information on outcome, which prevents to draw conclusions on the clinical relevance of the three newly identified groups.

The TC classification, the seven group and the ten group models described so far used either different criteria or genes to define the proliferation-gene signature. Additionally, in a recent study Hose et al. used as well their microarray data of Heidelberg/Montpellier laboratory datasets to build a gene expression-based proliferation index (GPI) that accounts for 50 proliferation genes differentially expressed between proliferating (HMLCs and plasmablasts) and non-proliferating cells (normal PCs and memory B cells) (Table 16.1) [33]. In this study, a significant correlation was evidenced between high proliferation indexes and the presence of disease progression-associated gain of 1q21 or deletion of 13q14.3, as well as between low proliferation indexes and hyperdiploid status. Moreover, the Authors demonstrated the capability of proliferation score to predict EFS and OS, allowing highly predictive risk stratification of patients independent of other clinical prognostic factors. Overall, albeit calculated using different methods and with minimally overlapped genes, the microarray-based proliferation indexes are able to discriminate a high-risk prognostic group.

A new gene-risk model was proposed by Decaux et al. from the "Intergroupe Francophone du Myelome", who studied the transcriptional profiles of purified

PCs from 250 MM patients at diagnosis from a proprietary cohort (182 used as training and 68 as test set from IFM-99 trial), and identified 15 genes able to determine a risk score associated with OS (Table 16.1) [34]. The data were also validated in more than 800 independent samples from UAMS [28], Mayo Clinic [35], and Millennium Pharmaceuticals [36]. The 15-gene model was build first by assessing the correlation with survival for each of the transcript represented on the array; then, principal component analysis was used to summarize the 15-gene list information and define the equation that lead to the unique score. Patients were then divided according to the score into high-risk group, characterized by the overexpression of genes involved in cell cycle progression and its surveillance, and low-risk group, characterized by hyperdiploid signature. OS at 3 years in low-risk and high-risk groups was 90.5% and 47.4%, respectively, and the gene-risk model was independent of traditional prognostic factors. The Authors also suggested that the combination of three independent prognostic entities [the 15-gene model, serum β2 microglobulin (sβ2M) and t(4;14) translocation] provided an accurate tool to identify the highest risk group (12.4% of patients) with high-risk score, sβ2M 5.5 and/or t(4;14), showing a 3-year rate of survival of 34%. However, when competed with the UAMS 17-gene model, the IFM model lost independency in the TT2 data set and failed to identify the early disease-related deaths in the TT3; the 15-gene model preserved its value of significant independent variable in the other non-UAMS data sets examined by the Authors.

Of note, although UARK 98–026 and UARK 03–033, used to derive the UAMS 17-gene model, and IFM-99 trials have included patients undergoing high-dose therapy, the two models actually do not share common genes. This might undoubtedly be related to the biological complexity of the tumor; however, it also indicates that an effective use of microarray expression data for diagnostic purpose will require significantly more studies and consensus. Furthermore, the functional association between these various genes could be considered in a more composite model.

Recently, our group suggested a different approach for the identification of prognostic model, which takes advantage of a composite of data sets from multiple institutions [37]. In our study, we started from the hypothesis that the selection of predictive genes based on their putative regulatory role, rather than solely on their expression levels or outcome data as done in previous studies, will substantially improve the reliability and robustness of prognostic signatures. However, conventional analysis of gene expression data is rarely capable of distinguishing between "master regulators" and target genes or between direct transcriptional interactions and indirect ones. This level of complexity can instead be accessed using methods that allow the reverse engineering and the reconstruction of regulatory networks. We have thus reconstructed gene regulatory networks in a panel of 1,883 samples from MM patients derived from proprietary and six other publicly available gene expression sets profiled on GeneChip® microarray platforms. To this aim, we used ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks), a robust procedure for reverse engineering of transcriptional data developed at Columbia University [38]. For more effectiveness, we applied a computational

procedure, derived from network theory, to identify those nodes (i.e., genes) that play a critical role in the stability and functioning of the network. This approach provides important information concerning genes that, although not necessarily associated with any particular molecular feature, are involved in the transcriptional control of several genes and might contribute to determine the characteristics of certain disease subtypes. The critical analysis of network components was thus applied to identify genes playing an essential role in transcriptional networks, which are conserved between datasets. Our analysis confirmed the crucial role in MM biology of *CCND1* and *CCND2*, which were identified as the most critical genes. Moreover, we validated two robust gene signatures with prognostic power; the first one ("critical-gene" model) including four of the most critical transcripts (*FAM53B*, *KIF21B*, *WHSC1* and *TMPO*) and the second including shared primary "connections" of the critical genes. Specifically, this "neighbor-gene" model included the *BLNK*-shared neighbors *CSGALNACT1* and *SLC7A7* (Table 16.1). Both the models predicted survival in three datasets with available follow-up information, independent of other prognostic parameters and of the other tested gene-risk model (UAMS, IFM and UK models) [37].

Finally, although partly beyond the scope of this narrative, it is worth mention that gene expression profiling studies has been promisingly applied in MM to investigate the effects of treatments or therapeutic regimens in the biology of PCs, either *in vitro* [39] or in primary tumor dataset [36], as well as in the understanding of the role of microenvironment in the disease [40].

2 Integrating Transcriptional and Genome-Wide Profiling Data Allow Uncovering Critical Aberrations

The wide perspective offered by all of the mentioned gene expression studies could be undoubtedly enlarged further, should the transcriptional data be analyzed in the context of the chromosomal alterations underlying gene expression aberrations. Appropriate analyses of gene expression data might allow unraveling chromosomal disruptions or recombinations that lead to aberrant expression of involved transcripts, and which might nonetheless be cryptic based on other analytical approaches. Those analyses of microarray data lie on the identification of outlier profiles, which might be suggestive of rearrangements that aberrantly juxtapose the promoter and/or enhancer elements of one gene to another [41]. In the circumstance of a proto-oncogene, this causes altered expression of an oncogenic protein. This type of rearrangement is exemplified by the apposition of immunoglobulin (IG) promoter elements to *MYC*, which lead to activation of this oncogene in B cell malignancies. In MM, we firstly demonstrated that spiked expression, as assessed by microarray, of the genes deregulated by primary *IGH@* translocations in MM could be used as surrogate of the cytogenetic detection (e.g. by Fluorescence *In-situ* Hybridization, FISH) of the corresponding translocation [42].

DNA microarrays have been used by several investigations to describe the relationship between genomic copy number aberrations and expression imbalances, demonstrating the occurrence of specific gene-dosage effects on physically adjacent genes; overall, the integration of parallel microarray approaches for studying the cancer genome scenery are becoming crucial to obtain a more insightful comprehension of key genes and/or alteration driving tumorigenicity [43]. The high-density microarray analysis of the whole genome allows generating detailed profiles of allelic imbalances. Among the most widely used procedure, both the array-comparative genomic hybridization (aCGH) and the Single-nucleotide polymorphism (SNP)-genotyping allow the detection of copy number alterations (CNAs); whereas only SNP-genotyping allows a fine detection of loss-of-heterozigosity (LOH).

Several studies have been aimed at characterizing the recurrent genomic features of MM and analyzing the transcriptional *milieu* in the context of amplified/deleted chromosomal regions and/or occurrence of LOH. Primary analyses were performed on representative subsets on HMCLs, and integrated gene expression profiling with FISH assays and conventional comparative genomic hybridization [44] or SNP-genotyping [45]. Overall, the results of the investigations by Largo et al. and Lombardi et al. are concordant in highlighting the huge genomic heterogeneity of HMCLs. The transcriptional profiles of HMCLs could be easily distinguished by those of primary tumors, particularly for overexpression of transcripts related to cell cycle progression and proliferation pathways; however, differently from primary tumors, the HMCLs could be only marginally stratified into subgroups sharing common transcriptional features, with the exception of a fraction of genes characterizing cell lines harboring t(4;14) or *MAF*/*MAFB* translocations. The HMCLs analyzed show a large number of non-recurrent CNAs; the two studies are in agreement in the definition of regions (namely on chromosome arms 1q, 8q, and 18q) showing in at least two cell lines both amplification and the corresponding overexpression of some residing genes, among which *BCL2* [44, 45].

Later on, different laboratories have used either aCGH or SNP-genotyping to investigate the genomics of MM primary tumors and integrate these data with the transcriptional profiles of the corresponding samples.

Carrasco et al. used high-resolution aCGH to describe the CNAs in the genomes of purified PCs from 67 newly diagnosed and untreated MM patients included in the UARK98-026 protocol (TT2 therapy) [29]. In line with what observed in HMCLs, their analysis revealed a highly rearranged MM genome, with a large numbers of distinct CNAs; however, in their dataset they defined 87 discrete minimal common regions (MCRs) within focal, recurrent, and high-amplitude CNAs. The frequency profile of chromosomal imbalances indicated frequent gains (>20%) at 1q arm and the entire chromosomes 3, 5, 7, 9, 11, 15, 19, and 21, as well as frequent losses of 1p, 6p, 8p, 14q and 16q arm and the entire chromosome 13, resembling and in part extending previous evidences using conventional cytogenetic procedures, FISH or CGH [8, 46–51]. To determine whether MM patients that share common genomic features could identify subgroups showing meaningful genetic and/or clinical pattern, the Authors performed an unsupervised analysis with a modification of nonnegative matrix factorization (named "gNMF") as leading algorithm used to

extract distinctive genomic features from aCGH profiles. The analysis generated consensus matrices that showed stable cluster assignments (with rank parameter, namely the number of clusters obtained, set from 2 through 4), suggesting the presence of up to four distinct genomic patterns in their dataset. The major distinction was between hyperdiploid and non-hyperdiploid samples, although no correlation with outcome was evidenced. The Authors hypothesized that this was due to heterogeneity within the groups defined by NMF when a binary division of the cluster was chosen. Indeed, the division into four stable cluster led hyperdiploid MM cases to be stratified into two subclasses, with a clear survival advantage for patients characterized by both the presence of chromosome 11 gain and the absence of 1q arm gain or chromosome 13 loss. Carrasco et al. also integrated genotyping with transcriptional profiling data and evaluated whether the expression of genes mapped to amplified MCR showed a copy number-correlated pattern and significant overexpression in tumors relative to normal PCs. The comparisons for each gene of the mRNA levels in tumors with and without CNAs in the region of interest led to the identification of strong candidates likely targeted by amplification of MCRs, among which are genes with a putative role in MM pathogenesis (e.g. *MYC*, *MCL1*, *IL6R*, or *HGF*). Our group reinforced and extended the evidence of two distinguishable transcriptional entities within hyperdiploid group, one characterized by deletion of chromosome 13 and extra-copies of 1q, and the other by trisomy of chromosome 11. Notably, the former was characterized by overexpression of *CCND2*. In the same study, we demonstrated that hyperdiploidy is reflected in transcriptional imbalances of wide chromosomal regions using a self-developed model-free statistical method (LAP) that analyze the gene expression data in the context of the physical localization of the genes in the genome. Particularly, the LAP procedure revealed a consistent gene-dosage effect distributed over most of the length, if not the whole, of trisomic chromosomes [52].

The recurrence of the CNAs in MM primary tumors, firstly described by high-density microarrays in the study of Carrasco et al. was confirmed by subsequent studies, also based on high-throughput microarray technologies. This can be clearly observed by comparing the skylines of the frequency plots of gains and losses depicted by Carrasco et al. (Figure 4 of their publication) with those reported in the studies from Avet-Loiseau et al. [53] Walker et al. [54] and our group [55], all of them performed on high-density Affymetrix GeneChip® Human Mapping platforms (exemplar plot on the proprietary data in Fig. 16.1). The former of these studies analyzed purified PCs from ten normal donors and 192 cases of newly diagnosed MM included in the IFM-99 clinical trial on 500 K Array set. Their analyses revealed that 1q amplification and 1p, 12p, 14q, 16q, and 22q deletions were the lesions most frequently associated with poor prognosis, whereas the recurrent gains of chromosomes involved in hyperdiploidy are associated with favorable prognosis. Multivariate analysis of chromosomal imbalances identified 1q23.3 and 5q31.3 amplifications and deletion at 12p13.31 as independent variables. However, only del(12p)(13.31) and amp(5q)(31.3) retained independency when adjusted to the established prognostic variables t(4;14), del(17p), and sβ2M, which allowed the Authors to define a prognostic index based on amp(5q)(31.3), del(12p)(13.31) and sβ2M [53].

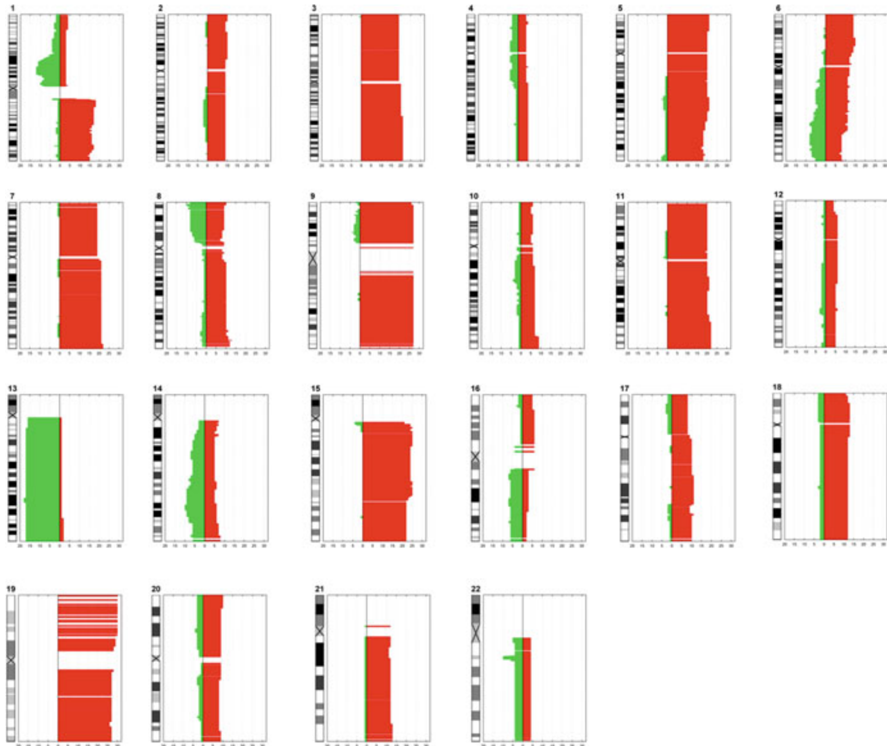

Fig. 16.1 Frequency plots showing the recurrence of chromosome copy number alterations in multiple myeloma, as inferred on the proprietary dataset of 45 samples (Agnelli et al. Genes Chromosomes Cancer 2009) Gains are depicted in *red* color, losses in *green*

Our group investigated a panel of 45 patients at diagnosis using combined FISH and microarray approaches. We generated the genome-wide profiles of 41 MMs and four PCLs on 50 K Array platform. Despite the lower resolution of the high-density array set, the accuracy of our genotyping analysis took advantage of a self-developed procedure to infer exact local copy numbers (CN) for each sample, named "FISH-based normalization" (FBN, also available as R package at http://www.r-project.org). Particularly, due to the intrinsic properties of SNP-genotyping technology, the scaling of the estimated CN raw profiles failed to reflect correctly the real CN profile in case of marked aneuploid status (i.e., near-tetraploidy). To overcome biases we developed, and validated in a proprietary dataset of 25 HMCLs, the FBN procedure that incorporates the local ploidy information assessed by FISH in the final stage of data normalization of mapping arrays. This allowed the identification of a significant fraction of MM patients (more than 15%) showing a marked aneuploidy status. A clustering analysis based on NMF-algorithm, similarly to that applied by Carrasco et al. [29] revealed that the most significant consensus matrix was defined by five group, likely corresponding to the four described by

Carrasco et al. plus that including those patients showing near-tetraploid or anyhow marked aneuploid genomic configuration. Based on these results, we performed an integrative analysis of mapping and gene expression data profiled on Affymetrix GeneChip® HG-U133A array and identified more than 1,000 transcripts whose expression strongly correlated with the underlying CNAs of the corresponding *loci*.

Overall, this scenario opened the issue on how to consider effectively those DNA portions with $CN = 2$ in the context of a near-tetraploid status, which might be traced back to the occurrence of a deletion. This aspect requires attention whenever the allelic configuration might imply gene loss or gain of function despite a virtually normal genomic configuration, namely in the presence of uniparental disomy (UPD); naturally, this is particularly relevant whenever the involved regions encompass oncogenes or tumor suppressors. The SNP-microarray technology is helpful to unravel these events through the ability of combining the CN analysis with LOH investigation. Our analysis supported the occurrence in MM of several different pictures, summarized in Table 16.2, that account for the profound genomic heterogeneity and complexity of the disease. Particularly, the LOH analysis revealed both recurrent events of UPD (mostly on 1p arm and chromosome 13 of the near-tetraploid group) and the absence of LOH with inferred CN indicating allelic loss (suggestive of the presence of subclones) [55]. This latter aspect was also confirmed by Walker et al. who integrated expression and SNP mapping array data and used a quantitative short tandem repeat polymerase chain reaction procedure to check copy number and allele correspondence in one patient with 13q loss. Their assay indicated that such sample was heterozygous, but it had half the amount of each allele compared to the control sample [56]. In the same study, the Authors considered more than 3,000 genes mapped to the regions of LOH that recurred in more than 10% of the total and compared this list to that previously identified in the progression of normal through myeloma plasma cells [17]. Among the 47 matched transcripts, a fraction between 62 and 70% was identified as downregulated in association with the progression (i.e. MGUS and MM tumors compared with normal donors and MM compared with MGUS cases). The same Authors deeply analyzed the role of LOH in MM using integrated FISH analysis with transcriptional profiling and SNP-genotyping [57]. Specifically, they demonstrated the adverse prognostic role of deletion at 16q, independent of other poor-risk cytogenetic factors [i.e. t(4;14) and del(17)], and identified 2 genes, the WW domain-containing oxidoreductase gene *WWOX* mapped at common fragile site FRA16D and the *CYLD* deubiquitinase, a negative regulator of the NF-*k*B pathway, whose expression is associated with LOH at 16q23 and 16q12, respectively. In our previously described analysis [55], we confirmed the significant association between the expression of *WWOX* and the occurrence of LOH. In addition, in a following report using integrated analysis of microRNA expression profiles and SNP-genotyping data we also demonstrated that the occurrence of LOH involves the downregulation of the microRNA *miR-140-3p* at 16q22.1-q23.1 [58]. The *miR-140-3p* expression was also significantly correlated with that of the WW domain-containing gene *WWP2*, involved in ubiquitination, similarly modulated by LOH [55].

Table 16.2 Genomic configurations as conceivable based on CN and LOH analysis of SNP-microarray data

LOH	Local inferred CN	Assumption
Yes	0	Biallelic deletion
	1	Deletion of single allele
	2	Uniparental disomy
	3	Uniparental trisomy
	>3	Uniparental amplification
No	1	Occurrence of PC subclones harboring the two different alleles
	2	Wilt-type condition
	3 or >3	Gain or amplification of at least one of the two alleles, both being present

The UK group have recently provided a comprehensive analysis of the genomic profiles of 114 patients on 500 K Array set and of the expression profiles of 258 samples on Affymetrix GeneChip® HG-U133 Plus 2.0 arrays, all included in UK MRC Myeloma IX study [54, 59]. Differently from previous analogous studies, the virtue of their SNP-genotyping analyses lies in the availability of 84/114 matched non-tumor DNA to compute accurate CNAs and acquired LOH. The Authors defined a spectrum of minimally deleted regions in which relevant genes of prognostic importance are mapped (at 1p, 1q and 17p), and provided an exhaustive summary of the other recurrent alteration identified [54]. Next, in the following report, they analyzed the occurrence of homozygous deletion (HZD) in myeloma samples, and identified 170 genes whose loss of expression is correlated with HZD [59]. Frequently occurring HZDs were identified at 1p32.3 (in the region encompassing *FAF1* and *CDKN2C* genes), 11q (*BIRC2* and *BIRC3*), 14q (*AMN* and *TRAF3*), and 16q (*CYLD*). Among the recurrent HZDs identified by Dickens et al. those involving *BIRC2* and *BIRC3* genes at 11q have been also previously reported by *Largo* et al. who described HZDs in MM primary tumors using high-density aCGH [60]. However, only 29 genes had HZD in 5% or more samples of UK dataset. The large parte of recurrent HZDs seem to occur in genomic regions carrying hemizygous deletions occur (1p, 6q, 8p, 12p, 13q, 14q, 16q, 20p, and 22), albeit not exclusively; in an univariate analysis, the 6 cases with HZDs affecting *CDKN2C/FAF1* at 1p32 or the 12 with *ATP8A2* at 13q12.13 had impaired OS. Dickens et al. then evaluated whether a common transcriptional profile characterized HZDs. They carried out an analysis of differential gene expression between the samples with and those without HZD of cell death-associated genes (uncorrected *t*-test at $P < 0.001$) and generated an expression-based signature of 97 genes associated with shorter survival in 258 patients (Table 16.1). They validated the 97-gene signature in two independent datasets including 800 samples, and demonstrated that it was independent of other known prognostic factors, including sβ2M, serum albumin, and cytogenetic factors. Moreover, because the regular use in the clinical setting of a microarray-based 97-gene model is indeed impractical, the Authors sought the list to derive a more readily applicable 6-gene cell death signature capable of identifying those patients with poor outcome. Using univariate and multivariate regression analyses and comparing

the ratios of expression of genes included in the 97-gene model, they identified three pairs of genes (Table 16.1) whose relative expression provides a classifier able to recognize 12% of patients having short OS, being the simple rule for the detection of poor outcome cases the ratio of ≥ 1 in any one of these pairs. Their 6-gene signature resulted as independent of other published signatures (UAMS and IFM) and other conventional prognostic factors [59].

Finally, another intriguing approach to identify risk variants for MM was that shown by Broderick et al. [61] who integrate transcriptional with SNP-microarray data in genome-wide association study based on the hypothesis that a possible explanation of higher risk of MM in relatives of subjects with MM might be the coinheritance of multiple lowrisk variants. The Authors used Illumina OmniExpress BeadChips technology to conduct genome-wide association study of two cohorts of MM cases, and identified two loci of increased risk for MM at 3p22.1 and 7p15.3, with a promising association at 2p23.3. Although without obtaining statistically significant result, the Authors integrated the obtained results with the mRNA expression profiles of PCs from 191 MM cases included in Myeloma IX trial, to investigate the occurrence of putative *cis*acting regulatory effects on the surrounding genes of the involved SNP variants.

3 Coordinated Methylation and Transcriptional Analyses Indicate that Hypomethylation Is Associated with Progression in MM

Differently from transcriptional and genome-wide profiling approaches, large-scale studies of methylation profiles in MM are still limited, despite increasing evidence of a role for epigenetic mechanisms in the pathogenesis of MM [62–65]. Herein, we focus on two recent publications that used high-throughput technologies to investigate differential methylation profiling in plasma cell dyscrasia.

Salhia et al. studied the differential CpG methylation of 1,500 genic loci assessed using GoldenGate Methylation Cancer Panel I (Illumina) [66]. The panel of investigated specimens included highly purified PCs from six healthy donors and from MGUS, SMM, and MM tumors (totaling 179 samples), together with four HMCLs. The Authors identified 245 unique loci corresponding to the union of all differentially methylated loci (DML) from each tumor group, which encompassed 176 genes, and demonstrated that the median methylation level of all 245 DML significantly decreased from normal PCs (β values = 0.68) to MM (0.34) through MGUS and SMM samples (both 0.44). Conversely, hypermethylation was found rarely, as only 22 loci were hypermethylated in the entire data set, resulting in about 3.5% of MGUS-specific DML, about 9% of SMM-specific DML and about 6% of MM-specific DML. Eight DML were validated by pyrosequencing technology on 16 MM samples and four normal PCs to confirm the methylation status of the genes identified. Combined with methylation profiling, Sahlia et al. used aCGH (performed on Agilent 244A microarrays) to determine the genomic profiles of the

analyzed samples, which allowed demonstrating that neither hyperdiploid status nor 1q amplification, nor 17p deletion, nor chromosome 13 monosomy were related to overall methylation levels. Similarly, overall methylation levels were unrelated to whether a patient was treated or newly diagnosed [66]. The results illustrated by Salhia et al. were in line with previous investigations from our group demonstrating hypomethylation in repetitive DNA elements in the progression from normal PCs phenotype to PCL through MM, as well as in HMCLs [67]. In this study, we integrated gene expression profiling with methylation data obtained through pyrosequencing to correlate methylation patterns with the mRNA levels of DNA methyltransferases (DNMTs), codifying for enzymes responsible for CpG methylation. In particular, we identified a progressive and significant increase of *DNMT1* expression from controls to MMs, PCLs and HMCLs.

Walker et al. investigated the genome-wide methylation profiles of 6 normal B cells, 3 normal PCs, 4 MGUS samples, 161 symptomatic myeloma (included in Myeloma IX MRC clinical trial), 7 PCL, and 9 HMCLs using Illumina Infinium humanmethylation27 BeadArray [68]. In line with what observed by Salhia et al. the number of differentially methylated loci increased with tumor progression, and most of them were hypomethylated. Specifically, their analysis indicated that few methylation changes occur between normal PCs and the MGUS phenotype, whereas the large majority of hypomethylated probes (3,407 CpG loci, encompassing 1,428 genes) distinguished MGUS from symptomatic MM. Importantly, an unsupervised hierarchical clustering of their dataset revealed three main clusters, the first including nonmalignant PCs, the second grouping HMCLs and t(4;14) samples, and the third encompassing all of the other MM samples. Within this latter group, the analysis revealed the occurrence of discrete methylation clusters based on cytogenetic abnormalities, although partially interspersed. To evaluate whether the observed heterogeneity in global methylation within symptomatic MM samples could be attributable to the presence of different cytogenetic subgroups within the sample set, they compare the methylation levels of each translocation subgroup, and confirmed that the major differences were associated with t(4;14), being more than 2,500 CpG probes ($\sim$10%) hypermethylated and 302 probes hypomethylated in comparison with samples with no split *IGH@* locus. To further elucidate the methylation pattern of t(4;14) cases, Walker et al. integrated the methylation data with the transcriptional data of the Myeloma IX MRC dataset, and generated a list of 353 differentially expressed probe sets between t(4;14) and no split *IGH@* locus samples (as assessed on Affymetrix HG-U133Plus 2.0 arrays) with the corresponding gene differentially methylated. These data underlie that t(4;14) myeloma have the greatest impact on DNA methylation, although further investigation are undoubtedly required to elucidate this finding. Finally, of note, two distinct groups of hyperdiploid samples are distinguishable based on clustering analysis. The difference between them are not related to cytogenetic markers, e.g. 1q or del(13q), which have been previously associated with specific hyperdiploid subgroups [29, 52], whereas a significant difference was found in the OS of the two groups, suggesting that methylation might have both clinical and biologic effects within the hyperdiploid patients [68].

4 Characterization of microRNA Expression in Different Molecular Subtypes and in the Prognostic Stratification of MM

The most recent advances are uncovering microRNAs as crucial actors in the pathophysiology of the disease. microRNAs (miRNAs) are small non-coding RNA that act as post-transcriptional regulators and bind to complementary sequences on target transcripts, usually resulting in translational repression or mRNA target degradation and gene silencing. Aberrant expression of miRNAs has been implicated in numerous disease states and tumors, and miRNA-based therapies are currently under investigation [69]. A peculiar behavior of several miRNA transcripts has been identified also in MM, which prompt investigators to focus their attention on miRNA role within the plasma cell dyscrasia [70].

However, so far, few studies used high-throughput technologies and comprehensive integrative genomics analyses on representative cohorts of primary MM tumors to investigate the miRNA involvement in the disease.

Ronchetti et al. [71] firstly investigated MM cell lines and primary patients integrating gene expression and genome profiling data generated on Affymetrix oligonucleotide microarrays with miRNA expression levels obtained by Quantitative Real-Time PCR (Q-RT-PCR). A significant correlation was identified between the expression levels of *MEST*, *EVL*, and *GULP1* and those of the corresponding intronic miRNAs *miR-335*, *miR-342-3p*, and *miR-561*. The SNP-genotyping analysis of a subset of 20 HMCLs indicated lack of correlation between the expression levels of the three paired host genes/intronic miRNAs and the local copy number variations of the residing *loci*. Subsequently, Pichiorri et al. studied the microRNA expression profiles of purified PCs from six normal donors, 6 MGUS and 16 MM tumors, and 49 myeloma cell lines, using custom-made arrays and Q-RT-PCR. The Authors revealed by univariate analysis of the miRNAs represented on their platform the overexpression of *miR-21*, *miR-106b ~ 25* cluster, *miR-181a* and *miR-181b* both in MM and MGUS samples if compared with normal cases, and the selective up-regulation of *miR-32* and *miR-17 ~ 92* cluster in MM tumors. Moreover, they demonstrated in a luciferase assay that *miR-19a* and *miR-19b* mimics inhibited the expression of *SOCS-1*, while mutation of the predicted miRNA-binding site on the 3'-UTR of the gene abrogated the inhibition; and that SOCS-1 protein was significantly upregulated in U266 and JJN3 cells transfected with antisense oligonucleotide for *miR-19a* and *miR-19b* [72]. In a similar approach, Roccaro et al. studied the expression profiles of 318 miRNA transcript using liquid phase Luminex microbead miRNA profiling assay in purified PCs from 15 patients with relapsed or refractory MM tumors, healthy donors and three HMLCs. Particularly, their analysis allowed to identify the specific downregulation of *miR-15a* and *miR-16* in MM samples; further functional validation revealed their role in the regulation of proliferation and growth of MM cells *in vitro* and *in vivo* [73].

Our group provided a comprehensive analysis of high-density microarray data from a panel of 38 primary MM tumors and two PCLs, integrating miRNAs,

gene expression and genotyping profiles. Specifically, Lionetti et al. characterized the miRNA expression profiles of the different molecular subtypes of MM using Agilent Human miRNA Microarray v2 (specific for 723 human mature miRNA transcripts) [58]. Notably, a limited number of miRNAs was able to discriminate between the TC subgroups; particularly, the t(4;14) cases were characterized by the specific upregulation of three clustered miRNA (*miR-99b*, *let-7e*, and *miR-125a-5p*, mapped to 19q13.33), while 10 miRNA were overexpressed in TC5 samples, among which is worth noting *miR-150* and *miR-155* [74, 75]. Then, Lionetti et al. integrated the miRNA expression profiles with the previously generated [55] CN values of the corresponding miRNA genes, showing that several miRNAs mapped to chromosomal regions affected by allelic imbalances, either numeric or involving LOH. The numeric alterations were associated with the expression of 49 miRNAs, most of them on chromosome 1 and on odd-numbered chromosomes involved in hyperdiploidy [58]. The other integrative analysis described in Lionetti et al. raised from the hypothesis that the consequence of a truly functional interaction between a miRNA and its predicted mRNA targets, if acting at the transcriptional level, should be outlined as anticorrelated expression profiles of miRNA/mRNA pairs. Based on this hypothesis, a network of more than 20,000 functional interactions supported by expression data potentially occurring in MM were reconstructed from the board of putative regulatory relationships predicted from sequence information (using MiRanda algorithm [76]). The different computational approaches used in our investigation are summarized in Fig. 16.2 [58].

A similar investigation, aimed at characterizing the molecular subgroups of MM and the putative miRNA/mRNA regulatory interactions, was carried out in a following study, in which different technical and computational approaches were yet applied to a similarly representative panel of MM tumors [77]. Specifically, Gutierrez et al. used Multiplex TaqMan® miRNA arrays for the quantification of 365 human miRNAs and the whole-transcriptome Human GeneChip® Gene 1.0 ST arrays and identified a number of miRNAs that were differentially expressed in cytogenetically normal PCs or in those harboring t(4;14), t(14;16), t(11;14), or *RB* gene deletion as a sole abnormality, as compared with healthy donors. Of the miRNAs identified in the different comparison, two miRNAs (*miR-214* and *miR-375*) were deregulated in all the myeloma samples irrespectively of cytogenetic features, whereas in line with results reported by us [58] PCs with t(14;16) overexpressed *miR-1* and *miR-133a.* To find out miRNA/mRNA regulatory interactions, the Authors combined a correlation analysis under the assumption of bivariate normal distribution with the target prediction analysis carried out using miRecords, a computational resource for miRNA-target interactions discovery that integrates DIANA-microT, MicroInspector, miRanda, MirTarget2, miTarget, NBmiRTar, PicTar, PITA, RNA22, RNAhybrid and TargetScan miRNA target prediction tools [78]. Moreover, to discriminate miRNA–mRNA interactions more susceptible to degradation processes, they applied further filtering rules from the combination of four algorithms (TargetScan, miRDB, Pictar and MiRanda), e.g. criteria of seed region complementarity, thus reducing false-positive predictions and providing miRNA–mRNA interactions likely more sensitive to a cleavage pathway.

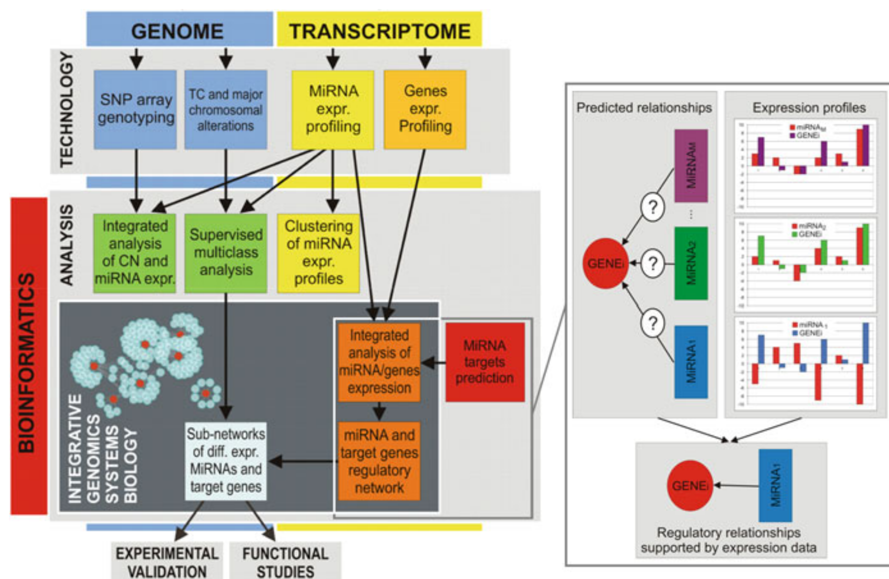

Fig. 16.2 Exemplar workflow of the integrative genomics analysis incorporating transcriptional, genotyping and microRNA expression data generated on high-density microarrays. (Modified from the original scheme kindly provided by Stefania Bortoluzzi, University of Padua)

Among the putative target genes identified, there were also transcripts that play a pivotal role in the biology of MM, such as the *CCND2* gene, up-regulated in the MM subtypes t(4;14), t(14;16), that showed significant interactions with several miRNAs that are significantly deregulated in the same MM subtypes.

Finally, Zhou et al. analyzed the miRNome of two healthy donors and 52 newly diagnosed MM patients using Agilent platform and integrated the miRNA expression profiles with the transcriptional profiles of the same cohort profiled on Affymwtriex HG-U133Plus 2.0 arrays [79]. The remarkable finding of their investigation was that the overall expression levels of miRNAs (namely, the mean levels of expressed miRNAs) could be associated with progression in MM. Specifically, the Authors integrated the Gene Set Enrichment Analysis (GSEA) procedure [80] with the results of the correlations between total miRNA expression level and expression levels of individual genes. GSEA was used to associate the correlated genes, ranked by correlation coefficients, with the gene sets included in the Broad Institute Molecular Signature database (MSigDB at http://www.broadinstitute.org/gsea/msigdb/index.jsp). The GSEA analysis outlined enrichment in high-risk gene sets suggestive of enhanced cell proliferation and related to undifferentiated cells, including genes up-regulated in several types of undifferentiated cancers, plasmablasts or different types of stem cells. In line with this, Zhou et al. suggested a positive correlation between 28 miRNAs and the 70-gene risk model defined by the same Authors [28] and between two miRNAs (*hsa-miR-18a* and *hsa-miR-107*) and the proliferation index, thus indicating that the global miRNA expression profiles

in MM is possibly implicated with prognosis. The Authors finally investigated the putative role of miRNA upregulation in MM onset through the silencing of the constitutive expression of *EIF2C2/AGO2* and *DICER1*, two crucial components of miRNA biogenesis machinery, in HMCLs, which led to decreased cell viability, cell cycle arrest and the induction of apoptosis; however, although included in the 70-gene model and thus associated with high-risk MM, the expression of *AGO2* was unrelated to the total miRNA expression, suggesting that other factors than *AGO2* might contribute to the global up-regulation of miRNAs in high-risk MM [79].

5 Conclusions

So far, the wide-scale "–omic" profiling technologies have unquestionably shed lights in the biology of MM, whose comprehension could further take advantage of more and more studies being aimed at integrating the different approaches to reconstruct the biological *milieu* of the concerted interactions between transcriptome, genome and miRNome in the disease. In this context, of high importance is the application of the novel massive parallel sequencing technologies, which in a recent report have provided earliest insights into the genetics of MM [81] and undoubtedly warrant further investigations. However, as previously stated, this kind of analyses is subjected to generate long list of outputs (genes, miRNAs, or allelic aberrations) and consequently false positive calls are expected. Robust statistics, validations (e.g. Q-RT-PCR or biochemical assays), and above all functional analyses are mandatory to select true aberrations with possible meanings in the biology of the disease.

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Chapter 17
Genome-Wide Analysis and Gene Expression Profiling of Neuroblastoma: What Contribution Did They Give to the Tumor Treatment?

Gian Paolo Tonini

Abstract In the last decade geneticists are exploring the possibility to trace specific signatures for each type of cancer by using the powerful microarray technology. This technology allows us to analyze in a single round the genome and/or transcriptome of cancer cells. Genome-wide analysis can be performed by array Comparative Genomic Hybridization (aCGH) in order to identify chromosome gain and/or loss while microarray gene expression profiling is used to identify cancer cell gene signature associated with disease progression. Furthermore, the Omics study of cancer cells greatly improved the identification of candidate molecules for targeting therapy of both adult and pediatric cancers. So, translational research fuels therapeutic innovation and represents an indispensable link between basic and clinical research. Neuroblastoma is a pediatric cancer that shows clinical and biological heterogeneity. The tumor can onset as localized disease with a good outcome or as metastatic cancer with an unfavorable course for patients over 1 year of age. The first and more consistent aberration discovered in neuroblastoma was *MYCN* gene amplification. *MYCN* amplification is observed in 20% of cases and is the stronger prognostic factor. Actually, the SIOPEN as released several therapeutic trials in which *MYCN* amplification is a decisional factor for personalized medicine. Afterwards, genome-wide study of tumor cells identified several numerical and/or structural chromosome aberrations. Loss of chromosome 1p, 2p, 9p, 11q and 14q and gain of 17q are the most frequent abnormalities observed in neuroblastoma. More recently, SIOPEN has released the LINES, the first European trial in which therapy in a subgroup of patients is tailored according to the presence of structural chromosome aberrations. Although the gene signature is not used yet as prognostic factor, more than one gene signature has been found associated with unfavorable outcome. Since the major challenge for oncologists is the cure of high risk patients, the search for neuroblastoma associated genes is greatly improved in the last years. The *ALK* gene

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is the first discovered gene associated to neuroblastoma predisposition. *ALK* is a tyrosine kinase receptor activated by point mutations that are located in the tyrosine kinase domain. The alk receptor is a good candidate for target therapy and pilot trials are started employing *ALK* small molecules inhibitors. Finally, the Next Generation Sequencing technology will be employed to search candidate neuroblastoma gene and building up databases collecting as many samples as possible. In the next future, the discovery of new genome point mutations could give the possibility to identify druggable genes for innovative therapies.

The present paper shows how tumor genome data have been used to tailor new therapy for neuroblastoma patients demonstrating how information from genome analysis is transferred to the patient's bed.

1 Introduction to Genome of Neuroblastoma

Among pediatric cancers, neuroblastoma is one of the most studied from the genomic point of view. The first observations about the chromosome abnormalities in neuroblastoma started in 1973 when Biedler and coworkers [1] reported the presence of double minutes chromosomes in human neuroblastoma cell lines. At that time nobody knew that these structures contained several copies of *MYCN* oncogene. Afterwards, Brodeur et al. [2] observed chromosome 1p deletion in both neuroblastoma cell lines and primary tumors opening the way to the study of neuroblastoma genome. An important turning point was in 1983 when Schwab and collaborators [3] discovered *MYCN* gene amplification in human neuroblastoma cell lines. Two years later, Seeger et al. [4] demonstrated a close relation between *MYCN* oncogene amplification and tumor aggressiveness in patients at different stages of disease.

Now, we know that neuroblastoma cells are characterized by several non-random chromosome abnormalities including: deletion of chromosome 1p, 11q, 14q, and gain of 2p, 17q. However, not only structural chromosome aberrations are present in neuroblastoma, but also numerical chromosome extracopies have been found in neuroblastoma cells.

Most observations were performed using the classical metaphase kariotype technique; but since 1988 the metaphase comparative genomic hybridization (mCGH) has been used and afterwards the mCGH was substituted by the microarray CGH (aCGH) technology. The introduction of the latter allowed scientists to move from the detection of gross chromosome abnormalities to the identification of chromosome microlesions. The aCGH permits to break down at the gene level and to identify deletion less than 10–20 bp large [5, 6]. The genome analysis by aCGH generates a huge amount of data and requires the use of diverse algorithms to obtain univocal information about genome aberrations. Thanks to aCGH analysis of neuroblastoma genome, some candidate tumor genes associated with tumor development have been proposed. Moreover, aCGH was very useful to identify genomic profiles significantly associated with disease outcome

Microarray technology has been also used to study the overall gene expression profiling of neuroblastoma. Gene expression study produces tumor gene signatures associated with diverse clinical stages. Since more than 100,000 probes defining 30,000 genes are located on the microarray, this information is managed with complex algorithms. Supervisor and unsupervisor analysis are usually employed to identify gene expression profiling associated with tumor biological subtypes or tumor aggressiveness. More than one gene signature correlated with poor disease outcome has been proposed and some of them will be employed in clinical practice.

2 Copy Number Variation in Localized and Metastatic Neuroblastoma

Neuroblastoma shows variable clinical aspects: some tumors may regress spontaneously even if they show a diffuse disease with hepatic metastases; others, namely the localized tumors are less aggressive and patients have a benign clinical course. Finally, a very aggressive metastatic tumor onset in about 50% of cases and only 25% of these patients survive at 5 years [7, 8]. Wide-genome study shows diverse chromosome profiles of these tumors.

A great revision in the treatment of neuroblastoma occurred in 1995 when the SIOPEN (International Society of Paediatric Oncology European Neuroblastoma) released the first Localized European Neuroblastoma (LNESG1) trial in which the *MYCN* status (amplified *vs* not amplified) discriminated the therapy of patients with localized disease [9]. Patients in stage 1 with localized tumor without *MYCN* amplification were included in a “wait and see” program rather than treated with chemotherapy as happened in previous trials. On the contrary, patients whose tumor had *MYCN* amplification were treated with standard chemotherapy. A significant decrease of drug toxicity was observed. Afterwards, the following trials: UR (unreseactable), INES (Infants Neuroblastoma European Study Group), LNESG2 (Localized European Neuroblastoma Study Group 2) were released. In each trial, the analysis of *MYCN* gene status was mandatory for therapy.

MYCN gene gives a malignant advantage to tumor cells and makes them more aggressive. Particularly, this was observed in localized tumors were *MYCN* amplification greatly increases the tumor aggressiveness and patients have a poor outcome. The overall frequency of *MYCN* amplification in neuroblastoma is 20% but only 4–5% occurs in localized tumors. Nevertheless, some patients with localized tumor with *MYCN* single copy have a poor outcome as well as those with *MYCN* amplification.

Apart *MYCN*, tumor of advanced metastatic stages have shown several copy number aberrations (CNAs) and diverse genomic platforms have been used to identify CNAs in neuroblastoma. The platforms with bacterial artificial chromosomes (BACs), phage artificial chromosomes (PACs) and cosmids as probes yield a resolution of 1–1.5 Mb, whereas cDNAs yield an average resolution of less

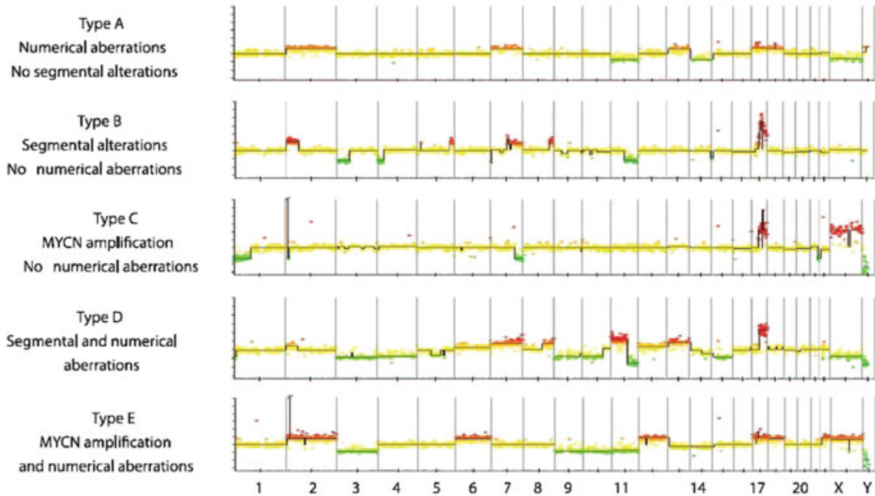

Fig. 17.1 Genomic profile from Type A to Type E of neuroblastoma. Type A has neither segmental nor numerical aberrations whereas Type E has structural aberrations including *MYCN* gene amplification. Type A is associated with tumor whose patient has favorable outcome. Type E is observed in tumor of patients having a rapid disease progression. Intermediate Types show intermediate clinical situations. The genomic pattern of Type E shows several chromosome loss (*green*) and gain (*red*) (Reproduced in part from Janoueix-Lerosey et al. [16])

than 1 Mb. Several studies have proved the utility of BAC, PAC or cDNA-based microarrays for the CGH profiling of NB [5, 10–12]. Our group [13, 14] and others [10, 15] have studied the genome of neuroblastoma by oligonucleotide microarray technology using Agilent Technology.

The wide-genome analysis of neuroblastoma shows that this tumor can be grossly divided into 3 groups: tumors without CNAs, the so called "flat profile"; tumors with numerical CNAs and tumors with both numerical and structural CNAs. The latter group includes those tumors with *MYCN* gene amplification. More recently, Janoueix-Lerosey et al. [16] have used BAC array to refine these categories of tumors, identifying five groups of neuroblastomas with diverse chromosome aberrations: type A, numerical alterations only; type B, segmental alterations without numerical alterations (without *MYCN* amplification); type C, *MYCN* amplification without numerical alterations; type D, both segmental and numerical alterations (without *MYCN* amplification); and type E, *MYCN* amplification with numerical alterations (Fig. 17.1).

All information have contributed to set up the LINES (Localized Intermediate Neuroblastoma European Study) in which, for the first time, the genomic profile is used to discriminate the treatment in some subgroups of patients. In LINES, that started in 2012 not only *MYCN* gene amplification but also structural CNAs are used to evaluate the patients risk and patients are differently treated according their tumor genome profile.

Thus, after 17 years and several preclinical studies, we have moved from single gene (*MYCN* amplified) to genome (structural chromosome abnormalities) risk factor.

The genomic aspect of LINES is a paradigmatic example of translational research. Some groups involved in the management of neuroblastoma patients used Agilent microarray (usually of 44 or 105 K) as diagnostic tool. However, due to the relatively high cost of microarray technology, some other groups used the PCR-based multiplex ligation-dependent probe amplification (MLPA). The aCGH profiles of tumor's patients circulate among the members of a biological committee for the review control, then the genomic profiles are stored in a European Register (SIOPEN-R-NET) [17] and finally they are sent to oncologists.

This system provides all biological and clinical information for each patient enrolled in the study.

On the other hand, if the aCGH is performed as genomic study unrelated to any clinical trials, it is possible to upload the aCGH raw data in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi). Microarray data are identified by an access number and are accessible to the scientific community.

Briefly, the aCGH protocol can be summarized as follows: 2 μg of tumor and reference DNA are sufficient for a microarray of 105 K hybridization. Then microarray is scanned using a G256BA SureScanner (Agilent technology) while the image is elaborated by feature extraction software (v9.5, Agilent Technologies). The analysis is carried out using the CGH Analytics software (v. 3.5.14, Agilent Technologies) applying Z-score algorithm. Probes with a log ratio value of >2 were considered as amplified. It is important to proceed with a quality control for each microarray that can be assessed using the quality metrics provided by CGH Analytics [13, 18].

3 Gene Expression Profiling as Prognostic Factor for Patient Risk Evaluation

As reported above, the DNA profile is a strong prognostic factor already introduced in neuroblastoma trials for personalized treatment. However, microarray technology is also used to evaluate the gene expression of neuroblastoma cells. One of the first studies was performed by Khan et al. [19]. They employed cDNA platform and the artificial neural network algorithm to identify new diagnostic categories based on gene expression signature.

In 2005, Ohira et al. [20] using a similar cDNA platform, performed a risk evaluation of neuroblastoma with an accuracy of 90% and then provided a customized "mini-chip" for routine purpose. Oberthuer et al. [21] designed an 11 K custom dual-color oligonucleotides chip and tested more than 200 tumors. The oligonucleotides were composed of 60 bases linked to the silica microarray wafer with a chemical linker. The chip was scanned with an Agilent instrument and the procedure of dye flipped Cy3- and Cy5-labeled was applied. The raw microarray data were processed

using software packages from the *R*-project (www.r-project.org) and Bioconductor (www.bioconductor.org). Finally, they used the supervised class prediction analysis (prediction analysis for microarrays: PAM) [22] to perform test and validation sets. The 11 K chip gave one of the first gene signatures suitable to discriminate between intermediate- and high-risk patients. Afterwards, several data from gene expression profiling of neuroblastoma have been stored in public database and are available for bioinformatics studies.

De Preter et al. [23] used microarray data of 933 neuroblastoma samples from eight independent studies and identified 42 genes able to predict the clinical outcome of neuroblastoma patients. In 2009, Vermeulen et al. [24] analyzed several gene expression profiling by public databases and selected 59 candidate genes. Then performed real time quantitative PCR of 579 neuroblastoma samples and demonstrated a great sensitivity and robustness of the 59-gene signature as independent marker predictor of disease outcome. Finally, Oberthuer et al. [21] used 144-gene signature to evaluate the patient's risk and the cytotoxic risk of drug treatment. In the study, patients were classified as favorable or unfavorable by the 144-gene classifier, established previously on a set of patients with neuroblastoma. PAM classification were compared with those of common current prognostic markers. The authors used the already known dual color 11 K chip with the same dye flipped procedure.

It is interesting to note that since several gene signatures have also been performed by Affymetrix technology [25, 26] Agilent introduced a one-color microarray [18] making this platform comparable with Affymetrix.

Although more than one signature has been provided to predict the patient's outcome, none of them has been employed in clinical trials until now. The reason of this delay can be found in the following major points: (a) RNA is more unstable than DNA and very often RNA purified from the tumor tissue results degraded; (b) very often the time interval from the tumor exseresis to the RNA purification is too long and RNA is damaged; (c) neuroblastoma tissue heterogeneity can impair the RNA tumor purity because it is diluted in RNA of non-malignant cells. However, when the tumor tissue is homogeneous, composed by small undifferentiated neuroblastoma cells, the gene expression profiling is constant through diverse tissue areas [27].

More recently, Coco et al. [18] and Stgliani et al. [28] demonstrated that tumor of high-risk neuroblastoma patients, namely patients older than 1 year of age with metastatic disease, displays more structural CNAs than those found in younger ones (Fig. 17.2). Furthermore, tumor cells of high-risk patients show the activation of telomerase genes. The genome-transcriptome integration analysis shows a clear relation between loss of DNA loci and low gene expression level of genes located in the lost DNA region.

We conclude that genome profile is useful to predict the risk of patients with low- and intermediate disease, but is not still consistent to predict the outcome of patients with high-risk neuroblastoma. Moreover, so far there are no reliable gene signatures able to predict the patient risk in clinical trials as prognostic factor.

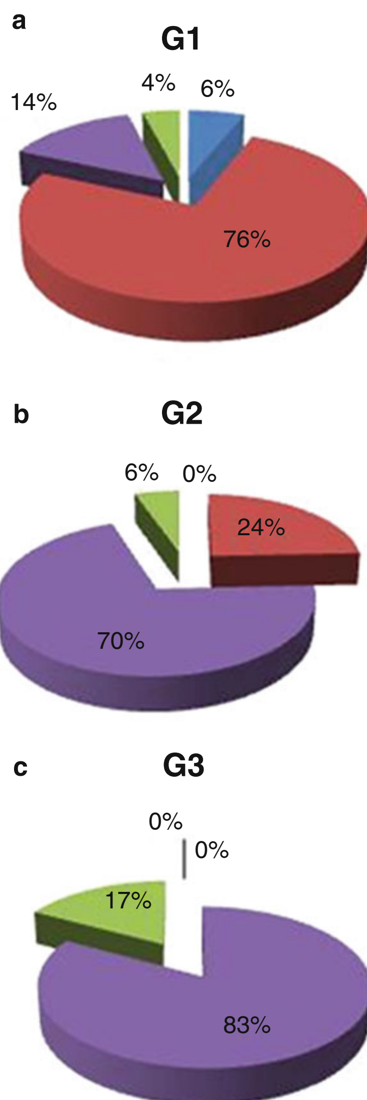

Fig. 17.2 The figure shows the distribution of numerical and structural chromosome aberrations in three groups of metastatic neuroblastomas. G1 are tumors with *MYCN* single copy of patients at stage 4S, a disseminated tumor occurring in patients under 12 months of age. G2 are tumors with *MYCN* single copy of patients under 18 months of age and stage 4 disease. G3 are tumors with *MYCN* amplification of patients older than 18 months of age and with stage 4. *Blue color*: no CNA, *red*: numerical CNA, *purple*: numerical plus structural CNA and green: structural CNA. The combination numerical plus structural CNA increases in frequency from stage 4S tumor (14%) less aggressive to stage 4 tumor (83%) more aggressive phenotype. On the contrary, numerical aberrations are not present in aggressive tumor of patients older than 18 months of age with stage 4 disease (Reproduced by Coco et al. [18])

4 Personalized Medicine of Neuroblastoma

The genomic era has allowed researchers to perform precise profiles of tumor genome and transcriptome. As described above, the goal of the most advanced clinical trials is to treat patients according to the genome abnormalities detected in the tumor [29, 30]. This avoids over treatment with toxic agents for those

patients which tumor has a flat profile or very few CNAs. On the contrary, patients with tumor with dramatic DNA damage and recurrent chromosome structural aberrations should be treated heavily to reach the complete remission. The LINES is the paradigmatic example showing that preclinical genomic studies in a large number of cases are very useful to find a robust genomic prognostic marker. It is to underline that although a huge amount of data was generated by microarray DNA and RNA of neuroblastoma, the discovery of neuroblastoma associated genes remains elusive. Some candidate neuroblastoma genes such as: *SURVIVIN* [31], *BARD1* [32], *LMO1* [33], and *LIN28B* [34] have been proposed. However, the most important neuroblastoma-associated gene was discovered thank to the study of familial neuroblastoma.

Candidate chromosome loci [35] selected by linkage analysis of several members of families with recurrent neuroblastoma combined with wide-genome SNPs array analysis allowed us to identify *ALK* (anaplastic lymphoma kinase) as the first neuroblastoma predisposition gene [36]. In several neuroblastoma familial cases and in about 8–10% of sporadic neuroblastomas *ALK* gene synthetizes a tyrosine kinase receptor that is activated by autophosphorilated. Autophosphorilation of tyrosin kinase domain is caused by point mutation and the most frequent mutations are 1174L and 1275Q. The ALK activation is directly correlated with an increase of neuroblastoma cell growth and ALK down regulation by hsRNA results in an inhibition of cell proliferation.

ALK activation has been found more frequently in advanced disease than in localized ones, giving an additional chance to treat this aggressive tumor [37]. ALK is an orphan membrane receptor and it is a good druggable target, in fact small molecule compounds such as Crizotinib inhibit its expression. Crizotinib is one of the most known compounds able to inhibit *ALK* expression in NSCLC. In this tumor, *ALK* is autophosphorilated as the result of juxtaposition of *ALK* with *EML4* gene [38]. About 80% of patients with *ALK-EML4* activation reached the complete remission after treatment with Crizotinib, indicating that *ALK* is a major gene associated with tumor progression [39]. Although in neuroblastoma *ALK* is activated by missense mutation rather than gene translocation, the use of Crizotinib has been proposed in the Phase I/II trial.

Apart alk receptor, others tyrosine kinase are activated and give their contribution to neuroblastoma carcinogenesis. *TRKB* gene is highly expressed in unfavorable neuroblastoma but has rarely been found mutated. Camoratto et al. [40] have shown a good inhibition activity of the synthetic compound CEP-751 versus *TRKB* both in vitro and in vivo. Moreover, *TRKB* expression has been found associated to drug resistance [41], an aspect to be taken into account for biological therapy.

In conclusion, personalized treatment of neuroblastoma has been introduced at two levels: (1) investigation of the genome tumor profiling to classify the patient's risk and then to decide the best treatment, (2) identification of target druggable molecules to treat patients with new compounds [42].

5 Deep Re-sequencing of Genome Neuroblastoma

Up to now, the genome-wide analysis by microarray technology has greatly improved the knowledge of neuroblastoma genome. Moreover, it has been demonstrated that tumor aggressiveness greatly depends on the number and the type of CNAs. Patients whose tumor has a flat profile have a better outcome than those whose tumor has several non-random structural chromosome aberrations.

Recently, the next generation sequencing (NGS) technology has been used to analyze the genome and transcriptome of neuroblastoma [43–45]. One of the first applications was the study of miRNA expression in neuroblastoma. Shulte et al. [46] analyzed five favorable and five unfavorable neuroblastomas using the SOLiD technology. Cluster analysis differentiated between favorable and unfavorable tumors, and the miRNA of these two groups were significantly differents. The miR17-92 and the miR-181 were overexpressed in unfavorable NBs whereas miR-542-5p and miR-628, were expressed in favorable NBs.

Recently, the results of neuroblastoma exon sequencing performed by Illumina technology were presented during the Advanced Neuroblastoma Research 2012 meeting held in Stockholm. Overall data confirm that tumors of metastatic neuroblastoma have more structural chromosome aberrations than tumors of patients with localized disease. Furthermore, many research groups have focused their attention to the so called Ultra High Risk (UHR) patients. These patients are refractory to any therapy and rapidly relapse and die. Possibly, the deep genomic study of UHR tumors will elucidate the resistance to drug treatment. Since the discovery of *ALK* mutations demonstrated that single nucleotide substitution cans active peculiar receptors leading to a major aggressiveness of tumor cells, we expect that other activated mutations of diverse tyrosine kinase molecule will be discovered. However, it is to note that preliminary data of exome sequencing indicates that the number of mutations is lower than expected. So, the possible presence of single nucleotide mutations in non-coding sequence makes the re-sequencing of the entire genome necessary.

Finally, it is evident that although NGS approach is expensive, we need to known the distribution of mutations in neuroblastoma genome in order to identify new targets for future advance of personalized treatments.

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