Molecular Markers and Targets in Melanoma

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Melanoma develops as a result of several genetic alterations, with UV radiation often acting as a mutagenic risk factor. Deep knowledge of the molecular signaling pathways of different types of melanoma allows better characterization and provides tools for the development of therapies based on the intervention of signals promoted by these cascades. The latest World Health Organization classification acknowledged the specific genetic drivers leading to melanoma and classifies melanocytic lesions into nine distinct categories according to the associate cumulative sun damage (CSD), which correlates with the molecular alterations of tumors. The largest groups are melanomas associated with low-CSD or superficial spreading melanomas, characterized by frequent presentation of the BRAFV600 mutation. High-CSD melanomas include lentigo maligna type and desmoplastic melanomas, which often have a high mutation burden and can harbor NRAS, BRAFnon-V600E, or NF1 mutations. Non-CSD-associated melanomas encompass acral and mucosal melanomas that usually do not show BRAF, NRAS, or NF1 mutations (triple wild-type), but in a subset may have KIT or SF3B1 mutations. To improve survival, these driver alterations can be treated with targeted therapy achieving significant antitumor activity.

melanoma molecular pathways markers target therapy BRAF NRAS KIT NTRK

1. Introduction

1.1. Epidemiology

Melanoma is the most aggressive and deadly skin cancer. Its incidence has increased steadily in the last decades, especially in the Caucasian population, posing a heightened challenge to the global healthcare system [1][2]. Relevant geographical variations exist, depending on the clinical phenotype, the genetic background of individuals, and the extent of ultraviolet (UV) radiation exposure [3]. Currently, it is one of the most frequent cancers in fair-skinned people, especially those with blond or red hair, who have light-colored eyes. Unlike other solid tumors, melanoma mainly affects young and middle-aged people [4]. Melanoma-related mortality has increased in parallel with the increase in the incidence rate over the years, reaching a mortality rate of one in four deaths [5]. Nevertheless, the therapeutic landscape of unresectable stage III and IV melanoma has been revolutionized by immunotherapies and targeted therapies. Both strategies have shown markedly improved survival compared with the use of chemotherapy (ChT) regimens [6]. Melanoma mortality has decreased significantly since the US Food and Drug Administration (FDA) approved ipilimumab in 2011, the first immune checkpoint inhibitor (ICI) to improve

survival in the advanced setting [7][8], and vemurafenib, a v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) tyrosine kinase inhibitor, first in class [9][10].

Melanoma develops from cutaneous melanocytes, located in the basal layer of the epidermis. UV radiation represents a major contributor to cutaneous melanomagenesis through its harmful effects on the skin and direct DNA damage [11], and it triggers the acceleration of tumorigenesis. Intense and intermittent sun exposure, as well as exposure to UV-A rays from artificial sources, has also been linked to an increased risk of melanoma development [12].

Host risk factors, such as the number of nevi, both congenital or acquired, genetic susceptibility, and a family history of melanoma, are relevant risk factors for the development of melanoma. About 25% of cutaneous melanomas arise from a nevus [13]. Polymorphisms of the melanocortin 1 receptor (*MC1R*) gene represent the most relevant gene for susceptibility to melanoma [14].

A family history of melanoma is present in 5–15% of patients with cutaneous melanoma, but true hereditary melanoma due to a transmitted genetic mutation is less common, such as familial atypical multiple mole-melanoma (*FAMMM*) syndrome and its variant, melanoma-astrocytoma syndrome. Germline mutations in cyclin-dependent kinase inhibitor 2A (*CDKN2A*) and, less common, mutations in cyclin-dependent kinase 4 (*CDK4*) are the most frequent genetic abnormalities identified in these families [15]. Other inherited conditions, such as xeroderma pigmentosum, familial retinoblastoma, Lynch syndrome type II, and Li–Fraumeni cancer syndrome, may also be related to an increased risk of melanoma development [16].

2. Molecular Pathways of Melanoma Development

Cancer results from uncontrolled cellular growth of malignant tumor cells caused by a combination of genetic alterations that lead to neoplastic transformation and escape from the inhibitory signals. Several steps in this process are known as the hallmark of cancers [17].

Several key molecular pathways have been discovered to be involved in the onset, proliferation, survival, progression, and invasion. In this section, researchers summarize the major signaling pathways that are currently known to be dysregulated and involved in melanoma disease.

2.1. MAPK Pathway

Melanomagenesis occurs after mutational events that produce signaling pathways critical for cell survival. Mitogen-activated protein kinase (*MAPK*) is a signal transduction pathway, involved in a variety of physiological programs, such as cell proliferation, differentiation, development, migration, apoptosis, and transformation, and is the most relevant in the development of melanoma (**Figure 1**) [18]. The *MAPK* pathway is activated by the binding of a growth factor to a receptor tyrosine kinase (RTK) on the cell surface and stimulates the guanosine triphosphatases (GTPase) activity of *RAS*. The signal propagates through the RAF, mitogen-activated protein kinase kinase 1

(*MAP2K1*), and extracellular signal-related kinase (*ERK*) cascade, which enters the nucleus to activate transcription factors and promote the cell cycle (**Figure 1**) [18].

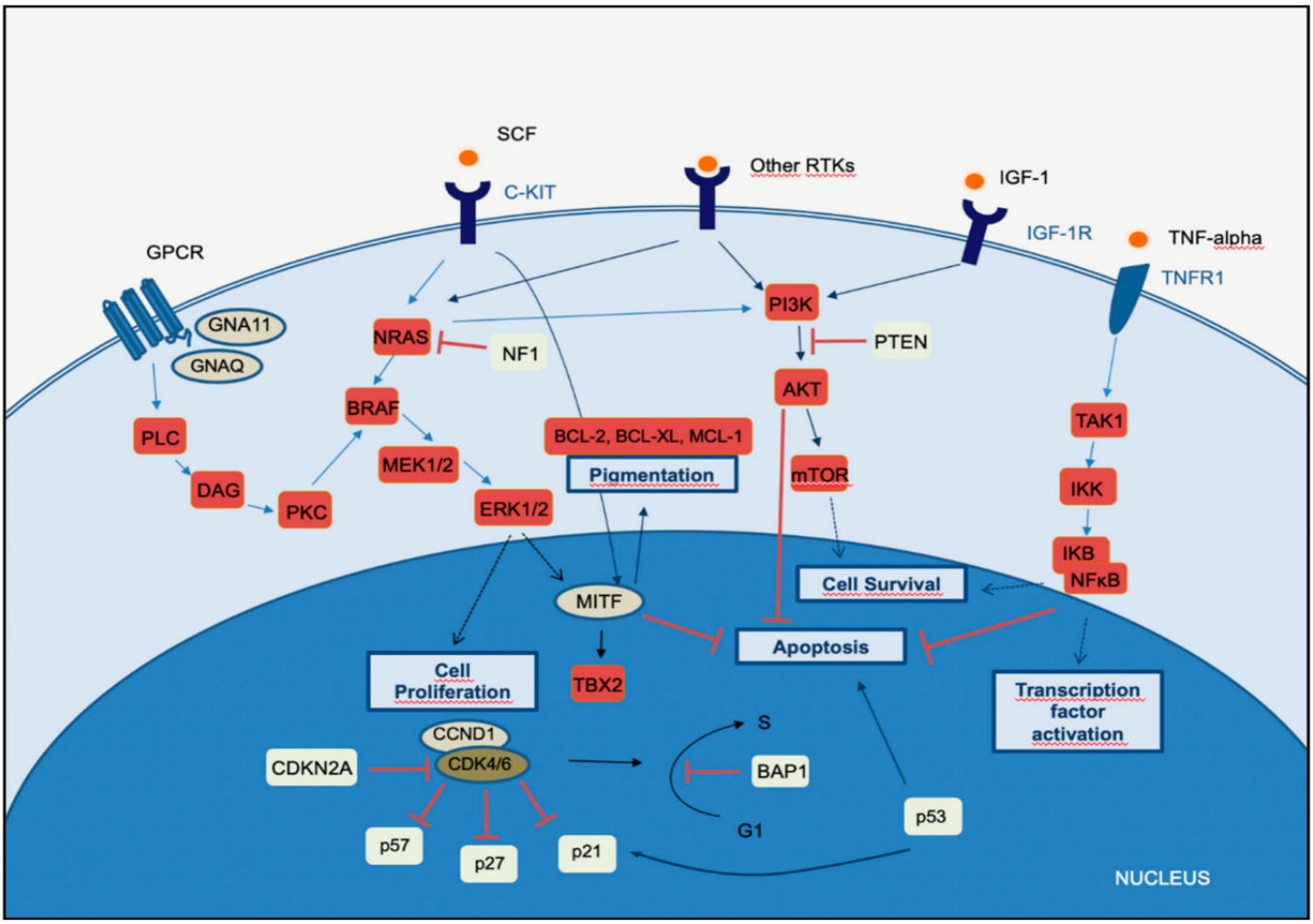

Figure 1. Melanoma key signaling pathways.

The *MAPK*, *PI3K*, and *NFκB* pathways intersect significantly in melanoma pathogenesis. Briefly, in the *MAPK-ERK* pathway, stimulation of *GPCR* results in activation of PLC. This promotes DAG and then activates *PKC*, which stimulates the *MAPK* pathway. Receptor tyrosin kinases (RTKs) are activated by binding of extracellular growth factor ligands and activate the tyrosine kinase activity of the cytoplasmic domain receptor, starting the cascade of signals. Activated *RAS* activates the protein kinase activity of *RAF* isoforms (*RAF1, BRAF, ARAF*). Each *RAF* isoform possesses a distinct capacity to activate *MEK*, with *BRAF* being the strongest activator. *MEK* phosphorylates and activates downstream proteins, such as *ERK1* and *ERK2*. *ERK* can translocate to the nucleus and phosphorylate different transcription factors, which leads to the control of cell cycle progression. *MITF* is a target of *ERK* and controls the production of the pigment melanin, cell cycling, and survival. The binding of the ligand to *KIT* (SCF) results in activation of the *MAPK* and *PI3K* pathways. In the *PI3K-AKT* pathway, ligand binding to the RTK leads to dimerization and autophosphorylation of the receptor and activation. Activated RTK recruits *PI3K* to the plasma membrane. *PI3K* activates *AKT*, whereas *PTEN* antagonizes this process. *PI3K* may also be activated by *GPCR*, *IGF-1R*, and *RAS*. Both *ERK* and *AKT* activate the *mTOR*-signaling pathway, which mediates cell survival and proliferation. In the *TNFR* pathway (canonical *NF-κB* pathway),

binding of the TNF-alpha cytokine to its receptor *TNFR1* results in *TAK1* activation. *TAK1* leads to the aggregation of a downstream kinase complex, the *IKK* complex. Phosphorylation of *IκB* by the *IKK* complex results in the release of *NFκB*. *NFκB* translocates to the nucleus and activates genes involved in cell survival and anti-apoptosis.

Fourteen *MAPKs* have been identified in mammals, and these kinases are typically divided into three main subfamilies: *ERKs*, c-Jun N-terminal kinases (*JNKs*), and *P38* kinases. Each of these *MAPKs* is activated through phosphorylation by an *MAPK* kinase (*MAP2K*), which in turn is activated by an *MAPKK* kinase (*MAP3K*) [18]. The *ERK* pathway is the best-characterized *MAPK* pathway, which has a relevant role in the development and progression of melanoma. On this *MAPK* axis, the role of *MAP3K* is played by the *RAF* family of serine/threonine kinases, which is characterized by an RAS/GTP-binding domain. RAS proteins vHa-ras Harvey rat sarcoma viral oncogene homolog (*HRAS*), *NRAS*, and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) are small GTPases located in the plasma membrane that act as activators in several pathways, apart from *MAPK*. Additionally, activation signals via *RAS* on the inner surface of the cell membrane increase *ERK* activity. Consequently, there is an increase in cellular proliferation, greater cell survival, and resistance to apoptosis. Activated *ERK* can also induce the metastatic potential of melanoma through the expression of integrins that promote tumor invasion [19].

In melanoma, dysregulated *MAPK* signaling and sustained *ERK* activation can eventually lead to cascade hyperactivity and subsequent cell proliferation, survival, invasion, metastasis, and angiogenesis. The *BRAF* gene is frequently mutated in several cancers, and *BRAF*V600 is the most common mutation of the skin. Mutated *BRAF*V600 leads to elevated *BRAF* kinase activity and sustained activation of downstream targets, in addition to unresponsive negative feedback mechanisms [20]. The mutant *KRAS*Q61, the most frequent mutation of *KRAS* in melanoma, leads to an important decrease in its intrinsic hydrolytic activity and a sustained active state of *KRAS.* Mutations in other molecules may also lead to *RAS* overstimulation, such as loss-of-function mutations in neurofibromin 1 (*NF1*). In most melanomas with altered *NF1*, a loss-of-function mutation is found, in which neurofibromin loses its ability to inactivate *RAS* and promotes stimulation of the *RAF* and its downstream targets, leading to stimulation of the *MAPK* pathway and consequent cell proliferation and survival [21].

Telomerase reverse transcriptase (*TERT*) promoter mutations frequently occur in melanoma and, according to The Cancer Genome Atlas (TCGA) data, mainly in the mutated subtypes *BRAF* (75% of cases), *RAS* (72% of cases), and *NF1* (83% of cases), suggesting a link between *MAPK* activation and *TERT* expression. The active *MAPK* pathway promotes phosphorylation and activation of the ETS1 transcription factor by *ERK* (the mutated *TERT* promoter bears ETS-binding sites) [22].

2.2. PI3K-AKT Pathway

The phosphatidylinositol-3-kinases (*PI3Ks*) comprise a family of lipid kinases with regulatory roles in many cellular mechanisms, including cell survival and growth, differentiation, proliferation, transcription, and translation. The pathway transduces signals from a variety of growth factors and cytokines and is the major downstream effector of RTKs and G-protein-coupled receptors (*GPCRs*) (**Figure 1**). Activated *PI3K* leads to the formation of

phosphatidylinositol-3,4,5-trisphosphate (PIP3) through phosphorylation of phosphatidylinositol-4,5-diphosphate (PIP2) in the plasma membrane. PIP3 is essential for the recruitment of the serine-threonine protein kinase *AKT* to the plasma membrane. *AKT* is crucial in this signaling pathway, transmitting signals by phosphorylating different downstream effector targets [23]. Once *AKT* is phosphorylated and fully activated, it turns on a major downstream effector of the *PI3K* pathway, inhibiting or activating a variety of targets and regulating important cellular processes, such as apoptosis, DNA repair, cell cycle, glucose metabolism, cell growth, motility, invasion, and angiogenesis. The main target of *AKT* is the mammalian target of rapamycin (*mTOR*), which has a central role in the *PI3K-AKT* pathway and cancer disease. *mTOR* plays a crucial part in regulating cell growth and proliferation by monitoring nutrient availability, cellular energy, oxygen levels, and mitogenic signals.

PI3K-AKT signaling has negative regulators, to control any persistent and long-term activation. A major regulator of *PI3K-AKT* signaling is the tumor suppressor phosphatase and tensin homolog (*PTEN*), which antagonizes the *PI3K* activity through its intrinsic lipid phosphatase activity, converting PIP3 back to PIP2. Loss of *PTEN* results in constitutive activation of *AKT* and has been largely associated with tumor development in malignant melanoma. Indeed, *PTEN* loss has been shown to be predictive of shorter overall survival (OS) [24][25].

The *PI3K* signaling cascade is upregulated in different types of cancer, including melanoma. More than two-thirds of primary and metastatic melanomas show high levels of phosphorylated *AKT*, suggesting that this alteration is an early event in melanoma pathogenesis. Oncogenic events that activate *PI3K-AKT* may include mutations or copy number variations in certain components of the pathway. *RAS* gene mutations and mutated or amplified expression of RTK may also hyperactivate the *PI3K-AKT* pathway [20]. Mutations in the *mTOR* gene are present in approximately 10% of melanomas, and this molecular event leads to shorter survival and worse prognosis [26]. *PI3K-AKT* signaling may also be activated in melanoma due to loss of function of the negative regulator *PTEN*, which occurs in 10–30% of cutaneous melanomas, leading to constitutive activation of the *PI3K* pathway. Interestingly, *PTEN* gene alterations are mutually exclusive with *NRAS* mutations, and approximately 20% of melanomas with loss of *PTEN* function also have *BRAF*V600E mutations [27].

2.3. CDKN2A, Cell Cycle, and Apoptosis Regulation

The *CDKN2A* gene encodes two proteins, $p16^{CDKN2A}$ and $p14^{CDKN2A}$, which have a tumor suppressor function. The cyclin proteins bind and activate CDKs, which has catalytic kinase activity. Several cyclin/CDK complexes have been identified that functionally act in different cell cycle phases: in the pre-replicative stage (G1), DNA duplication (S), and promotion of progression through the S phase to mitosis (**Figure 1**) [28]. $p16^{CDKN2A}$ and $p14^{CDKN2A}$ proteins have an inhibitory function, interfering with the activity of the cyclin/CDK complexes. $p16^{CDKN2A}$ inhibits the cyclin D1 (*CCND1*)/*CDK4* complex, which, in turn, phosphorylates pRb and allows progression through the G1–S checkpoint. $p14^{CDKN2A}$ is an antagonist of the mouse double minute 2 homolog (MDM2) protein. This protein degrades p53 and eliminates p53 control of cell growth. The $p14^{CDKN2A}$ protein inhibits the oncogenic actions of *MDM2* by blocking its actions on p53 [28]. p53 is a transcription factor that functions as a major negative regulator of cell proliferation and survival. Inactivation of the *TP53* gene results in intracellular accumulation of genetic damage, which promotes melanoma development and progression. *TP53* can

be inactivated through silencing or mutation, the latter occurring most frequently in high-cumulative solar damage-associated (CSD-associated) melanomas [29].

Somatic impairment of the *CDKN2A* gene in melanoma can occur by genetic deletions, inactivated mutations, or promoter hypermethylation and leads to a decrease of the function of $p16^{CDKN2A}$ and/or $p14^{CDKN2A}$ proteins, with consequent loss of cell cycle control. This situation is associated with a higher melanoma invasion potential and metastases [30].

As mentioned above, mutation of the *CDKN2A* gene at the germline level is the most frequent genetic alteration in patients with a strong familial history of melanoma. In addition, variants of the *MC1R* gene increase the melanoma risk in *CDKN2A* mutation carriers [31].

The *CCND1* and *CDK4* genes are found to be altered in a minority of melanomas, representing less than 5%, and depend on the melanoma type. *CCND1* gene amplifications affect about 30% of acral melanomas, 11% of lentigo maligna melanomas, and 6% of superficial spreading melanomas. *CDK4* gene amplification is frequently found in acral and mucosal melanomas [32].

2.4. MITF Pathway

The microphthalmia-associated transcription factor (*MITF*) acts as a master regulator of melanocyte development, function, and survival by modulating differentiation and cell cycle progression genes [33]. It is involved in the differentiation and maintenance of melanocytes and modulates melanocyte differentiation and pigmentation (**Figure 1**). In melanomas, *MITF* can behave as an oncogene, and in approximately 20% of melanomas, it amplifies and promotes the proliferation of tumor cells. Its amplification correlates with a worse prognosis and a lower OS and ChT resistance [33]. *MITF* is activated by the *MAPK* and cAMP pathways and regulates the transcription of three major pigmentation enzymes (TYR, TYRP1, and DCT) [34]. In melanoma, *ERK* activity stimulated by *BRAF* is associated with *MITF* ubiquitin-dependent degradation. *BRAF* can modulate intracellular MITF protein through two opposite mechanisms. On the one hand, it can degrade the MITF protein; on the other hand, *BRAF* can stimulate transcription factors that increase the expression of the MITF protein. About 10–15% of melanomas harbor the *BRAF* mutation along with *MITF* amplification, suggesting that additional mechanisms are involved in *ERK*-dependent degradation of *MITF*.

3. The Integration of Histology and Molecular Diagnostics of Melanoma

Despite recent molecular advances in melanoma characterization, paramount to diagnosis of a melanocytic skin lesion is the integration of several histopathological criteria with the clinical features. In many cases, general morphological criteria for atypia are often the subject of disagreement and inter-observer variability, especially in non-conventional lesions [35]. The World Health Organization (WHO) recognizes these challenges and incorporate the known molecular pathways in the latest WHO melanocytic tumor classification, introducing the concept of

"intermediate" lesions. As stated in a recent review on the topic, this multidimensional classification showed that the view of melanocytic tumors as either benign or malignant might no longer be the proper approach [36]. Thus, WHO 2018 indicates nine categories/pathways leading to melanoma, each with specific genetic drivers (**Table 1**). Furthermore, melanomas can be clustered in three main subtypes, according to the degree of CSD (**Table 1** and **Figure 2**) [37]. The largest group are melanomas associated with low-CSD or superficial spreading melanomas, which often arise on the trunk and proximal areas of the extremities. The most frequent molecular alteration in these melanomas is the *BRAF*V600E mutation [38]. In addition, *TERT* promoter mutations and *CDKN2A* mutations are also found in the majority of cases. *PTEN* and *TP53* are commonly observed in advanced tumors. Lentigo maligna and desmoplastic melanomas are considered tumors associated with high-CSD. These melanomas arise on heavily sun-damaged skin, such as the face or hands, and affect older people. Molecularly, they often have a high mutation load and may harbor *NRAS, BRAF* non-V600E, or *NF1* mutations. *TERT* promoter mutations and *CDKN2A* are also frequently found in these melanomas, and *KIT* mutations are found in a subset of cases. Interestingly, the number of mutations increases with the CSD grade (**Figure 2**), and desmoplastic melanomas harbor the highest tumor mutation burden. The category of "low to non-UV exposure/CSD" melanomas includes Spitz melanomas, acral melanomas, mucosal melanomas, melanomas developed from congenital nevi and blue nevi, and uveal melanomas. These melanomas rarely harbor *BRAF*, *NRAS*, or *NF1* mutations (triple wild-type) [37]. A subset of acral and mucosal melanomas may have *KIT* mutations, in addition to gene amplifications and structural rearrangements, most frequently of the *CCND1* gene and *SF3B1*. Therefore, genomic studies have subsequently exemplified that acral and mucosal melanomas are biologically distinct from their cutaneous counterparts at sun-exposed sites. Spitz melanomas show a particular oncogenic signaling pathway involving tyrosine kinase or serine-threonine kinase fusions, and melanomas in blue nevus and uveal melanomas are characterized by *GNA11* or *GNAQ* mutations [38].

Arising from Skin High CSD	Arising from Skin Low CSD	Arising from Acral Surfaces	Arising from Mucosal Surfaces	Uveal Melanoma
*BRAF*nonV600E mut.	*APC* mut.	*ALK* rearr.	*BRAF* mut.	*BAP1* mut.
EGFR mut.	*BAP1* mut.	*BRAF* mut.	*CCND1* amp.	*CYSLTR2* mut.
ERBB2 mut.	*BRAF*V600E mut.	*CCND1* amp.	*CDK4* amp.	*EIF1AX* mut.
KIT mut.	*CDKN2A* mut.	*GAB2* amp.	*CDKN2A* mut.	*GNA11* mut.
MAP2K1 mut.	*CTNNB1* mut.	*HRAS* mut.	*KIT* mut.	*GNAQ* mut.
MAP3K1 mut.	*MAP2K1* mut.	*KIT* mut.	*KRAS* mut.	*PLCB4* mut.
MET mut.	*NRAS* mut.	*KRAS* mut.	*MDM2* amp.	*SF3B1* mut.
NF1 mut.	*PRKAR1A* mut.	*NF1* mut.	*NF1* mut.	
NFKBIE mut.	*PRKCA* mut.	*NRAS* mut.	*NRAS* mut.	
NRAS mut.	*PTEN* mut.	*NTRK3* rearr.	*SF3B1* mut.	
PIK3CA mut.	*TERT* mut.	*TERT* mut.		
PTEN mut.	*TP53* mut.			
PTPN11 mut.				
RAC1 mut.				
TERT mut.				
TP53 mut.				

UV Signature
High TMB

Lack UV signature
Low TMB

Figure 2. Genomic alterations of melanoma subtypes defined by UV exposure. Abbreviations: amp, amplification; CSD, cumulative sun damage; rearr, rearrangement; TMB, tumor mutational burden; UV, ultraviolet.

Table 1. The classification of melanomas (modified from 2018 World Health Organization Classification).

UV Exposure	Categories	Melanoma Subtype	Key Molecular Genes	
Low UV/CSD	I	Superficial spreading melanoma	*BRAF*V600 mut *CDKN2A* mut *NRAS* mut	*TERT* mut *PTEN* mut *TP53* mut
High UV/CSD	II	Lentigo maligna melanoma	*NRAS* mut *BRAFnon-V600E* mut *KIT* mut *TERT* mut	*CDKN2A* mut *PTEN* mut *TP53* mut
Low or no UV/CSD	III	Desmoplastic melanoma	*NF1* mut *NFKBIE* mut	*NRAS* mut *PIK3CA* mut
	IV	Spitz melanoma	*ALK* rearr *NTRK1* rearr *NRTK3* rearr	*CDKN2A* mut *HRAS* mut
	V	Acral melanoma	*KIT* mut *NRAS or* *BRAF* mut *ALK* rearr *NRTK3* rearr	*CDKN2A* mut *CCND1* amp *TERT* mut
	VI	Mucosal melanoma	*KIT* mut *NRAS or* *BRAF* mut *CDKN2A* mut *SF3B1* mut	*CCND1* amp *CDK4* mut *MDM2* amp
	VII	Melanoma in congenital nevus	*NRAS* mut	*BRAFV600E* mut
	VIII	Melanoma in blue nevus	*GNA11* mut *GNAQ* mut *CYSLTR2* mut	*BAP1* mut *EIFAX* mut *SF3B1* mut
	IX	Uveal melanoma	*GNA11* mut *GNAQ* mut *CYSLTR2* mut *PLCB4* mut	*BAP1* mut *EIFAX* mut *SF3B1* mut

Abbreviations: amp, amplification; CSD, cumulative sun damage; mut, mutation; rearr, rearrangement.

Certainly, to reduce diagnostic uncertainties and maintain a diagnostic approach based on the WHO 2018 classification, histological assessment should be accompanied by basic immunohistochemistry (IHC) and molecular tests. Recent recommendations of the European Society of Pathology, the European Organization for Research and Treatment of Cancer, and the EURACAN for the diagnosis of intermediate melanocytic proliferations and melanoma variants indicate that most pathology laboratories should perform basic IHC tests, such as: HMB-45; SOX10; MITF, tyrosinase, MART-1; P16; Ki-67/MIB1; BAP1 (BRCA1-associated protein 1); β-catenin; PRAME; and at least one molecular method to detect *BRAF* codon 600 and *NRAS* mutations [36]. The most difficult cases that require complementary studies should be analyzed in specialized referral centers, where laboratories can

determine a higher grade in a given lesion or the identification of molecular targets that can benefit from targeted therapy.

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