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Chapter 2

Targeted Therapies in Melanoma: Successes and Pitfalls

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1. Introduction

Incidence of melanoma is steadily rising worldwide [1]. Lifetime risk of developing melanoma in Caucasians is estimated as 1 in 50 individuals [2-3]. The incidence of melanoma varies according to the geographical origins of the population and the extent of sun exposure. In Australia and United States, an incidence of melanoma higher than observed in the European countries (with the notable exception of Sweden) has been reported [4-5]. There is a gradient of melanoma incidence from north to south in Europe, with highest frequencies in the northern counties. This suggests that initiation and development of melanoma is due to a combination of the damaging effects of UV and a predisposing genetic background [5].

Melanoma arises from melanocytes, neural crest-derived cells that are located in the basal layer of the epidermis and skin appendages in humans. Melanocytes, by synthesizing melanin pigments and exporting them to adjacent keratonocytes play a key role in protecting the skin from the damaging effects of ultraviolet (UV) and other solar radiation [6]. Melanocytes can proliferate to form nevi (common moles), initially in the basal epidermis (junctional nevus) and later by limited local dermal infiltration (compound nevus). Nevi develop during embryonic life (congenital nevus) and in children and adults, (acquired nevus) partly as a result of solar exposure in the latter two populations. Further progression of melanocytic tumors relates to factors that include intermittent exposure to UV radiation (though a direct relationship between risk of melanoma and UV exposure remains somehow unclear), a history of sunburn and endogenous factors such as skin type and elevated numbers of nevi (especially dysplastic nevi, also known as atypical moles) [7-8].

Considering the growth patterns, four histological types of melanoma have been historically recognized: superficial spreading melanoma (SSM), lentigo maligna melanoma (LMM), nodular melanoma (NM), and acral lentiginous melanoma (ALM) [9]. Comparative genomic hybridization revealed that several genomic regions (mostly, 11q13, 22q11-13, and 5p15) were abnormally amplified in ALM [10]; such regions were different from those found altered in superficial SSM or NM (mainly, 9p21 and 1p22) [11]. Recently, a new classification of melanoma including the site of primary tumour and the degree of chronic sun-induced damage of the surrounding skin has been introduced [12]. Based on these criteria, melanomas are classified into four groups; melanoma on skin with chronic sun-damage (CSD melanoma), melanoma on skin without chronic sun-damage (non-CSD melanoma), melanoma on palms, soles and nail bed (acral melanoma), and melanoma on mucous membrane (mucosal melanoma) [12]. Non-CSD melanomas are characterized by high frequency of BRAF or NRAS mutations (which are mutually exclusive), while CSD, acral, and mucosal melanomas show a low frequency of BRAF/NRAS mutations but a high incidence of alterations in additional genes, such as mutations of receptor tyrosine kinase KIT gene, amplifications of cyclin D1 (CCND1) and cyclin-dependent kinase 4 (CDK4) genes [7, 12-13]. All genes affected into the different types of melanoma are involved in regulating cell-cycle progression and cell survival [12-13]. On the other hand, such a difference of genetic alterations indicates distinct genetic pathways in the pathogenesis of melanoma depending on the anatomical site of the primary lesion. Trying to merge the two classifications, it could be affirmed that non-CSD melanoma roughly corresponds to SSM, CSD melanoma to LMM, and acral melanoma to ALM. Since NM may arise at any anatomical site, this histological type can not be included in any of the subgroups of the latter classification (indeed, no distinct genetic pathway has been so far correlated with NM).

During recent past years, melanocytic transformation is being demonstrated to occur as a sequential accumulation of genetic and molecular alterations [13-14]. In this sense, it is becoming an unquestionable certainty that molecular classification of melanoma patients could be achieved through the assessment of the molecular profile of primary tumors and/or the correspondent metastases, by unveiling which gene or pathway is truly affected. Although pathogenetic mechanisms underlying melanoma development are still largely unknown, several genes and metabolic pathways have been shown to carry molecular alterations in melanoma.

2. Main genes and related pathways

2.1. *BRAF* and MAPK pathway

The *mitogen-activated protein kinase* (MAPK) signal transduction pathway regulates cell growth, survival, and invasion. MAPK signaling is initiated at the cell membrane, either by receptor tyrosine kinases (RTKs) binding ligand or integrin adhesion to extracellular matrix, which transmits activation signals via RAS on the cell membrane inner surface (Figure 1).

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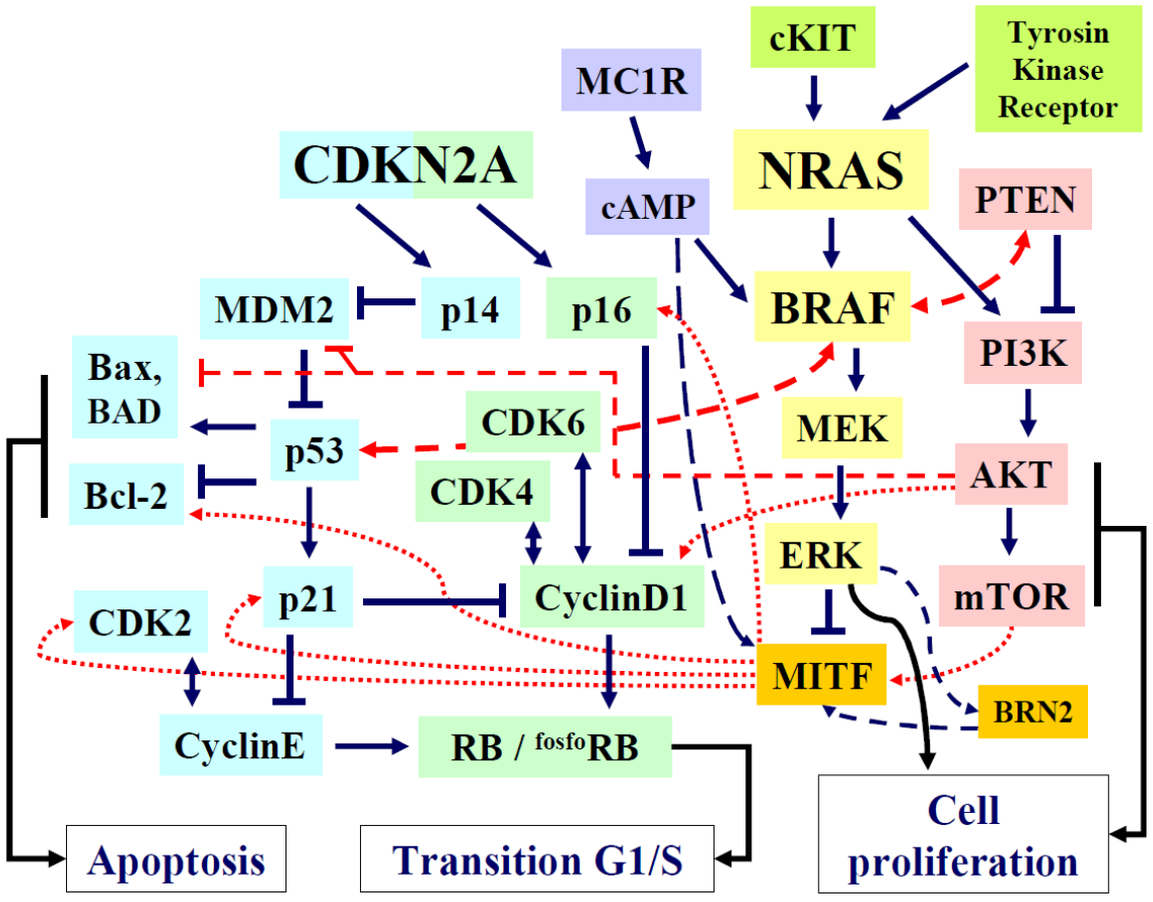

Figure 1. Main pathways involved in melanomagenesis. Arrows, activation signals. Interrupted lines, inhibition signals.

RAS proteins are small GTPases thatare activated by extracellular stimuli and regulate signal transduction of the BRAF-MEK1/2-ERK1/2 and AKT/PI3K pathways, controlling crucial cellular processes such as proliferation, differentiation, cell adhesion, apoptosis, and cell migration [14-16]. RAS gene is mutated in an estimated 20-30% of all cancers [17]. RAS proteins are constituted by three main isoforms - NRAS, KRAS, and HRAS, which present a similar function but a different specificity for tissue distribution [18]. On this regard, KRAS alterations mostly occur in gastrointestinal cancer [17-18], HRAS alterations are frequently observed in bladder cancer, and NRAS is mutated in 15-25% of melanomas [17-18]. Despite the huge amount of knowledge implicating RAS in tumour initiation and promotion, RAS itself has not become a successful target of therapy.

The RAF kinase family consists of three members - ARAF, BRAF, and CRAF, all of which can activate a series of protein kinases; such a signaling cascade culminate in the phosphorylation and activation of the extracellular signal-regulated kinase (ERK) downstream protein [19]. In melanoma, the most commonly mutated component of this pathway is the *BRAF* gene; the prevalent *BRAF* mutation (in nearly, 90% of cases) being a substitution of valine with glutamic acid at position 600 (V600E) [20]. Mutated *BRAF* induces constitutive ERK activation; activated ERK then translocates to the nucleus and initiates the transcription of a variety of growth-related genes, stimulating cell proliferation and survival [20]. Indeed, the

increased activity of ERK seems to be implicated in rapid melanoma cell growth, enhanced cell survival and resistance to apoptosis [21]. High levels of activated ERK may further induce the metastatic potential of melanoma by increasing the expression of invasion-promoting integrins [22-23]. Presence of *BRAF* mutations in benign and dysplastic nevi supports the hypothesis that activation of the RAF/MEK/ERK pathway is an early event in melanoma progression [24-25]. In other words, *BRAF* activation is necessary but not sufficient for the development of melanoma and additional co-operating genetic events are required to achieve full malignancy.

In a study aimed to better define the role of BRAF in melanomagenesis, a transgenic zebra fish expressing ^{V600E}BRAF showed dramatic development of patches of ectopic melanocytes (designated as fish-nevi) [26]. Remarkably, activated *BRAF* in p53-deficient zebra fish induced the formation of melanocytic lesions that rapidly developed into invasive melanomas that resembled human melanomas in terms of their histology and biological behaviors [26]. These data provide direct evidence that the p53 and BRAF pathways interact functionally during melanomagenesis.

The *BRAF* gene also cooperates with the cyclin-dependent kinase inhibitor p16^{CDKN2A} (see below). Activating *BRAF* mutations have been reported to constitutively induce up-regulation of *p16*^{CDKN2A} and cell cycle arrest (this phenomenon appears to be a protective response to an inappropriate mitogenic signal). In particular, mutant BRAF protein induces cell senescence by increasing the expression levels of the p16^{CDKN2A} protein, which, in turn, may limit hyperplastic growth caused by *BRAF* mutations [25]. Therefore, inactivation of *p16*^{CDKN2A} gene may promote the melanocytic proliferation depending on oncogenic *BRAF*. In this sense, several factors seem to be able to induce the arrest of the cell cycle and cell senescence caused by *BRAF* activation [27-28].

Finally, it has been showed that primary melanomas arising from chronically sun-damages skin and from mucosal sites, which typically do not harbour *BRAF* and *NRAS* mutations, have increased copy number of the *CCND1*/*Cyclin D1* gene [12]. In contrast to primary melanomas, a subset (>15%) of metastatic melanoma samples with *BRAF* mutations also exhibit amplification of *CCND1*/*Cyclin D1*. These melanomas are resistant to BRAF inhibitors highlighting the need for combination therapy [29-30].

2.2. *CDKN2A* and senescence/apoptosis pathways

The cyclin-dependent kinase inhibitor 2 (*CDKN2A*; at chromosome 9p21) gene encodes two proteins, p16^{CDKN2A} and p14^{CDKN2A} (a product of an alternative splicing), that are known to function as tumor suppressors [31-33]. The cyclin proteins are regulatory effectors able to bind and activate the cyclin-dependent kinases (CDKs) that bear catalytic kinase activity. Several distinct cyclin/CDK complexes have been identified and functionally assigned to specific phases of the cell cycle: Cyclin D/CDK4 complex leads the passage from the pre-replicative (G1) to the DNA duplication (S) phase; the Cyclin E/CDK2 complex promotes the progression through the S phase and the Cyclin B/CDK1 complex induces cells to enter mitosis [31-32]. In such a functional network, proteins like p16^{CDKN2A} and p14^{CDKN2A} act as inhibitors of the cell cycle, negatively interfering with the activity of the

cyclin/CDK complexes and, in this way, ensuring the control of the cell replication [33]. In particular, $p16^{CDKN2A}$ is part of the G1–S cell cycle checkpoint mechanism that involves the retinoblastoma-susceptibility tumor suppressor protein (pRb). The $p16^{CDKN2A}$ inhibits the Cyclin D/CDK4 complex, which, in turn, phosphorylates pRb and allows progression through the G1–S checkpoint (Figure 1) [33]. The *Cyclin D* (*CCND1*) and *CDK4* genes are found altered in less than 5% of total melanomas [12], though with an heterogeneous prevalence according to the distinct types of melanoma (see above). Somatic *CDK4* amplification is relatively common in acral and mucosal melanomas [12], whereas germline *CDK4* mutations are observed in a limited fraction of melanoma-prone families [34]. The *CCND1* gene amplifications is primarily found in ALM lesions (more than one third of cases) and to a lesser degree in other types (11% for LMM and 6% for SSM) [35]. Regarding the alternative *CDKN2A* gene product, $p14^{CDKN2A}$ is an antagonist of the murine double minute 2 (MDM2) protein, which targets p53 to degradation by ubiquitination and proteasome processing, thus abrogating p53 control of cell growth (Figure 1) [32-33]. In particular, the $p14^{CDKN2A}$ protein exerts a tumor suppressor effect by inhibiting the oncogenic actions of the downstream MDM2 protein, whose direct interaction with p53 blocks any p53-mediated activity and targets the p53 protein for rapid degradation [32-33]. The p53 is a transcription factor that functions as a major negative regulator of cell proliferation and survival, being activated by different adverse signals (i.e. growth factors withdrawal, DNA damage, oncogenic aberrations, hypoxia, etc.) and driving cells to either interrupt progression into the cell-cycle or enter apoptosis program, in order to avoid reproduction of altered cells [33, 36]. In normal conditions, expression levels of p53 within cells are low. In response to DNA damage, p53 accumulates and prevents cell division. Therefore, inactivation of the *TP53* gene results in an intracellular accumulation of genetic damage which promotes tumor formation [36]. In melanoma, such an inactivation is mostly due to functional gene silencing since the frequency of *TP53* mutations is low (less than 10% of cases) [37]. Impairment of the $p14^{CDKN2A}$-MDM2-p53 cascade, whose final effectors are the Bax/Bcl-2 proteins, has been implicated in defective apoptotic responses to genotoxic damage and, thus, to anticancer agents (in most cases, high expression levels of Bcl-2 protein have been demonstrated to reduce apoptosis and sensitivity of melanoma cells to proapoptotic stimuli, contributing to further increase tumor aggressiveness and refractoriness to therapy) [33].

More in general, genetic loss or rearrangement in the CDKN2A locus may result in impairing or silencing $p16^{CDKN2A}$, $p14^{CDKN2A}$ or both genes, with the consequence of losing the mechanisms controlling cell proliferation and/or survival. In melanoma, the *CDKN2A* gene is somatically inactivated by genomic deletions (approximately 50% of cases) or point mutations (about 10% of cases); in addition, this gene is often transcriptionally silenced by promoter hypermethylation [38]. A reduced expression of the $p16^{CDKN2A}$ protein seems to be strictly associated with malignant tumor invasion, varying from 5% to about 15% in benign melanocytic lesions, from 10% to about 50% in primary melanomas, and from 50% to about 60% in melanoma metastases [39]. The *CDKN2A* gene is frequently mutated at germline level in patients with a strong familial history of melanoma (three or more affected family members), indicating that it represents a key susceptibility gene for

familial melanoma [40]. In melanoma, CDKN2A mutations typically occur in the $p16^{CDKN2A}$ gene, either alone or in combination with $p14^{CDKN2A}$ gene (some families harbor however mutations only in this latter gene) [33, 40].

A recent meta-analysis of studies conducted in independent populations indicated that multiple variants of the melanocortin-1 receptor (*MC1R*) gene increase the melanoma risk in *CDKN2A* mutation carriers [41]. The *MC1R* gene encodes a G-protein coupled receptor. In the skin, two types of melanin pigment, dark-protective eumelanin and red-photo reactive pheomelanin, are present [42]. MC1R plays an important role in determining the ratio of eumelanin and pheomelanin production. After stimulation by UV, keratinocytes produce alpha melanocyte stimulating hormone (MSH) that binds to the MC1R on melanocytes and shifts the balance of these two pigments in the direction of eumelanin [42]. In particular, stimulation of MC1R by MSH mediates activation of adenylate cyclase, subsequent elevation of cAMP levels, and activation of the microphthalmia transcription factor (MITF; see below). Activated MITF binds to a conserved region found in the promoters of the *tyrosinase* (*TYR*), *tyrosinase-related protein 1* (*TYRP1*), and *DOPAchrome tautomerase* (*DCT*) genes, stimulating the transcriptional up-regulation of these proteins and inducing maturation of the melanosomes [43]. This ultimately results in increased eumelanin production and darkening of the skin or hair.

New findings have shed light on the mechanisms by which MC1R contributes to melanoma risk. In vitro studies showed that acute UV irradiation of melanocytes with impaired MC1R results in an increased production of free radicals [44]. Melanomas that arise on body sites only intermittently exposed to sun, and which therefore lack marked signs of chronic solar damage, were found to have a high frequency of *BRAF* mutations [12]. One could speculate that induction of *BRAF* mutations may occur only when solar exposure is not sufficiently prolonged to induce the striking tissue changes that generate the hallmark signs of solar damage. Several *MC1R* variants, that impairing relevant protein function, have been associated with *BRAF* mutation in melanoma arising in Caucasian populations from United States and Europe [45-48]. On the basis of such indications, it is possible that increased production of free radicals following UV exposure is combination with impairment of *MC1R* may induce mutations in the *BRAF* gene.

Additional mechanisms promoting susceptibility to pathogenetic mutations of the *BRAF* gene may however exist since there is no demonstrable association between germ line *MC1R* status and the prevalence of somatic *BRAF* mutations in melanomas from Australian population, even after classifying the melanomas by their location relative to intermittent and chronic sun-exposure [49].

2.3. *PTEN* and mTORC pathways

Phosphatase and tensin homolog deleted in chromosome ten (PTEN) has a key role in cellular signal transduction by decreasing intracellular phosphatidylinositol [3,4-bisphosphate (PIP2) and 3,4,5-triphosphate (PIP3)] that are produced by the activation of phoshoinosite 3-kinase (PI3K) [50]. In the absence of extracellular growth stimuli mediated by cell surface receptors and G-proteins, PTEN dephosphorylates PIP3 generating the PIP2 phospholipid,

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unable to stimulate phosphorylation of the PI3K protein; this in turn maintains suppression of cell cycle progression and cell growth. In other words, there is a balance between PIP2 and PIP3 which is maintained by the opposite activities of PTEN and PI3K, which instead converts PIP2 into PIP3 [50]. Upon growth stimulation, mainly obtained by triggering the RAS kinase, PI3K is constitutively activated (Figure 1), resulting in an increase of intracellular levels of PIP3 and a consequent activation of AKT by phosphorylation [50-51]. Activated AKT in turn phosphorylates its substrate, the serine/threonine kinase mTOR, leading to increased synthesis of target proteins that promote cell division and survival as well as apoptotic escape [51]. The mechanisms associated with the ability of AKT to suppress apoptosis include the phosphorylation and inactivation of many pro-apoptotic proteins, such as BAD (Bcl-2 antagonist of cell death) and MDM2, as well as the activation of NF-kB [52] (Figure 1).

Three *AKT* genes have been described in humans: *AKT1*, which is involved in apoptosis and protein synthesis; *AKT2*, which is involved in controlling the glucose metabolism; and *AKT3*, whose increased activity (often associated with the amplification of the *AKT3* locus at chromosome 1q43-44) is mainly involved in stimulating cell growth and has been implicated in many cancers including melanoma [51-52]. More than two thirds of primary and metastatic melanomas exhibit higher levels of phosphorylated AKT [52], suggesting that such an alteration might be considered as an early event in melanoma pathogenesis.

Overall, PI3K expression is higher in malignant melanomas, as compared to nevi, and seems to correlate with a worse prognosis [53]. In primary melanomas, since activating mutations of PI3K are quite rare (about 1%), and comparative genomic hybridization did not reveal amplification at this gene locus [12, 37], activation of the PI3K pathway is mostly due to functional silencing of the tumour suppressor gene PTEN. Inactivation of *PTEN* gene is mainly due to hypermethylation-based epigenetic mechanisms, with a low incidence (less than 10%) of somatic mutations and/or allelic deletions; loss of (or reduced) PTEN protein is observed by immunohistochemistry in 20-40% of melanoma tissues [54-55]. Consistent with its role in the PI3K-AKT pathway, vast majority (more than 80%) of melanoma samples with loss of PTEN protein presents a significant increase in expression of phosphorylated AKT [37].

PTEN inactivation has been mostly observed as a late event in melanoma, although a dose-dependent down-regulation of PTEN expression has been implicated in early stages of tumorigenesis, often occurring in conjunction with mutations in *BRAF* gene (which have been demonstrated to indeed play a role in induction of the melanocytic proliferation and early steps of melanoma development) [50]. PTEN downregulation In addition, alterations of the BRAF-MAPK pathway are frequently associated with PTEN-AKT impairment [7, 56]. In summary, the combined effects of the inactivation of *PTEN* gene and activation of the PI3K-AKT effectors may result in aberrant cell growth, apoptosis escape, and abnormal cell spreading and migration [33, 50].

2.4. *MITF* and melanocytic differentiation

The microphthalmia-associated transcription factor (MITF) is a transcription factor that is involved in differentiation and maintenance of melanocytes, playing a role in melanoma de-

velopment and pathogenesis [43, 57]. MITF is activated by the MAPK pathway as well as by the cAMP pathway (Figure 1), and leads to transcription of genes involved in pigmentation (TYR, TYRP1, and DCT; see above) as well as cell cycle progression and survival [43]. The *MITF* gene is amplified in melanoma (about 20% of cases); *MITF* amplification correlated with increased resistance to chemotherapy and decreased overall survival [57].

The connection between MITF and melanoma development is complex because it plays a double role of inducer/repressor of cellular proliferation. High levels of MITF expression lead to G1 cell-cycle arrest and differentiation, through induction of the cell cycle inhibitors $p16^{CDKN2A}$ and p21 [58-59] (Figure 1). Very low or null MITF expression levels predispose to apoptosis whereas inter-mediate MITF expression levels promote cell proliferation [57-59]. Therefore, it is thought that melanoma cells have developed strategies to maintain MITF levels in the range compatible with tumorigenesis. It has been shown that constitutive ERK activity, stimulated by ^{V600E}BRAF in melanoma cells, is associated with MITF ubiquitin-dependent degradation [60]. Nevertheless, continued expression of MITF is necessary for proliferation and survival of melanoma cells, because it also regulates CDK2 and Bcl-2 genes [61-62]. It has been recently shown that oncogenic BRAF may control intracellular levels of the MITF protein through a fine balance of two opposite mechanisms: a direct reduction of MITF levels, by inducing protein degradation, and an indirect increase of MITF levels, by stimulating transcription factors which increase protein expression levels [63]. Oncogenic *BRAF* mutations are associated with *MITF* amplification in a low fraction (10-15%) of melanomas [63], suggesting that other mechanisms are likely to be involved in ERK-dependent degradation of MITF.

2.5. *iNOS* and *NF-kB* pathways

Human melanoma cells are known to express the inducible nitric oxide synthase (iNOS) enzyme, which is responsible for synthesis of nitric oxide (NO), a free radical involved in several physiological processes such as neurotransmission, vasodilation, and regulation of immune responses [64]. The iNOS enzyme has been found to be frequently expressed in melanoma [65-66] and the subsequent increased concentrations of NO have been demonstrated to contribute to melanomagenesis through a sustained protection of the tumour from apoptosis [67]. However, the role of iNOS in melanoma progression remains controversial. Higher levels of iNOS have been found in subcutaneous and lymph node metastases of non-progressive melanoma as compared to metastases of progressive melanoma [68], however, iNOS was found to be expressed to a lesser extent in metastases as compared with nevi and primary melanomas [69]. Nevertheless, the expression of iNOS in patients with lymph nodes and in-transit metastases (stage III disease) has been proposed as an indicator of poor prognosis [70].

Recently, it has been reported that the constitutive iNOS expression in melanoma cells might be induced by activation of the MAPK pathway through stimulation of the activity of the Nuclear Factor-kB (NF-kB) [71-72]. NF-kB is a protein complex that acts as a transcriptional factor and regulates the transcription of several genes involved in many critical pathways [73]. In a quiescent status, proteins of the NF-kB complex are localized into the

cytoplasm. NF-κB exists as cytoplasmic hetero- or homodimers associated with members of the inhibitor-of-kB (IkB) proteins (IkB*a*, Ik*Bb* and IkB*e*), which form complexes sequestering NF-kB into the cytoplasm [74]. Upon appropriate stimulation, the phosphorylation of IkB proteins is promoted, triggering their ubiquitination and degradation in the proteasome [74]. As a consequence, NF-kB may translocate to the nucleus where it binds to target DNA loci and induces transcription of several genes - including *iNOS* - associated with immune and inflammatory response, angiogenesis, cell proliferation, tumor promotion, and apoptosis [73-74].

Regarding the role of NF-kB in tumorigenesis, there are compelling evidence that activation of NF-kB controls multiple cellular processes in cancer due to its ability to promote cell proliferation, suppress apoptosis, promote cell migration, and suppress cell differentiation, opening the way for new therapeutic approaches against such a target [75-76]. In melanoma, NF-kB is constitutively activated since expression of the IkB proteins seems to be significantly reduced in comparison to nevi [77].

2.6. *cKIT* and tyrosinase kinase receptors

cKIT is a member of the transmenbrane receptor tyrosine kinase family that comprised five immunoglobin-like motifs, a single transmembrane region, an inhibitory cytoplasmic juxtamenbrane domain, and a split cytoplasmic kinase domain separated by a kinase insert segment [78]. Under physiological conditions, binding of the cKIT ligand stem-cell factor (SCF) to the extracellular domain of the receptor leads its dimerization, activation of the intracellular tyrosine kinase domain through autophosporylation of specific tyrosine residue [78]. The intracellular signaling through cKIT plays a critical role in the development of several mammalian cells, including growth, differentiation, migration, and proliferation of melanocytes [79]. It has been defined that cKIT recruits and activates a number of intracellular signaling pathways implicated in tumor progression, such as MAPK, PI3K/AKT, Src, activators of transcription (STAT), and phospholipase-C (PLC) [79-80].

Although the role of cKIT in melanomagenesis is still controversial, several studies have reported its downregulation during melanoma growth and invasion (in vertical growth phase of melanoma and metastatic lesions) [81-83]. Indeed, the majority of highly metastatic human melanomas do not express detectable levels of the cKIT receptor [83]. As a confirmation of this, over-expression of cKIT in metastatic melanoma cell lines led to important reduction in tumor growth, while cKIT activation through exposure to cKIT ligands induced apoptosis [83].

Specific mutations within the cKIT gene cause constitutive phosphorylation and activation of the kinase domain resulting in uncontrolled cell proliferation. [84]. Although such mutations seem to be more rare than *BRAF* and *NRAS* mutations, these may reflect the important role of cKIT tyrosine kinase in melanocyte development [84]. Sequencing of cKIT exons 11, 13, 17, and 18 revealed the most prevalent mutation to be K642, L576P, D816H-V, V559A [84]. The cKIT mutations are more common in mucosal and acral melanomas compared with cutaneous melanomas and are in most cases accompanied by an increase in gene copy number (40% mucosal or acral melanomas - frequently, associated with amplification of *cy-*

clin D1 - as well as 30% of melanomas on skin with chronic sun-induced damage) [84]. High expression levels of cKIT and CDK4 proteins have been identified in another subset of melanomas lacking *BRAF* mutations [85].

2.7. CTLA-4 and T-cell activation

The above-mentioned main intracellular molecular pathways are thus involved in tumor growth and survival, actively participating to the different phases of development and progression of melanoma. Additional extracellular factors, mainly represented by different components of the tumor microenvironment, have been implied to play a role in melanoma tissue invasion and metastatic dissemination. As an example, changes in the expression of adhesion molecules such as MCAM/MUC18, E-cadherin, and integrins occur in the transition from the radial growth phase (RGP) to the vertical growth phase (VGP) of melanoma; they are induced by both intracellular modifications [i.e., activation of the focal adhesion kinase (FAK) and integrin linked kinase (ILK) patways or high levels of activated ERK (phospoERK$_{1\text{-}2}$)] and biological signals directly generated by the extracellular matrix (ECM), which is composed of proteins, glycoproteins, proteoglycans, and glycosaminoglycans in complex arrangements [22-23].

Among others, a block of the anti-tumor immune response induced by changes in pericellular microenvironment has been demonstrated to contribute to melanoma progression [86]. In recent past years, research has tried to better define the molecular mechanisms underlying the downregulation of the immune system by such pericellular components, in order to develop new therapeutic targets [87]. Actually, two immunomodulant antibodies, such as anti-CTLA4 and anti-PD1, have been demonstrated to be effective in inhibiting some down-regulators of the anti-tumor immune response [30, 88]. Moreover, drugs able to interfere with the differentiation of the myeloid-derived suppressor cells (MDSC) and T regulatory cells (Treg), which are both physiologically involved in controlling an abnormal immune response during the inflammatory processes and pathologically favoring tumor progression through suppression of T-cell activation, represent additional therapeutic strategies to be exploited [89].

For T-cell activation, melanoma antigens that are bound to the major histocompatibility complex (MHC) on antigen-presenting cells (APCs) require the costimulation of CD28 receptor on T-cells by CD80 or CD86 ligands on APCs [90]. The cytotoxic T-lymphocyte antigen-4 (CTLA-4) can bind with greater affinity to CD80 and CD86, and thus disrupt the necessary costimulatory signal provided by APCs [88, 90]. This led to the hypothesis that blockade of CTLA-4 function may allow for optimal costimulation of CD28 receptors on T-cells by APC CD80/86, and enhanced T-cell activation [88, 90]. Ipilimumab (Yervoy™ Bristol-Myers Squibb, New York, NY) blocks the costimulatory signal required for T-cell activation [30, 88]. In particular, Ipilimumab is a recombinant human IgG1 monoclonal antibody that binds to CTLA-4 and blocks binding to CD80 or CD86 on APCs, thus increasing activation and proliferation of T cells [88]. Two randomized phase III trials have indicated a significant advantage in disease-free survival (DFS) and overall survival (OS) in either monotherapy or combination therapy [91-92]. The first trial compared monotherapy with Ipilimumab 3 mg/kg, combination of Ipilimumab with gp100 vaccine, and gp100 vaccine alone; the study demonstrated a significant advantage in OS for patients treated with Ipilimumab (regardless the addition off gp100) in comparison to those receiving the

gp100 vaccine alone [median OS 10.0 vs 6.4 mesi; Hazard Ratio (HR) for death, 0.68; p<0,001) [91]. In the second study, Dacarbazine was administered as standard chemotherapeutic drug for melanoma patients, in association with Ipilimumab or placebo; the OS rate was significantly higher in the group of patients treated with Ipilimumab + Dacarbazine (11.2 *vs*. 9.1 months), with even more significant percentages of survival for such an association after one year (47.3% *vs*. 36.3%), two years (28.5% *vs*. 17.9%), or three years (20.8% *vs*. 12.2%) of follow-up [92]. The DFS and OS curves from the two studies have been indicated as largely overlapping, strongly demonstrating, for the first time in the history of medical treatments for the advanced disease, a clear survival benefit in metastatic (disease stage IV) melanoma. For these reasons, Ipilimumab has been recently approved by FDA and EMEA for the treatment of metastatic melanoma.

3. Melanoma subtypes and targeted therapeutic options

The different molecular *pathways* involved into the pathogenesis of melanoma are functionally linked each other (Figure 1). There is thus a need to consider such biological cascades as part of a functional web, and the alterations detected in distinct components of the various pathways must be globally considered for the effects determining in such a functional web. This new vision helps in clarifying the reasons by which some alterations may coexist or not in specific melanoma subtypes. As an example, *BRAF* mutations may be observed in conjunction with *PI3K* alterations, but none of them may coexist with *NRAS* mutations; since BRAF and PI3K kinases act downstream NRAS protein, occurrence of *NRAS* mutations activating both MAPK and PI3K-AKT pathways makes unnecessary the further activation of BRAF and PI3K (upstream effectors of the MAPK and PI3K-AKT pathways, respectively). Analogously, oncogenic *BRAF* mutations are able to more intensively activate ERK protein, main last effector downstream the MAPK pathway, when inactivation of the mechanisms controlling senescence and apoptosis concomitantly occurs.

In an attempt to simplify such complex processes underlying the different phases of development and progression of melanoma, the main pathogenetic molecular alterations may be grouped in the following way:

- oncogenic *BRAF* mutations, genomic rearrangements (mainly represented by allelic deletions) at the 9p21 chromosome, and increased expression levels of the AKT3 protein are the main alterations involved into the phase of stimulation of the proliferation for normal melanocytes (*initial preneoplastic phase*);
- impairments of the mechanisms controlling the cell senescence, apoptosis and cell survival (which particularly include alterations in the different components of *CDKN2A* pathways: functional deficit of $p16^{CDKN2A}$ gene, amplification of *CDK4-Cyclin D1/CCND1* loci, inattivation of *TP53* gene through a deregulation of the $p14^{CDKN2A}$-MDM2-p53 functional cascade), oncogenic mutations in *NRAS* gene, activating mutations and, to a lesser extent, gene amplifications of *cKIT* are the main alterations involved into the phase of acquisition of the malignant phenotype which underlay the development of melanoma (*intermediate neoplastic phase*);

- complete silencing of $p16^{CDKN2A}$ gene, functional loss of PTEN, activation of the PI3K-AKT pathway, and amplification of the *MITF* gene are the main alterations involved into the phase of acquisition of a more aggressive and invasive phenotype which underlay the progression and dissemination of melanoma (*final metasticase phase*).

All these findings clearly indicate the existence of a complex molecular machinery that provides checks and balances in normal melanocytes, allowing a physiologically controlled cell proliferation. Progression from normal melanocytes to malignant metastatic cell in melanoma patients is the result of a combination of down- or up-regulations of the various effectors acting into the different molecular pathways. According to this, it has been proposed a linear model of pathogenesis of melanoma based on the sequential accumulation of most of the previously-described molecular alterations (Figure 2A) [7, 13, 93]. In a limited fraction of cases, it has been recently hypothesized a second non-linear model of melanomagenesis based on accumulation of the same genetic alterations in tissue stem cells, with generation of malignant cells directly forming RGP or VGP or metastatic melanoma lesions (Figure 2B) [94]. This latter hypothesis has been derived by the evidence of some inconsistencies of the linear model in subgroups of melanomas (i.e., incidence of *BRAF* mutations higher in VGP lesions than that found in RGP lesions [94]).

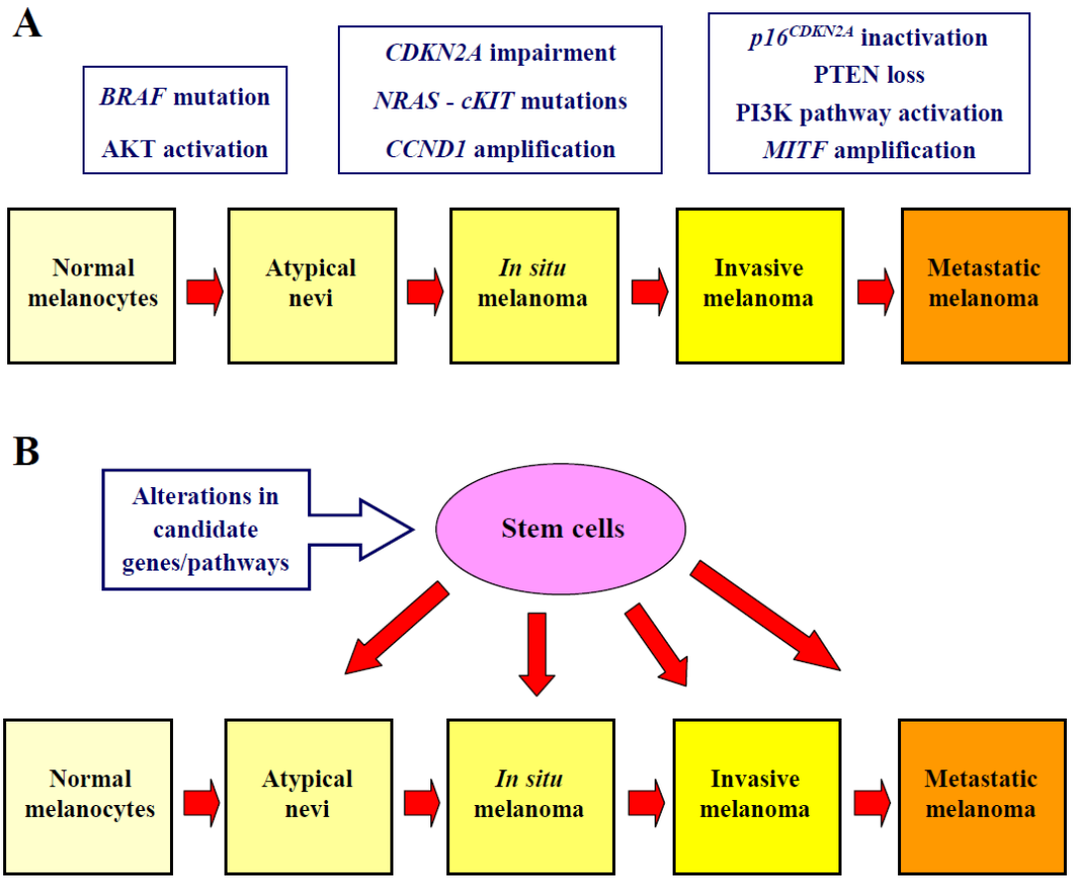

Figure 2. Models of development and progression for melanoma. A, sequential model. B, non-linear model.

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Nevertheless, all these evidence represent a strong indication that the different molecular pathways associated with the melanomagenesis does correspond to different subsets of melanoma patients, with distinguished biological and clinical behavior of the disease. Identification of such different patients' subsets should be introduced in clinical trials, in order to better assess the classification of all predictive and prognostic factors associated with the disease as well as more accurately address patients to the most effective therapeutic intervention according to their biological and molecular status.

On the basis of the presence of the specific molecular features, a discrimination of the main subtypes of melanoma, along with the more appropriate therapeutic option for each subtype, could be schematically reported.

1. Subtype MAPK

Prevalence of *BRAF* mutations, with tendency to increased expression level of activated ERK (phospoERK$_{1\text{-}2}$) in melanoma tissues. This subtype benefits by the treatment with inhibitors of BRAF.

After failure of BAY 43-9006 (which is not specific for mutated BRAF, but suppresses activity of several different kinases [95]), a second generation BRAF inhibitor (Vemurafenib, also known as PLX4032 or RO5185426) was highly specific for ^{V600E}BRAF mutation and appeared very promising from the clinical point of view [96]. Very recently, results from a phase III study comparing the vemurafenib to dacarbazine have been indeed reported, indicating a relative reduction of 63% in the risk of death and of 74% in the risk of either death or disease progression, as compared with dacarbazine [97]. Preliminary data using an additional ^{V600E}BRAF inhibitor compound, Dabrafenib (previously known as GSK2118436), seemed to point out that this molecule is also active on ^{V600K}BRAF and ^{V600D}BRAF mutations [98]; actually, treatment with such a compound is under evaluation in a phase III study among BRAF mutation positive stage III-IV melanoma patients.

However, preliminary data seem to indicate that a large variety of induced alterations may drive resistance to BRAF inhibitors: upregulation of the receptor tyrosine kinase (RTK) effectors [99], mutation in *NRAS* gene and platelet-derived growth factor receptor β (PDGFRβ) [99], amplification of the *CCND1*/*Cyclin D1* gene or lack of phosphatase-and-tensin homologue (PTEN) function [100], mutations in downstream *MEK* gene [101], activation of MAPK pathway agonists such as COT kinase [102], or enhancement of the IGF-1R/PI3K signaling [103]. These findings highlight the need for combination therapy.

The MEK inhibitors (AS703026, E6201, GSK1120212, GDC0973, MEK162) as single agents have activity against melanoma, in patients either carrying BRAF mutations and unexposed to prior BRAF inhibitor therapy or presenting NRAS mutations [30]. A new combination of MEK and BRAF inhibitors as first line therapy for BRAF mutated melanoma patients naïve to prior anti-BRAF treatment is showing great promise [30].

The *BRAF* mutations may coexist with additional molecular alterations, with subsequent constitution of further biological and molecular subgroups of melanoma patients:

a. Impairment of the $p16^{CDKN2A}$-CDK4/CCND1-RB or $p14^{CDKN2A}$-MDM2-TP53 pathways, with reduced expression of the $p16^{CDKN2A}$ protein and tendency to amplification of the *CDK4*/*CCND1* gene loci or inactivation of the TP53 gene (with consequent functional loss of the p53 protein), respectively. This subtype benefits by the treatment with inhibitors of the cyclin-dependent kinases.

Melanoma patients carrying genetic alterations affecting $p16^{CDKN2A}$ could potentially be treated with inhibitors of CDK4/CDK6. There are currently no validated therapeutic options for melanoma with mutated $p14^{CDKN2A}$. Conversely, several CDK4 inhibitors (Alvocidib, AT-7519, P1446A-05, PD-0332991, Flavopiridol/alvocidib/HMR 1275, P276-00, R547, SNS-032/ BMS-387032, UCN-01, ZK 304709/MTGI) are currently under investigation for a variety of cancer types, including metastatic melanoma, and results are awaited. For p53, there are currently no drugs, approved or in trials, against such a target. Conversely, an anti-sense agent (Oblimersen) targeted at nuclear Bcl-2 has been evaluated in trials, failing to demonstrate a significant clinical benefit among patients with melanoma [104].

b. Amplification of *MITF* ± associated with reduction of the protein expression levels.

No drug targeting MITF has been developed; however, expression of MITF has been demonstrated to be reduced by compounds inhibiting the multiple histone deacetylase (HDAC) complex [105]. Ongoing trials based on HDAC-inhibitors [LBH589 (Panobinostat) or Valproic acid (Vorinostat)] will elucidate whether a clinical benefit could be obtained by down-regulating intracellular level of MITF protein.

c. Activation of NF-kB.

Proteasome inhibitors, such as Bortezomib (Velcade, previously known as PS-341), represent a new class of anticancer therapeutic agents which inhibit degradation of important cell cycle and/or regulatory proteins, including IkB [106-107]. Bortezomid has been demonstrated to contribute in maintaining integrity of the complexes sequestering NF-kB into the cytoplasm, thus reducing the NF-kB activity [106-107]. Phase 2 studies combinating Bortezomib with other chemotherapeutic agents, including paclitaxel, carboplatin, or temozolomide equally have been established [108-109]. A compound that more directly targets the NF-kB pathway is BMS-345541 (4(2'-aminoethyl) amino-1,8-dimethylimidazo(1,2-a)quinoxaline), identified as a selective inhibitor of the catalytic subunits of IKK that binds at an allosteric site of the enzyme [110].

Since mutational activation of BRAF in human melanomas has been demonstrated to contribute to constitutive induction of NF-kB activity through an increase of the IKK activity [111], inhibition of BRAF signaling using the above mentioned inhibitors may decrease the NF-kB transcriptional activity and sensitize melanoma cells to apoptosis.

2. Subtype NRAS

Prevalence of *NRAS* mutation, with markedly increased expression level of activated ERK ($phospoERK_{1-2}$) and eventual activation of AKT. This subtype benefits by the treatment with inhibitors of MEK or mTORC.

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To date, two approaches have been considered in developing drugs against RAS. The first is based on the block of farnesylation. A small clinical trial using an inhibitor of the farnesyl transferase enzyme failed to be efficacious in a melanoma cohort; however, patients included into such a study were not selected on the basis of their *NRAS* mutation status [112-113]. In the light of recent successes of the target therapies based on anti-BRAF or anti-cKIT inhibitors, a more stringently selected cohort carrying alterations in *NRAS* gene would have increased responsiveness. On the other hand, a direct targeting of RAS has been demonstrated to be very difficult [114]; this is the reason why therapeutic strategies have focused on inhibiting downstream effectors into the pathways activated by RAS (i.e., MEK inhibitors for the MAPK pathway - see above - and mTORC inhibitors for the PI3K-AKT pathway - see below), which represent the second treatment approach against RAS.

3. Subtype cKIT

Prevalence of *cKIT* mutations ± gene amplification and/or increased protein expression levels. This subtype benefits by the treatment with inhibitors of cKIT (in particular, patients carrying gene mutations, with some sequence variants - such as K642E e L576P - which are highly responsive).

Activating cKIT mutations have been implicated in a variety of cancers, mainly represented by gastrointestinal stromal tumors (GIST) and chronic myelogenous leukemia (CML). This is the reason why several drugs targeting cKIT have been developed and tested in clinical trials, including Imatinib (approved for Philadelphia chromosome–positive CML and cKIT-positive GIST) and Sunitib (approved for advanced kidney cancer and Imatinib-resistant GIST) as well as Nilotinib and Dasatinib (approved for CML and Philadelphia chromosome-positive acute lymphoblastic leukemia).

The inhibitors of cKIT that may have a therapeutic benefit in melanoma, by inducing cell cycle arrest and apoptosis as well as significantly inhibiting cell migration and invasion of tumor cells, are:

Imatinib mesylate, formerly known as STI571, is designated chemically as 4-benzamide methanesulfonate. The efficacy of imatinib varies with the site of cKIT mutation; moreover, this drug can inhibit both the wild-type receptor activated by ligand and mutated receptor in the absence of ligand. However, imatinib is less effective at inhibiting the receptor with mutations in the enzymatic site (exon 17 mutations) [79];

Nilotinib (AMN107), which has been rationally designed based on the imatinib mesylate scaffold to have a more selective action. On this regard, Nilotinib inhibits both wild-type and cKIT mutants in exon 11 (V560del and V560G), exon 13 (K642E), double mutants involving exons 11, 13, and 17 as well as imatinib-resistant cKIT mutant (V560del/V654A) cells [115];

Dasatinib, which is a piperazinyl ethanol exhibiting increased potency but reduced selectivity compared with imatinib mesylate, has been demonstrated to inhibit both wild type and mutant cKIT in a dose-dependent manner, causing inhibition of cell migration and invasion through reduction of the phosphorylation of either Src kinase and FAK pathway [116-117].

4. Subtype mTORC

Prevalence of PTEN loss (± *PI3K* mutations, which are mostly infrequent) and phosphorylation of AKT, with absence of concurrent mutations in *BRAF* gene. This subtype mainly benefits by the treatment with inhibitors of mTOR.

In melanoma cells, three potential targets may be considered for therapeutic intervention against this pathway: AKT, PI3K and mTOR. Restoration of functional PTEN or interfering with AKT and PI3K activity would increase chemosensitivity to apoptotic agents and improve the efficacy of anti-tumor treatment. Several inhibitors of PI3K (BKM120, BEZ235, BGT226, GDC0941, PX-866, SF1126, and XL147) and AKT (GSK690693, MK2206, and VQD-002) have been developed; results of ongoing trials are thus awaited. To date, clinical trials using agents against the PI3K/AKT pathway have failed to demonstrate significant efficacy [118]. However, one therapeutic approach which seems to inhibit this pathway is based on the use of mTOR inhibitors [119]: rapamycin, Temsirolimus (CCI-779), Everolimus (RAD001), Sirolimus and AZD8055. While controversial data have been reported for rapamycin (suppressing disease progression in some patients with glioblastoma but ineffective in controlling the disease in others) [120], a limited advantage in response rates has been so far described for Temsirolimus [121]. It is to be underlined that none of the trials with mTOR inhibitors included patients specifically selected for alterations in the AKT/PI3K pathway. Several clinical trials are investigating specific combinations of mTOR inhibitors and chemotherapy drugs in the treatment of melanoma.

5. Subtype GNAQ/GNA11

Prevalence of *GNAQ*/*GNA11* mutations, with increased expression level of phosphoERK. This subtype benefits by the treatment with inhibitors of MEK.

The *GNAQ* and *GNA11* genes encodes specific GTP binding proteins that mediate signal transduction from the inner cell surface to the MAPK pathway through activation of the protein kinase C (PKC) enzyme [122]. Somatic mutations in *GNAQ* gene have been observed in about 90% of blue naevi, 50% of malignant blue naevi and 50% of primary uveal melanoma; conversely, the *GNA11* mutations have been found in less than 10% of blue nevi, about one third of primary uveal melanomas, and about 60% of metastatic uveal melanoma [122]. Since mutations in these two genes have non been detected among all the remaining types of melanoma (cutaneous, acral, mucosal), a clinical trial aimed at testing efficacy of a MEK inhibitor, Dabrafenib/GSK1120212, has been focused on patients with metastatic uveal melanoma carrying *GNAQ* and/or *GNA11* mutations [123].

4. Diagnostic panel of molecular alterations

The most prevalent molecular alterations within the heterogeneous patterns of biological features which characterize the distinct subtypes of melanoma are here summarized, according to the anatomical site of melanoma onset, the degree of exposure to the sun, and the histologic characteristics of the tumor lesions.

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- Acral melanoma

Mutation ± amplification of *cKIT*; amplification of *CDK4* or *CCND1* (associated with increased expression levels of the related proteins); amplification of the 11q13, 22q11-13, and/or 5p15 genomic loci.

- Melanoma of the head and neck district and melanoma on skin with chronic sun-induced damage (CSD)

Amplification of *CDK4* and/or *CCND1*; increased expression levels of p53 protein; mutation ± amplification of *cKIT* (in about 5% of cases).

- Melanoma of the trunk and melanoma on skin without chronic sun-induced damage (non-CSD)

Mutation of *BRAF* or, alternatively, *NRAS* (with eventual coexistence of molecolar alterations which may ba associated with BRAF mutations; see above); tendency to reduced expression of the p53 protein; occurrence of specific polymorphisms in *MC1R* gene.

- Mucosal melanoma

Mutation ± amplification of *cKIT*; amplification of *CDK4* or *CCND1* (associated with increased expression levels of the related proteins); mutation of *BRAF* (in less than 10% of cases).

- Uveal melanoma

Mutation ± amplification of *cKIT*; mutation of *GNAQ* and/or *GNA11*.

Knowledge of the principal molecular alterations to be tested in patients with such distinct subtypes of melanoma will be of great clinical importance, because it is likely to result in separate targeted therapeutic approaches and prevention strategies. To date, it has been already developed a panel of molecular tests to be performed in patients with melanoma from different anatomical locations (Figure 3). This initial "*flow-chart*" will surely become more detailed and enriched on the basis of the progressive identification and validation of additional genetics and molecular alterations correlated with the disease.

Finally, recent meta-analyses tried to define the prognostic role of majority of molecular alterations previously described [124-125]:

- negative prognostic factors

loss of p53; over-expression of iNOS, AP-2, MMP-2 and metallothioneine; increased proliferation index (high expression levels of Ki-67);

- positive prognostic factors

reduced expression or loss of $p16^{CDKN2A}$; over-expression of Bcl-2 and ATF-2 (± associated with simultaneous increases expression of beta-catenin, fibronectin and p21 proteins).

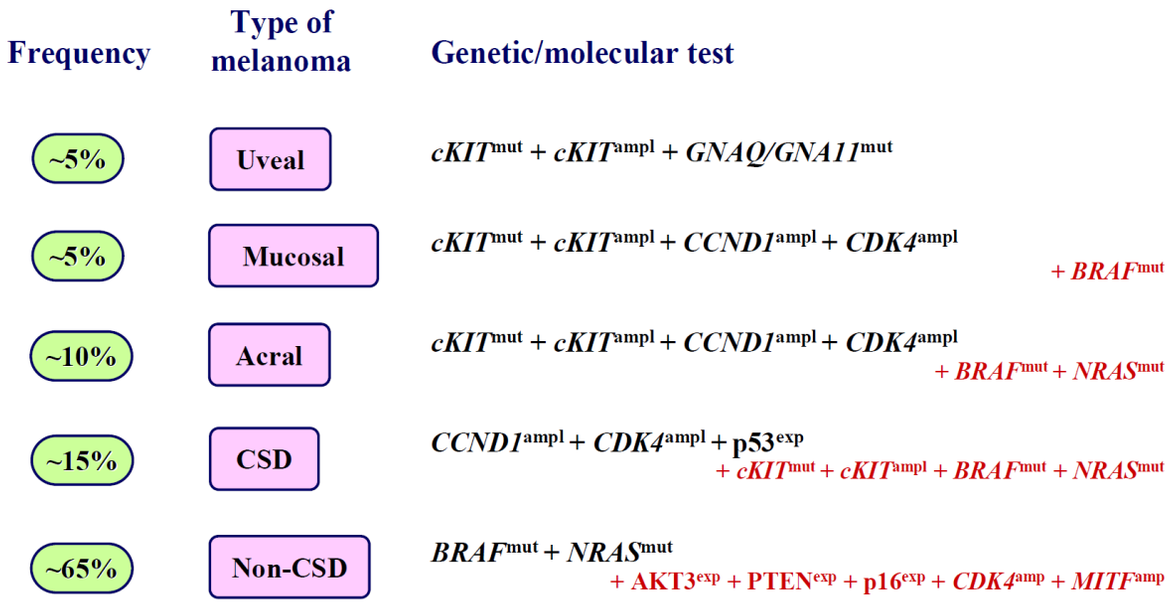

Figure 3. Principal genetic and molecular tests on tumor tissues for the different types of melanoma. Amp, gene amplification detected by *fluorescence in situ hybridization* (FISH) analysis. Exp, protein expression level detected by immunohistochemistry. Mut, gene sequence variation detected by mutation analysis. In red, tests for less prevalent alterations.

5. Conclusion

Taken together all the described molecular mechanisms involved in melanoma genesis and progression, data seem to emphasize the fact that in melanoma, but probably in all types of cancer, it is unlikely that targeting a single component in the signalling pathway will yield significant anti-tumour responses. For this purpose, molecular analyses could help clinicians to define the prognosis (prognostic value) as well as to make a prediction, identifying the subsets of patients who would be expected to be more or less likely to respond to specific therapeutic interventions (predictive value).

In other words, it is becoming evident that combination therapies targeting simultaneously several signaling pathways might be a winning therapeutic strategy to treat melanoma patients. Preclinical studies using combination of anti BRAF and AKT3 siRNA demonstrated a significantly higher reduction of tumor growth compared to single agent administration [126-127]. There is also evidence of synergism among MEK and PI3K inhibitors as well as promising results have been obtained by combinations of the mTOR inhibitors and sorafenib or MEK inhibitors [121, 128-129]. In contrast to single agent activity, these combinations of target drugs resulted in recovering of apoptosis by complete down-regulation of the anti-apoptotic proteins Bcl-2 and Mcl-1. Cooperation between BRAF and MEK inhibitors has also been demonstrated in preclinical studies with a consistent increase of apoptosis and abrogation of ERK activation compared to BRAF inhibitor alone [130]. Such cooperation was based on the observation that MEK activation was not abrogated in melanoma cells that develop

resistance to BRAF inhibitors [131-134]. BRAF and MEK targeted synergic therapy is currently tested in a phase I clinical trial (NCT01072175) which combines the selective RAF inhibitor GSK2118436 with the MEK inhibitor GSK1120212 in patients with BRAF mutant tumors [135].

After decades without perspective, the history of medical treatment for the advanced melanoma is rapidly changing. Combined therapeutic approaches do represent the next challenge for treatment of patients with such a disease.

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