Chapter 2
Molecular Landscape Profile of Melanoma

Giuseppe Palmieri, Maria Colombino, Milena Casula, Maria Cristina Sini, Antonella Manca, Marina Pisano, Panagiotis Paliogiannis, and Antonio Cossu

Abbreviations

AKT	RAC-alpha serine/threonine-protein kinase
ARAF	A-Raf proto-oncogene, serine/threonine kinase
ARID2	AT-rich interaction domain 2
ATM	ATM serine/threonine kinase
BAP1	BRCA1 associated protein 1
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BRCA1–2	BRCA1–2, DNA repair associated
CCND1	cyclin D1
CDH1	cadherin 1
CDK4	cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2A
CHEK2	checkpoint kinase 2
CRAF	Raf-1 proto-oncogene, serine/threonine kinase
CSD	cumulative solar damage
CTNNB1	catenin beta 1
DDX3X	DEAD-box helicase 3, X-linked
EGFR	epidermal growth factor receptor
ERBB1–4	erb-b2 receptor tyrosine kinase 1–4

G. Palmieri (✉) · M. Colombino · M. Casula · M. C. Sini · A. Manca · M. Pisano
Unit of Cancer Genetics, Institute of Genetic and Biomedical Research (IRGB), National Research Council (CNR), Sassari, Italy
e-mail: mariacristina.sini@cnr.it; antonella.manca@icb.cnr.it; marina.pisano@cnr.it

P. Paliogiannis · A. Cossu
Department of Medical, Surgical and Experimental Sciences, University of Sassari, Sassari, Italy
e-mail: cossu@uniss.it

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ERK	extracellular signal-regulated kinase
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FISH	fluorescent in situ hybridization
GDP	guanosine diphosphate
GNA11	G protein subunit alpha 11
GNAQ	G protein subunit alpha q
GTP	guanosine triphosphate
HER2	human epidermal growth factor receptor 2
HRAS	HRas proto-oncogene, GTPase
IDH1	isocitrate dehydrogenase (NADP+) 1, cytosolic
KDR	kinase insert domain receptor
KIT	KIT proto-oncogene receptor tyrosine kinase
KRAS	KRAS proto-oncogene, GTPase
MAPK	mitogen-activated protein kinase
MC1R	melanocortin-1 receptor membrane receptor
MEK	MAPK/ERK kinase
MET	MET proto-oncogene, receptor tyrosine kinase
MITF	microphthalmia-associated transcription factor
mTOR	mechanistic target of rapamycin kinase
NF1	neurofibromin 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	next-generation sequencing
NRAS	NRAS proto-oncogene, GTPase
PALB2	partner and localizer of BRCA2
PD-L1	programmed death ligand 1
PDGFRA	platelet-derived growth factor receptor alpha
PI3K	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha
POT1	protection of telomeres 1
PPP6C	protein phosphatase 6 catalytic subunit
PREX2	phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2
PTEN	phosphatase and tensin homolog
RAC1	Rac family small GTPase 1
RAF1	Raf-1 proto-oncogene, serine/threonine kinase
RB1	RB transcriptional corepressor 1
RET	ret proto-oncogene
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SF3B1	splicing factor 3b subunit 1
SNX31	sorting nexin 31
SOS1	SOS Ras/Rac guanine nucleotide exchange factor 1
SPRED1	sprouty-related EVH1 domain containing 1
STK19	serine/threonine kinase 19
TERT	telomerase reverse transcriptase

TET	tet methylcytosine dioxygenase
TME	tumor microenvironment
TP53	tumor protein p53
UV	ultraviolet
VEGFR2	vascular endothelial growth factor receptor 2

Introduction

Cutaneous melanoma is mostly diagnosed at an early stage of disease and, although its incidence is continuously increasing in the population from western countries, it can be effectively treated by surgical excision [1]. Conversely, a large fraction of advanced stages remains refractory to systemic therapies [2]. Despite the impressive advancements into the treatment of the disease during the recent past years, clinical outcomes are still hardly predictable in melanoma patients due to the marked heterogeneity of the disease from the biological and molecular point of view [3, 4]. Therefore, the need to obtain a classification of the various tumor subtypes with distinct genetic and molecular characteristics becomes mandatory, definitively overcoming the concept according to which melanoma—as for all cancer subtypes—can be considered a single disease.

Given the central role of protein kinases in mediating different cell pathways, it is not surprising that aberrant kinase activity is a common feature of cancer cells and that kinase inhibitors are used and researched as anticancer therapies, including melanoma [5]. When constitutively activated, some kinases can be oncogenic and directly drive tumor growth, while other kinases can play an indirect role, acting as regulators of oncogenic intracellular signals or promoting extracellular effects into the tumor microenvironment such as the induction of angiogenesis or mechanisms for invasion and immune escape [6, 7].

From the genetic point of view, the pathogenesis of melanoma—like all other forms of malignant neoplasms—is based on the acquisition of sequential alterations affecting specific chromosome loci and genes involved in metabolic and molecular pathways controlling all such cellular homeostasis mechanisms [8, 9]. In other words, melanoma pathogenesis and, more in general, tumorigenesis may be actually considered as due to a process of sequential accumulation of mutations and changes in specific genes and DNA regions [8, 9].

Molecular Complexity of Melanoma

Cutaneous melanoma (CM) has a high prevalence of somatic mutations, both in primary lesions and—to a greater extent—in metastatic lesions, with an average mutation rate estimated to be much greater than 20 mutations per megabase of genomic DNA [10, 11]. Considering data from studies on CM with NGS-based

mutation analysis, majority (up to 70–80%) of DNA sequence variations is represented by C > T substitutions (including a small fraction of <5% cases constituted by CC > TT transitions). These variants are due to the mutagenic effects of the ultraviolet (UV) radiations on exposed skin, and the entire set of them is usually indicated as the UV mutation signature [12, 13]. The UV effects on mutagenesis may thus contribute to determine that CM displays one of the highest mutation load compared to that from other cancer types [14]. On this regard, it appears clear that the threshold of the tumor mutation burden (TMB) may vary across cancer types, probably modified by the intervention of multiple factors linked to distinct tumor microenvironments (such as immune cell infiltration or exclusion, expression levels of cytokines and/or checkpoint molecules, and clonality rates) [11]. All these factors are involved into the different response rates and clinical benefits to immune checkpoint inhibitors across all cancer types [11, 14]. Although TMB assessment is not a standardized biomarker that affects treatment decisions, efforts are being conducted to implement TMB measurement assays and uniform the interpretation of the data [15].

As a confirmation of the UV impact on the increase of the TMB levels in the skin, noncutaneous (i.e., ocular and mucosal) melanomas present a markedly lower mutational load and lack the UV signature [16, 17]. Moreover, the mutation rate in melanomas occurring on chronically sun-exposed skin was found to be at least five times higher than those on the skin not subject to sun damage (ratio of >20 mutations per megabase vs. ≤5 mutations per megabase, respectively) [8, 18]. Finally, there is clear epidemiological evidence of a relationship between nevus number, sun exposure, and C > T mutations [19].

Over the past few years, specific oncogenic mutations have been identified in genes encoding for RAS/RAF/MEK/ERK kinases belonging to the so-called mitogen-activated protein kinase (MAPK) signal transduction cascade, which regulate the main processes of cell proliferation and cell survival [10, 16, 20]. On the basis of in-depth mutational analyses through several next-generation sequencing (NGS) approaches [10, 12, 16, 21, 22], CM patients are currently classified into the following distinct molecular subtypes according to their mutational status:

- Cases with mutations activating the *BRAF* oncogene
- Cases with mutations activating the *RAS* oncogenes (including the three isoforms: *HRAS*, *KRAS* and, mainly, *NRAS*)
- Cases without mutations in these two oncogenes (with occurrence of activating mutations in *KIT* and increased frequency of mutations inactivating the *NF1* gene)

However, additional genes may be mutated at different prevalence within such CM subtypes, contributing to the molecular heterogeneity of the disease at somatic level. According to the mutation frequency reported in studies with NGS-based mutation analysis in CM samples, the mutated driver genes associated with these three melanoma subtypes could be divided into three groups: one (*TP53*, *NF1*, *CDKN2A*, and *ARID2*), with mutation frequency between 10% and 20%; the second (*PTEN*, *PPP6C*, *RAC1*, and *DDX3X*), with mutation frequency ≥5% and <10%; and the third one (up to 20 genes), with mutation frequency <5% [12, 23]. In

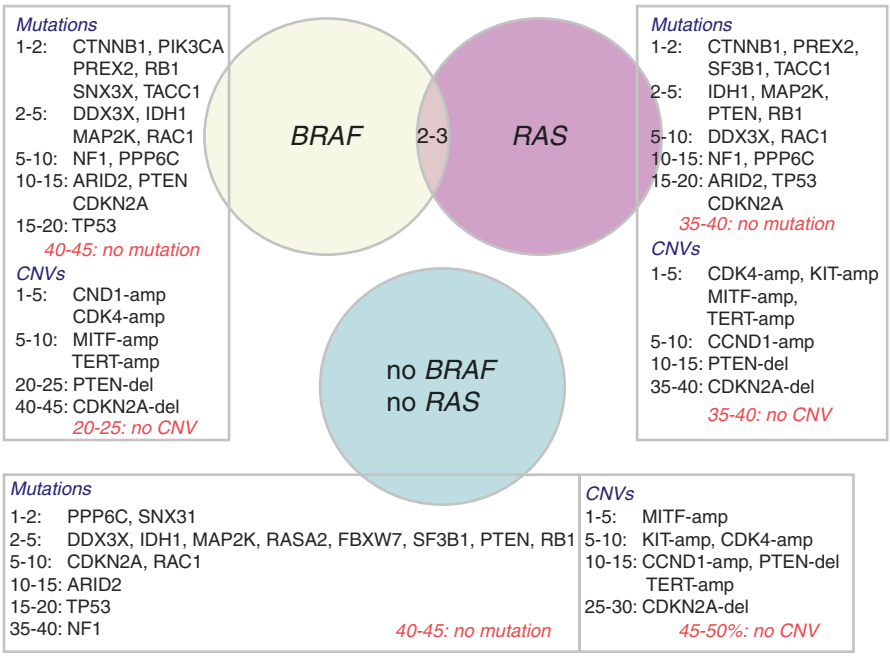

Fig. 2.1 The three main (BRAF^{mut}, RAS^{mut}, and non-BRAF^{mut}/non-RAS^{mut}) melanoma subtypes. Additional altered genes are reported for each subgroup. Numbers indicate the mutation frequency. *CNVs* copy number variations, *ampl* amplification, *del* deletion

Fig. 2.1, the three main mutational subtypes of melanoma and the frequencies of the coexisting mutated genes are summarized.

Overall, a complex model of tight interactions between such candidate genes and their signaling cascades whose alterations are important for the development of melanoma is emerging, including pathways mediating protection against ultraviolet-induced DNA damage and DNA repair, telomere maintenance, immunity, melanocyte differentiation, and cell adhesion. Some of them are involved in melanoma susceptibility and, therefore, in the increase of the risk for disease onset.

Genetic Integrity and Melanoma Susceptibility

The CM induction and development are extremely complex involving genetic and environmental factors, such as specific predisposing germline mutations, skin color, number and type of nevi, and sun exposure [24, 25]. About one tenth of melanomas occurs in patients with disease recurrence in family and less than half of these familial cases have been attributed to inheritance of a mutation in a highly penetrant predisposition gene [26]. In the majority of familial melanomas, a pattern of sequence variations in low- or very-low-penetrance predisposition genes are thought

to contribute to the melanoma inheritance [27]. In other words, mutations in multiple high-penetrant genes or the presence of several moderate-to-low risk alleles may explain the heterogeneity of presentation of the various melanoma pedigrees, as well as the multiple melanoma phenotypes [27, 28]. In a more general view, a combination of inheritance of familial patterns of variants/polymorphisms in multiple genes and different levels of exposures to environmental mutagens participate into the development of melanoma [28, 29].

In addition to a "melanoma-dominant" pattern of inheritance, melanoma can also occur as a "subordinate" neoplasm in the context of mixed cancer syndromes [27–29]. The significant increase of the melanoma risk in mixed cancer syndromes is often caused by mutations in genes involved in DNA repair by homologous-recombination mechanisms such as those regulated by *BAP1*, *BRCA1*, *BRCA2*, and *TP53* genes [29, 30]. This represents a clear clue pointing at the importance of the maintenance of the genome integrity for cutaneous melanoma susceptibility. An association between multiple independent variants in the *TP53* gene and cutaneous melanoma has been described for a long time [31]. *TP53* responds to cellular stresses and early cancerogenic events by inducing DNA repair mechanisms, cell cycle arrest, apoptosis, and cellular senescence toward the elimination of extensively damaged cells [32]. Mutations/deletions enhancing dysfunction of TP53 or inducing up-regulation of HDM2 (mouse double minute 2, human homolog), whose gene product is the natural inhibitor of p53, may inactivate the p53—the so-called Guardian of the Genome [33]. In the skin, this results in clonal expansion of cells that carry accumulated mutations with an induced increase in both nevus density and cutaneous melanoma risk [29, 30]. A growing body of evidence is supporting a key role for telomere maintenance in cutaneous melanoma susceptibility, with partial involvement of *POT1* and *TERT* genes, as well as *CCND1* and *ATM* loci [29, 34, 35]. These genes play established roles not only in telomere maintenance but also in DNA repair and regulation of senescence [29]. As a further indication that controlling the telomere function/maintenance is somehow important in melanoma pathogenesis, predisposing mutations have been observed in *POT1* and *TERT* genes among few melanoma families with high recurrence of the disease [34, 35]. Among others, mutations in the *TERT* promoter (*TERTp*) represent a common mechanism for reactivating the telomerase reverse transcriptase protein and, thus, maintaining the telomere length in cancer cells among many solid tumors [36, 37]. The occurrence of such activating mutations may contribute to increase TERT expression levels, alterations into target transcription factor binding sites, telomere stabilization, and cell immortalization and proliferation [37]. Finally, inherited mutations in the *BAP1* gene, which were firstly reported as an inherited cause of uveal melanoma, have been associated with a risk of lung cancer and meningioma and now recognized to also increase the risk of cutaneous melanoma [38, 39].

Overall, the above-mentioned genes and other genes involved in melanocyte proliferation or differentiation (such as *CDK4*, *MITF*, *MC1R*, *PTEN*, *RB1*) are very rarely mutated in families with melanoma recurrence (altogether, less than 3% mutated cases in more than 2500 pedigrees) compared to the *CDKN2A* high-risk

susceptibility gene, which remains the only most prevalent familial melanoma gene (about 20% mutated cases within the same large pedigrees' series) [40].

The *CDKN2A* tumor suppressor gene encodes two proteins: $p16^{CDKN2A}$ and $p14^{CDKN2A}$; inactivation of both alleles is necessary for the development of melanoma [41]. In melanoma families, about one fifth of probands may carry germline mutations in *CDKN2A*, whereas about two-thirds of melanoma patients present a *CDKN2A* gene inactivation (by genetic or epigenetic mechanisms) at the somatic level [9, 29, 42]. Significant discrepancies in *CDKN2A* mutation frequency are reported in melanoma pedigrees from different geographical areas [43], including northern and the southern parts of Italy [44, 45]. This suggests that additional genetic factors tightly linked to the patients' origin (contributing to the so-called genetic background) may account for differences in the prevalence of germline mutations in *CDKN2A* gene [20, 29, 46]. Activation of the downstream CDK4-RB effectors through inactivation of $p16^{CDKN2A}$ has been reported to promote melanoma progression; indeed, prevalence of this activation significantly increases during transition from primary to metastatic melanomas and achieves the maximal level in melanoma cell lines [20]. Similarly, the inactivation of $p14^{CDKN2A}$ causes the reduction of the p53 protein levels, with consequent impairment of the cell-cycle progression control and inhibition of the apoptosis, contributing to increase the survival of melanoma cells [20]. Activation of the CDKN2A-dependent pathways may also be associated with the amplification of the *CyclinD1* (*CCND1*) gene, which is generally found in melanomas negative for *BRAF* and *RAS* mutations [12].

Finally, the *CDH1* gene, encoding E-cadherin, is specifically upregulated in both normal melanocytes and keratinocytes, playing a crucial role in cell-cell adhesion between these two cell types [47]. In melanoma, the expression levels of E-cadherin are markedly reduced or quite absent, promoting a concurrent switch into the type of cell-cell adhesion and a preferential association with fibroblasts and vascular endothelial cells [47, 48]. This loss of E-cadherin expression thus results in enhanced invasion and constitutes an independent factor of poor prognosis in melanoma patients [48]. Interestingly, germline variations leading to upregulation of the *CDH1* expression in melanocytes seem to act as a protective mechanism, limiting reactive oxygen-mediated apoptosis and allowing cells damaged by oxidative stress to survive in the skin [49].

Molecular Heterogeneity and Melanoma Pathogenesis

At somatic level, a specific core of genes and pathways has been shown to play a crucial role in the pathogenesis of melanoma: RAS-dependent BRAF-ERK pathway, RAS-dependent PI3K-AKT pathway, RAS-regulating NF1 and KIT genes (Fig. 2.2). Overall, less than one tenth of CMs has been found to be negative for any genetic alteration, including both deleterious mutation and copy number variation (CNV), as ascertained by NGS-based analyzes carried out in recent past years [12]. These findings further confirm that melanoma is a highly mutated malignancy.

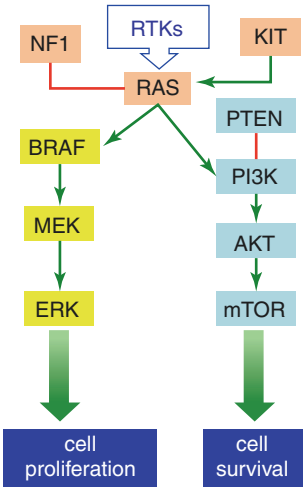

Fig. 2.2 Core signal transduction pathways involved in melanomagenesis. Green arrow, activating signals; red lines, inhibiting signals. *RTKs* receptor tyrosine kinases

Among the CNVs (Fig. 2.1), inactivation by deletion of *CDKN2A* and *PTEN* tumor suppressor genes has been confirmed to represent the structural rearrangement most frequently implicated in pathogenesis of all molecular subtypes of melanoma [12].

BRAF

The RAF kinase family consists of three proteins (ARAF, BRAF, and CRAF), all of which are part of the MAPK pathway; the formation of complexes by these different isoforms plays an important role in their activation [50]. In melanoma, the *BRAF* gene is mutated in 45–50% of cases; the most prevalent mutation (about 90% of cases) is represented by a substitution of a valine with glutamic acid at codon 600 ($BRAF^{V600E}$) [10, 12]. The remaining *BRAF* mutations mostly occur at the same codon: V600K (the most frequent; <10% of cases), V600D, and V600R; mutations in codons other than V600 are not common (among them, K601 is the most prevalently affected codon) [51]. The constitutive oncogenic activation of BRAF promotes a continuous, uncontrolled stimulation of cell proliferation [52]. There is an inverse relationship between the prevalence of the *BRAF* mutation and the age of melanoma onset: >50% of patients <30 years and only 25% ≥70 years harbor a $BRAF^{V600E}$ mutation. Inversely, non-V600E mutations (including V600K) are reported to steadily raise with the increase of the diagnosis age: <20% of patients <50 years and >40% in those ≥70 years [8]. The demonstration that *BRAF* is mutated in the majority (>50%) of common nevi suggests that its oncogenic activation is a necessary but not sufficient condition for the development of melanoma, being considered as an initiation event in the neoplastic transformation of melanocytes [18, 53]. The precise pathogenesis of *BRAF* mutations remains as yet unclear, but these observations suggest a complex relationship between intermittent sun

exposure and nevus formation [8, 31]. It has been questioned whether *BRAF* mutations might actually result from DNA damage consequent upon UV exposure. However, it is of note that neither *BRAF* nor *NRAS* mutations have the classical genetic signature of mutagenesis as a result of UV light exposure, which instead is associated with the BRAF/NRAS wild-type status [54].

Since common nevi are mutated in *BRAF*, alterations in other genes are therefore thought to cooperate with the *BRAF* mutations in inducing transformation and neoplastic progression of the melanocytic cells [10, 18, 31, 53]. On this regard, melanomas carrying a *BRAF* mutation are characterized by coexistence of additional specific gene alterations, mainly loss of PTEN and inactivation of *CDKN2A* or *TP53* (Fig. 2.1).

The NGS analyzes have clearly indicated that oncogenic mutations in *BRAF*, *RAS*, and *KIT*—within the core gene pathways involved in melanomagenesis reported in Fig. 2.2—are mutually exclusive (≤3% of patients presents with coexistence of mutations in such oncogenes at the time of diagnosis) [12]. The proportion of coexisting mutations in these genes is deeply modified by the use of the combination of BRAF and MEK inhibitors for the treatment of the patients with a BRAF-mutant melanoma, as consequence of the acquisition of resistance to the target therapy [55]. Patients with advanced melanoma (American Joint Committee on Cancer [AJCC] stage IV or III inoperable [56]), as well as those with radically operated AJCC stage III melanoma, both carrying a BRAF-V600 mutation, may be addressed to the therapy with BRAF and MEK inhibitors [57–65]. Although with lower efficacy, even patients with rare (V600 and non-V600) *BRAF* mutations can respond to targeted therapy [66]. The assessment of the BRAF mutational status has become mandatory for molecular classification of patients with stage III or IV melanomas [67].

From the practical point of view, the evaluation of the *BRAF* mutational status in stage IV melanoma patients should be carried out on tissue biopsy from the metastasis, as it represents the most recently developed tumor lesion and consists of a preponderant population of neoplastic cells. When this is not possible and in stage III melanomas, the mutational investigation may be performed on the tissue sample from primary melanoma. In this sense, a good agreement has been demonstrated in the *BRAF* mutational patterns between metastatic lesions (mainly, lymph node sites) and primary melanomas [68, 69]. In consideration of a certain rate of intertumoral heterogeneity [68, 70], a *BRAF* mutation analysis providing a wild-type result on the primary tumor among advanced melanoma patients should be however repeated on tissue biopsy from an accessible metastasis.

The *BRAF* mutational status can be assessed using methodologies presenting different degrees of sensitivity and specificity, though the complexity of the genes and pathways involved in melanomagenesis strongly suggest to move toward innovative approaches using a multigenic screening based on NGS assays [71]. The enrichment of the tissue sample is thus fundamental and the percentage of neoplastic cells present in the tissue to be sent for molecular analysis should be really representative (never be less than 50%) [72]. In the case of melanoma associated with nevus, it is crucial that the sample enrichment is focused on the isolation of a pure

population of melanoma cells, as melanocytic nevi can be carriers of *BRAF* mutations at the same frequency found in melanomas (see above and [53]).

The recently increased importance of achieving the classification of the *BRAF* mutational status for other cancer types—mainly, lung [73, 74] and colorectal [75, 76] adenocarcinomas—have been demonstrated to be highly sensitive to the response to the treatment with combined BRAF and MEK inhibitors. According to recent publications [77–80], it has been suggested to define a sort of functional classification for the various BRAF variants:

- Class I *BRAF* mutations include V600 mutations, which are able to induce a constitutive activation of the MAPK signaling cascade without the need of dimerization and an upstream RAS activation.
- Class II *BRAF* mutations include variants in codons different from the V600 one (mainly, G464, G469, L597, and V601), which are still independent from the upstream RAS activation but require the protein dimerization to activate the signal transduction pathway.
- Class III *BRAF* mutations include variants in codons located outside the core kinase domains, which require either upstream activation and protein dimerization with CRAF or, in minimal part, with ARAF (see below).

RAS

The RAS family is composed of three tissue-specific gene isoforms: *HRAS*, *KRAS*, and *NRAS*. The latter gene is the one mostly mutated in melanoma [10]. *NRAS* mutations are found in about 25% of melanoma patients; they occur almost exclusively in a single gene codon (Q61, about 90% of cases); in the remaining 10% of cases, the mutated codon is G12 or G13 (31–33) [10, 81].

The oncogenic stimulation of RAS is able to activate specific cytoplasmic downstream proteins with kinase function: RAF and PI3K [81]. As previously mentioned, *RAS* mutations have been demonstrated to be mutually exclusive with *BRAF* mutations in nearly all cases (coexistence of the two genes mutated in a constitutive manner is reported in 2–3% of melanomas) [12]. Occurrence of RAS activation—both for the acquisition of mutations or functional oncogenic induction—may cause that the translation of the mitogenic signal in the MAPK pathway can be switched to dimerization of wild-type CRAF or, to a lesser extent, wild-type ARAF, which therefore acquires a key role in maintaining cell proliferation stimulation in this subset of melanomas [50, 82]. Interestingly, an increased activation of the NRAS-CRAF axis has been described as responsible for the acquired resistance to BRAF inhibitors [55]. On this regard, enhanced RAS-dependent RAF dimerization has also been involved into the pathogenesis of squamous cell carcinomas, as a side effect in subsets of patients treated with BRAF inhibitors [83]. These agents have been demonstrated to indeed activate MAPK pathway by inducing RAF dimerization in cells lacking *BRAF* mutations, leading to increased keratinocyte proliferation [84]. More in general, the enhanced RAF dimerization represents a process that

may be constitutively promoted by any form of activation—through genetic (mutations) or functional mechanisms—in any of the three isoforms of *RAS* [84].

To date, there are no clinical studies that support the use of specific target therapy for melanoma with *NRAS* mutation. Two clinical studies (a phase II trial and a randomized phase III trial) have shown only a minimal therapeutic efficacy of the MEK inhibitor binimetinib in patients with advanced melanoma carrying an NRAS mutation [85, 86]. Therefore, detection of the *NRAS* mutation is not actually required in clinical practice, but it can be useful for the insertion of patients in further clinical studies only.

KIT

A limited fraction of melanomas that are not mutated in *BRAF* and *RAS* may carry activating mutations in *KIT* [10, 12] encoding a tyrosine kinase receptor of the cell membrane and resulting in a continuous induction of cell proliferation, through functional stimulation, mainly of the MAPK pathway (Fig. 2.2). Among the *KIT* mutations, those most frequently associated with melanoma are represented by the L576P mutation in exon 11 and the K642E mutation in exon 13 (other mutations of the KIT gene reported in melanoma are V599A, D816H, D820Y) [87]. The frequency of KIT mutations has been reported in 1–3% of total melanomas, but it may deeply vary among the different melanoma subtypes: 15–20% mucosal melanomas (the highest prevalence is observed in anal melanoma); 10–15% acral melanomas, 3% melanomas on chronically photo-exposed skin, almost total absence in melanomas in unexposed skin areas) [87–90].

The evaluation of the KIT mutational status is thus strongly indicated in the acral and mucosal melanomas, though after the evaluation of the BRAF mutational status (again, mutations in both genes are mutually exclusive; see above). In advanced melanomas with KIT mutation, treatment with immunotherapy is actually indicated. Albeit limited, some clinical experiences with phase II studies have shown objective responses with the use of KIT inhibitors in melanomas harboring mutations in exon 9, 11, or 13 [87, 91–93].

NF1

The NF1 mutations cause an inherited multisystem genetic disorder, neurofibromatosis type 1, at germline level and promote cell proliferation mainly through activation of the MAPK pathway at somatic level [94]. As reported by The Cancer Genome Atlas, inactivating mutations of the NF1 gene occur in a subset of melanomas (approximately 15% of cases) [10]. Physiologically, NF1 encodes for neurofibromin, a RAS-GTPase-activating protein, which negatively regulates RAS signaling by stabilizing the RAS-GDP-inactive form; mutations functionally silencing the NF1 gene result in RAS activation and enhancement of the malignant transformation in melanocytes [94]. These data demonstrate that inactivation of NF1

may contribute to increase the activity of several RASopathy genes—such as SOS1, PTPN11, RAF1, and SPRED1—in melanomagenesis [95, 96]. Tumors with mutations in NF1 and constitutive activation of RASopathy genes are often associated with a higher mutational load and, consequently, a greater probability of generating neoantigens [97]. Therefore, NF1-mutated tumors—including the desmoplastic melanoma subtype, which is characterized by a high mutational load and frequent NF1 mutations—are thought to be more sensitive to immunotherapy and, in particular, to treatment with immune checkpoint inhibitors [98].

Although the *NF1* mutations are the most prevalent alterations in the group of melanomas with both wild-type *BRAF* and wild-type *RAS* (about 35% of these cases), they are also present in *BRAF*- and *RAS*-mutated melanomas (about 5% and 15% of such cases, respectively) [12]. Melanomas with *NF1* mutations generally occur on chronically sun-exposed skin or in elder individuals and, as previously affirmed, show a higher mutation burden (to this latter, the UV-induced mutagenesis also contributes) [94, 96, 98]. Finally, an increase in frequency of *NF1* mutations is observed among BRAF-mutant tumors intrinsically resistant to BRAF inhibitors, as well as in melanomas of patients acquiring resistance to BRAF inhibitors [55].

PI3K-PTEN

The PTEN-PI3K-AKT-mTOR kinase cascade represents the core pathway—mainly dependent on RAS activation—involved in regulation of cell survival (Fig. 2.2). Oncogenic activation of the PI3K/AKT pathway, including that underlying the acquired resistance to treatment with BRAF and MEK inhibitors, can occur through several mechanisms: mutation and/or amplification of RTK genes (i.e., the four ERB-B receptor tyrosine kinase family members: *ERBB1*/EGFR, *ERBB2*/HER2, *ERBB3*/HER3, and *ERBB4*/HER4) [99, 100], deletion of PTEN [101, 102], somatic alterations of *AKT* [103, 104], or activating isoforms of RAS [94–96]. The *ERBB* genes encode transmembrane proteins that are activated by either homo- or heterodimerization with other *ERBB* family members, resulting in activation of both PI3K/AKT and MAPK signal transduction pathways [18, 100]. As a confirmation about the tight interaction between the *ERBB* genes and the PI3K/AKT pathway, mutations in the *ERBB* family members are targetable with PI3K inhibitors [100]. In melanoma, the PTEN gene is deleted in about a third of cases, with complete loss of expression of the corresponding protein in 10–20% of primary melanomas; the level of this loss increases during neoplastic progression, up to 40–50% in melanoma cell lines [10, 12]. The activation of the PI3K pathway results in aberrant growth of melanoma cells and increased survival capacity with the acquisition of resistance to apoptosis, as well as to acquired resistance to the treatment with targeted therapies in various tumor types (in melanoma, to the combination of BRAF and MEK inhibitors) [29]. Loss of PTEN in melanoma has been associated with poor or absent T-cell-inflamed tumor microenvironment, thus affecting the response to immune checkpoint inhibitors [105] and correlated with poor prognosis (a decreased overall survival and higher tendency to develop brain metastasis) in stage III melanoma patients carrying a BRAF mutation [106].

Molecular Classification of Melanoma Subtypes

As summarized in Fig. 2.3, the findings from the multigenic mutation analyses of the NGS-based studies indicate that CMM patients may be divided in at least six main distinct molecular subgroups [12]. Starting from the definition of the BRAF mutational status at baseline of any diagnostic and therapeutic path among AJCC stage III and IV patients, the molecular classification of the melanoma can identify molecular subtypes according to the coexistence of pathogenic mutations in other genes associated or not to the mutated and wild-type BRAF/NRAS (Fig. 2.3). From a practical point of view, the characterization of these subtypes becomes extremely important for a more appropriate assessment of clinical and biological features of patients with melanoma, as well as for programming the most correct therapeutic approach in each patient's subgroup.

Moving toward the use of the NGS-based assays for multigenic mutation testing in clinical practice, the following additional genes demonstrated markedly implied in melanomagenesis are required to be incorporated in mutational screening at somatic level. In recent past, several aspects have been clarified in order to more easily conduct the NGS analyses in hospital laboratories and, thus, to transfer the use of NGS assays in clinical practice [72, 107]. In particular, melanoma-specific gene panels have become commercially available for detecting somatic mutations through their use on the two most common NGS platforms [Illumina Inc. (San Diego, CA, USA) and Life Technologies-Thermo Fisher Scientific (Waltham, MA, USA)]. They include the following additional genes involved in melanoma pathogenesis:

- ARID2, CDK4, CDKN2A, CTNNB1, ERBB4, EZH2, GNA11, GNAQ, GRIN2A, HRAS, IDH1, KIT, KRAS, MAP 2 K1, MITF, PREX2, RAC1, RB1, TERT, TP53, TYR (Oncomine Melanoma extended panel; Life Technologies-Thermo Fisher Scientific)

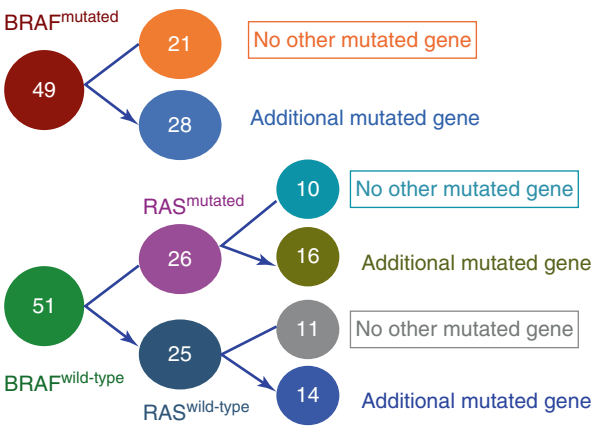

Fig. 2.3 Classification of 100 melanoma patients according to the gene mutation status distribution

- AKT, BRAF, CCND1, CDK4, CDK6, CTNNB1, ERBB2, ERBB3, ERBB4, GNA11, GNAQ, IDH1, KIT, KRAS, HRAS, MAP 2K1, MTOR, NRAS, PIK3CA, RAF1 (Focus Ampliseq panel, Illumina)

Here, we briefly summarize the characteristics of the main additional genes contributing to melanomagenesis.

CTNNB1

The *CTNNB1* gene encodes β-catenin, a scaffold protein interacting with components of the WNT signaling pathway, adhesion molecules (such as cadherin proteins and α-catenin), and epigenetic-transcriptional regulators (such as EZH2 and SMARCA) [108]. Among others, EZH2 positively regulates the WNT/CTNNB1 signaling in some cancer types, being essential for acquisition of cell motility [109]. On this regard, EZH2 has been found to positively regulate genes involved in cytoskeletal modifications underlying cell invasiveness and promotes CM motility and metastasis [109]. Coexistence of $BRAF^{V600E}$ mutation and *EZH2* activation is rather prevalent in melanoma by enhancing proliferation and survival of melanoma cells [110]. Activating *CTNNB1* mutations, as well as inactivating mutations in negative regulators of the β-catenin pathway, may determine effect into the tumor microenvironment by interfering with the T-cell priming and infiltration, favoring immune evasive mechanisms (including the suppression of chemokines and cytokine gene expression by tumor cells) [111–114].

PREX2—GRIN2A

Activating mutations in *PREX2*, a guanine nucleotide exchange factor involved in regulating the activity of the *PTEN* gene product [23, 115, 116], and in *GRIN2A*, a glutamate receptor participating to the control of cell proliferation [23, 116], have been reported in about 15% and 33% of melanoma samples, respectively. Oncogenic activation of both genes contributes to facilitate survival, growth, and invasion of melanoma cells. Patients with GRIN2A mutations may have a more aggressive disease and a poorer clinical outcome, though further studies are needed to confirm a role for such alterations as a prognostic marker [117].

RAC1

In CM, activating mutations occur in a specific dipyrimidine site of the RAC1 gene, representing a typical UV signature [21]. Unlike common *RAS* oncogenic mutations that impair or abolish intrinsic GTP hydrolysis ability and render the kinase constitutively active in terms of signaling, the RAC1 mutant protein abnormally accelerates the exchange from inactive GDP- to active GTP-isoform [118]. In melanoma,

mutated *RAC1* is often found in combination with additional gain-of-function mutations of other oncogenes (*BRAF* or *NRAS*) and/or loss of function mutations in tumor suppressor genes (*NF1*, *TP53*, or *PTEN*), suggesting that *RAC1* is not generally sufficient on its own to drive tumor formation [21, 119]. It has been suggested that coexistence of BRAF mutation and *RAC1* mutation in primary CM may be associated with thinner melanomas [120].

ARID2—IDH1

Somatic mutations in *ARID2* and *IDH1* genes, both involved in chromatin remodeling, have been found to be significantly associated with elevated levels of global DNA methylation in several malignancies, including melanoma [121, 122]. In particular, mutations in these two genes may cause important epigenetic dysfunction and hypermethylation of several target genomic loci, thus leading to aberrant gene expression in both primary tumor and metastases [123]. *ARID2* and *IDH1* somatic mutations have been found at a relatively high frequency (approximately 30%) in melanoma [10, 12].

MITF

The *MITF* gene acts as a master regulator of melanocyte development, function, and survival, by modulating various differentiation and cell-cycle progression genes [124]. The levels of expression of *MITF* are demonstrated to determine two different behavior profiles for melanoma cells. A proliferative profile, which is based on upregulation of *MITF* and other melanocytic genes (such as *TYR* and *DCT*), is associated with high rates of proliferation and low motility. Conversely, the invasive signature, which is based on downregulation of these same genes and upregulation of others ones (such as *INHBA* and *COL5A1*) involved in modifying the extracellular environment, is associated with lower rates of proliferation and high motility. *MITF* is amplified in a fraction of human melanomas, and its amplification rates increase in metastatic disease [124]. Coexistence of high MITF expression levels and *BRAF* mutations is able to transform human melanocytes; thus, MITF can function as a melanoma oncogene [124, 125]. Moreover, a reduction of *MITF* activity sensitizes melanoma cells to chemotherapeutic agents [125].

Conclusive Remarks

All these findings are strongly indicative of the existence of complex molecular regulatory mechanisms, which ensure the integrity and regularity of the various cellular functions in normal melanocytes. As melanoma progresses from benign nevi to invasive tumors, there are several changes into the genome, and molecular

pathways accumulate in cells and contribute to determine different biological features. In recent past years, concurrent intracellular alterations in molecular pathways have been found to even interfere with the homeostasis of the tumor microenvironment and interact with various extracellular factors participating in immune activity against the tumor. In the era of the precision medicine, an extensive mutation profile of the tumor becomes the first step toward the most accurate diagnostic classification of the patients, before taking the most appropriate therapeutic decision. More in general, the combination of multiple intracellular signaling pathways and extracellular modifications clearly indicates the need to evaluate more extensively the different molecular events underlying the biological and clinical behavior of the disease and the various actors playing a role in this complex scenario. An additional, practice changing advancement would be represented by characterization of the genetic and molecular assets not only at baseline but also during the course of treatment or follow-up in order to register any biological change of the disease due to its intrinsic and acquired intratumor heterogeneity. This will provide important clues to the clinician, dramatically improving the management of the melanoma patients.

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Conflict of Interest Giuseppe Palmieri has/had advisory role for Bristol Myers Squibb (BMS), Incyte, Merck Sharp & Dohme (MSD), Novartis, Pierre Fabre, and Roche-Genetech. All the remaining authors declare no conflict of interest.

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