Melanoma: Molecular pathogenesis and emerging target therapies (Review)

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Abstract. Malignant melanoma is an aggressive tumor of the skin with a poor prognosis for patients with advanced disease. It is resistant to current therapeutic approaches. In melanoma, both the Ras/Raf/MEK/ERK (MAPK) and the PI3K/AKT (AKT) signalling pathways are constitutively activated through multiple mechanisms. Mutations of BRAF have been proposed to contribute to melanoma development. Increased activity of the MAPK pathway prevents apoptosis and induces cell cycle progression. PTEN deletion results in Akt activation. Akt activation can result in the phosphorylation and inactivation of Raf. This decrease in downstream MEK and ERK activation may lead to loss of differentiation or senescence. This review summarizes the most relevant studies focused on the signalling pathways involved in melanomagenesis. New therapeutic strategies are also reported.

Contents

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1. Introduction

Melanoma is the most aggressive form of skin cancer (1). Its incidence has increased dramatically worldwide over the last 50 years (2); currently, the risk of developing melanoma is 1/58 for males in the United States and 1/25 for Australian males (3). It affects predominantly caucasian young and middle-aged adults (4). The main risk factors for cutaneous melanoma are intense and intermittent ultraviolet radiation exposures, phenotypic characteristics (fair skin and blond or red hair), melanocytic nevi's number, type and location (lower limbs in females, posterior trunk in males), personal or family history of melanoma (5).

If melanoma is diagnosed early it can be cured by surgical excision, and about 80% of cases are dealt with in this way (6). However, metastatic malignant melanoma is refractory to current therapies and has a very poor prognosis, with a median survival rate of 6 months (1). Recent discoveries in the complex networks involved in melanoma proliferation, progression and survival have created many opportunities for targeted drugs and new therapeutic approaches for this disease. These new targets include signal transduction pathways, oncogenes, growth factors and their receptors (7). In this review we summarize the most important studies on signalling pathways implicated in the pathogenesis of melanoma. New therapeutic strategies are also reported.

2. Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK pathway, also known as the MAPK (mitogen-activated protein kinase) pathway, is a signal transduction cascade relaying extracellular signals from plasma membrane to nucleus via an ordered series of consecutive phosphorylation events (8). In response to a variety of cellular stimuli, including growth factor-mediated activation of receptor tyrosine kinases (RTKs), Ras assumes an activated, GTP-bound state, leading to recruitment of Raf from the cytosol to the cell membrane where it becomes activated, likely via an Src-family tyrosine kinase (9-11). Activated Raf

causes the phosphorylation and activation of MAP kinase extracellular signal regulated kinases 1 and 2 (MEK1/MEK2), which in turn phosphorylate and activate extracellular signal-regulated kinases 1 and 2 (ERK1/ERK2) at specific Thr and Tyr residues (12-14). Activated ERK translocate to the nucleus and phosphorylate several nuclear transcription factors (Elk-1, Myc, CREB, Fos and others) which bind promoters of many genes, including growth factor and cytokine genes that are important for stimulating the cellular proliferation, differentiation, and survival of multiple cell types (15-35).

Dysregulation of Ras/Raf/MEK/ERK pathway plays a key role in pathogenesis of several human cancers (36); mutations at upstream membrane receptors, Ras and B-Raf as well as genes in other pathways (e.g., PI3K, PTEN, Akt), which serve to regulate Raf activity, promote constitutive ERK signalling, stimulating proliferation and survival and providing essential tumor growth and maintenance functions (37). Effects of PTEN deletion on PI3K/Akt and Raf/MEK/ERK activation in melanoma cancer are shown in Fig. 1. Therapies targeting mutant activity of components of the MAP kinase cascade could stop progression of malignant tumors by slowing tumor growth and inducing tumor cell death (36).

Abnormal activation of the MAP kinase cascade in melanoma. The MAPK pathway plays an important role in melanoma cell proliferation and survival, with ERK being constitutively activated in up to 90% of melanomas (38). In this disease, ERK hyperphosphorylation is most commonly due to mutations of NRAS (15-30%) and especially BRAF (50-70%) genes (39,40). The aberration of NRAS often is a substitution of leucine for glutamine at residue 61, this change impairs GTP hydrolysis and maintains the protein in a state of constitutive activation (41). Mutations in other Ras isoforms are rare in melanoma, suggesting an activity context dependent on specific Ras isoforms (42).

The most frequent BRAF mutation, which accounts for more than 90% of melanomas with alteration of B-Raf, is a glutamic acid for valine substitution at codon 600 in exon 15 (Val600Glu; B-RafV600E) (39); this mutation introduces a conformational change in protein structure due to glutamic acid that acts as a phosphomimetic between the Thr^{598} and Ser^{601} phosphorylation sites, leading to constitutive activation of the protein with a substantial increase in the basal kinase activity (43); the resulting hyperactivity of the MAP kinase pathway promotes tumor development (39,44,45). ^{V600E}BRAF also promotes vascular development by stimulating autocrine vascular endothelial growth factor (VEGF) secretion (46). Mutations in ARAF and CRAF have not been found in this tumor type. Likely, this pattern of mutations is due to the different mechanism of activation of the three Raf genes: BRAF requires one genetic mutation for oncogenic activation, while ARAF and CRAF require two mutations (47,48), and this is a very rare.

Interestingly, genetic alterations in NRAS and BRAF rarely coexist in melanoma (39,49,50), suggesting that mutant BRAF or NRAS alone is able to activate the MEK/ERK pathway. In Fig. 2 Raf/MEK/ERK and PI3K/Akt pathways and gene alterations that activate these pathways in melanoma are described.

3. Additional genetic insults involving other signalling networks are needed for melanoma tumorigenesis and progression

The mechanisms by which NRAS or BRAF mutations promote melanoma cell cycle progression and/or survival remain unclear. Several studies have shown the presence of the same mutations also in a high percentage of benign nevi, suggesting activation of the MAPK pathway is a necessary event for melanoma development, but it is not sufficient for malignant transformation (51,52). Therefore, oncogenic BRAF and NRAS must cooperate with additional genetic insults to induce invasive cancer development in melanocytes. Several candidate cooperative genetic changes have been identified in melanoma including MITF amplification and mutation and/or deletion of PTEN, p53 and $p16^{INK4a}$ (53).

Role of Microphthalmia-associated transcription factor (MITF) in melanomagenesis. The connection between MITF and melanoma development is complex. MITF acts as a master regulator of melanocyte development, function and survival (54,55); it plays a double role of inducer/repressor of cellular proliferation (56). High levels of MITF expression lead to G1 cell-cycle arrest and differentiation, through induction of the cell cycle inhibitors $p16^{INK4a}$ and $p21^{Cip}$ (57,58), whereas very low, or null, expression levels predispose to apoptosis (6). Only inter-mediate levels promote cell proliferation. 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 (59).

Nevertheless, continued expression of MITF is necessary for proliferation and survival of melanoma cells, because it regulates CDK2 and BCL-2 genes, respectively (60,61); furthermore, BRAF mutation is associated with MITF amplification in 10-15% of melanomas (62). However, other mechanisms likely counteract MITF degradation stimulated by ERK-dependent proteasomal degradation, since MITF amplification occurs only in few cases of melanomas in which BRAF and NRAS are mutated. MITF is a downstream target of ß-catenin, a key effector of the Wnt signalling pathway, able to stimulate growth of melanoma cells (63); thus, an alternative mechanism of MITF recovery could involve stabilizing mutations in ß-catenin leading to induction of MITF (64-66). Another mechanism could involve the same mutant BRAF; it has been recently shown that oncogenic BRAF controls MITF on two levels. It downregulates the protein by stimulating its degradation, but then counteracts this by increasing MITF expression through the transcription factor BRN2 (67).

Role of the PI3K pathway in melanoma tumorigenesis. In response to activated growth factor receptors, the phosphoinositide-3-OH kinase (PI3K) phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3), leading to activation of the major downstream effector of the PI3K pathway, Akt (68); once activated, Akt phosphorylates the downstream cellular proteins

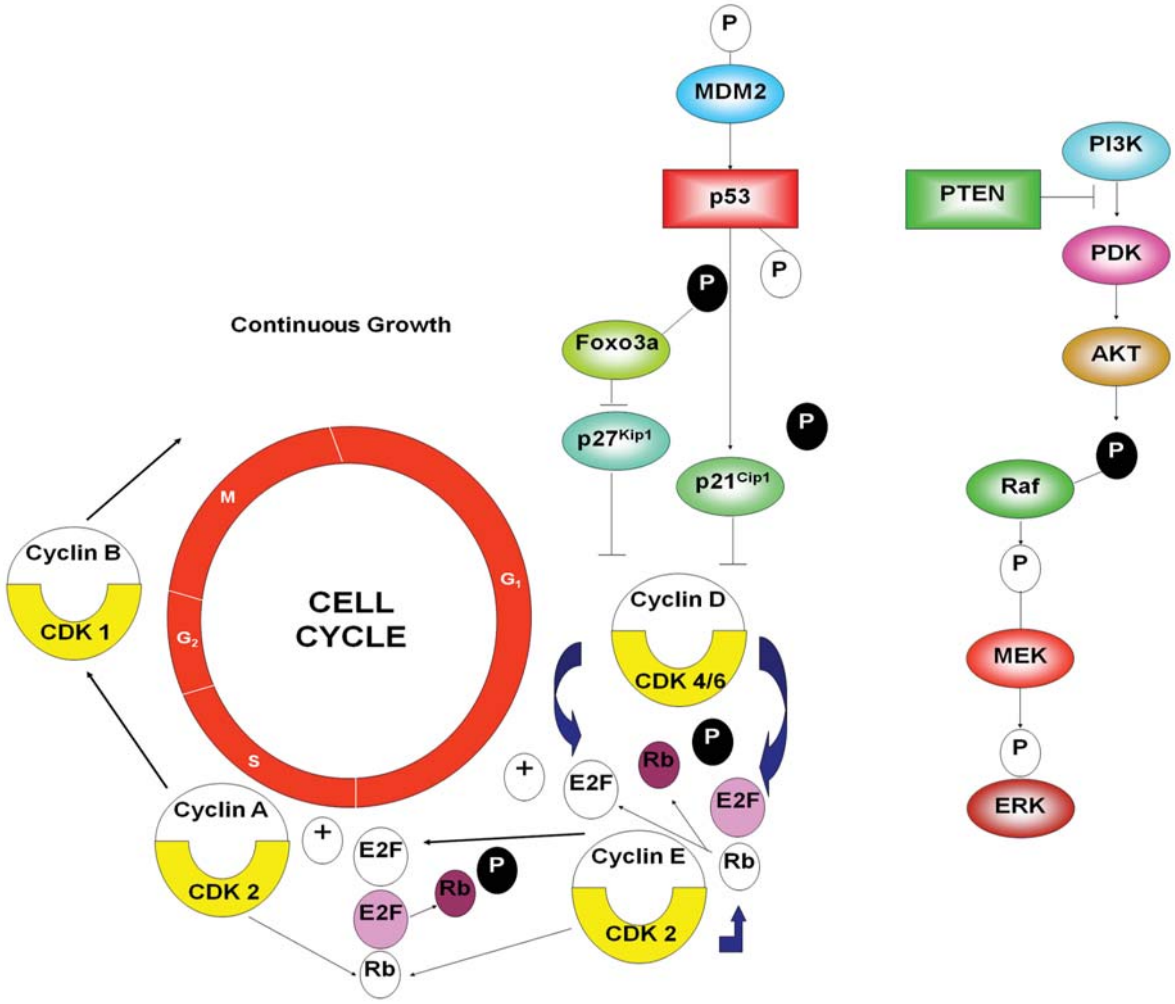

Figure 1. Effects of PTEN deletion on PI3K/Akt and Raf/MEK/ERK activation in melanoma. AKT activation results in inhibition of Raf and downstream MEK/ERK. Raf inactivation leads to decreased $p21^{Cip1}$ levels and increased cell cycle progression. Akt activation results in $p21^{Cip1}$ phosphorylation and FOXO3A phosphorylation which prevents transcription of $p27^{Kip1}$. Inactivation of Raf/MEK/ERK leads to decreased differentiation and proliferation of immature cells. Decreasing Raf/MEK/ERK cascade by PTEN deletion and Akt hyperactivation results in the continuous proliferation of immature melanoma cells.

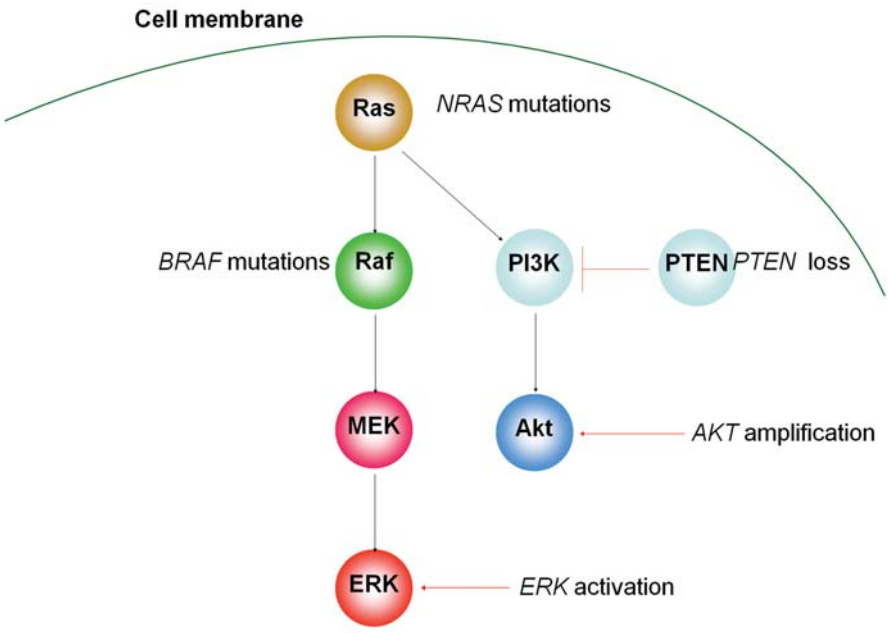

Figure 2. Raf/MEK/ERK and PI3K/Akt pathways and the mutations activating these pathways in melanoma.

that promote cell proliferation and survival (68-70). The lipid phosphatase PTEN negatively regulates this cascade through dephosphorylation of PIP3 (70).

Recent studies have revealed deregulation of the PI3K signalling in a high proportion of melanomas. Indeed, in about 45% of melanomas, PTEN is deleted and the downstream Akt gene is amplified (71,72). Both of these mutations result in overexpression of Akt3 (72), an isoform of Akt. Increased phospho-Akt expression in melanoma is associated with tumor progression and shorter survival (73,74). Oncogenic RAS can also bind and activate PI3K, resulting in increased AKT activity (75). These data suggest that loss of PTEN and oncogenic activation of RAS are largely equivalent with regard to their ability to increase oncogenic signalling through the PI3K pathway (76). This hypothesis is supported by the finding that PTEN somatic mutations are seen in melanomas harbouring mutations in BRAF but not NRAS (77). This is consistent with the ability of NRAS to activate both the PI3K and MAPK cascades, so in the presence of oncogenic NRAS additional mutations in BRAF and PTEN are unnecessary (6,74). In the recent evaluation of genomic alterations in primary melanomas, tumors with BRAF mutations had fewer copies of PTEN than those with NRAS mutations, suggesting that dual activation of the PI3K and MAPK pathways are important events in melanoma development (74,78). In Fig. 3 the frequency of NRAS, BRAF, PI3K mutations in our melanoma case series is reported. The preliminary data showed that the MAPK and AKT pathways were activated in all samples having gene mutations (Fig. 4). However, only the AKT pathway was highly expressed in tumor tissues with both PIK3CA and BRAF mutations suggesting that some PIK3CA mutations may block the MAPK pathway by activating AKT which phosphorylates and inactivates Raf.

Role of the p16(INK4a)-Rb (retinoblastoma protein) pathway in melanoma tumorigenesis. The p16(INK4a)-Rb pathway is a critical gatekeeper for cell cycle progression; in the Cdk4/6-mediated phosphorylated state, Rb drives cells towards G1/S-phase transition, while in the hypophosphorylated state, Rb binds and represses the E2F transcription factor and prevents the progression through the S-phase (79). $p16^{INK4a}$ stops cell cycle inhibiting the cyclin D/CDK4 complex, thereby preventing it from phosphorylating Rb (80).

The exit of cells from cell cycle is a physiological process; indeed, normal somatic cells have a finite lifespan, and after a finite number of divisions they exit from the cell cycle and enter a state known as senescence (6,81); senescence also occurs in response to oncogenic stress, so acting as a cellular protection mechanism against cancer formation (82,83). It has been shown that abnormally high activation of the MAP kinase pathway can inhibit cellular growth in a wide variety of normal and cancer cells by promoting cellular senescence (84,85); notably, ^{V600E}BRAF was recently found to induce $p16^{INK4a}$ expression and senescence in primary human melanocytes *in vitro* (84,86). Therefore, senescence can be overcome only if the p16(INK4a)-Rb pathway is not fully engaged, and this may occur when $p16^{INK4a}$ is inactivated (87,88). It has been reported that germline mutations in $p16^{INK4a}$ are linked to familial melanoma susceptibility (89-91); somatic mutations in gene encoding $p16^{INK4a}$ are also found in most sporadic melanomas (92,93). $p16^{INK4a}$ is inactivated by deletions, point mutations, promoter methylation (94,95) or through transcriptional silencing by overexpression of the transcriptional suppressor, inhibitor of differentiation 1 (ID1) (96). Given that $p16^{INK4a}$ needs to directly interact with the cyclin-Cdk complex in order to inhibit its protein kinase activity, changes in CDK4 that render it resistant to $p16^{INK4a}$ mimic $p16^{INK4a}$ loss (66). Both somatic and germline mutations in CDK4 have been detected in melanoma cell lines (97) and in familial melanomas (98).

Recently, another way to circumvent oncogene-induced senescence during melanoma progression has been discovered, Akt3 in early melanocytic lesions has been shown to phosphorylate ^{V600E}BRAF to reduce its activity and the MAP kinase pathway activity to levels promoting, rather than inhibiting, proliferation to overcome the senescence block (85,99).

4. Resistance to apoptosis and chemotherapy

It has been shown that melanoma cells have low levels of spontaneous apoptosis *in vivo* compared with other tumor cell types, and are relatively resistant to drug-induced apoptosis *in vitro* (6,100). As most chemotherapeutic drugs function by inducing apoptosis in malignant cells, resistance to apoptosis is thought to be the main cause of drug resistance in melanoma (100).

Dysregulation of the intrinsic (mitochondrial-dependent) apoptotic pathway form the basis for melanoma's resistance to apoptosis and chemotherapy (101-107). The p53/Bcl-2 signalling network is one of the most important regulators of cell apoptosis; the Bcl-2 superfamily includes proapoptotic (BAX, BAK, BAD, BID, Bim, NOXA, PUMA) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, BCL-w, and A1) members. In response to irreversible DNA damage, p53 becomes activated and induces the expression of proapoptotic members of the Bcl-2 family; these effectors promote mitochondrial membrane permeabilization and release of cytochrome c, which binds to Apaf-1 leading to the activation of effector caspases that result in apoptosis (108).

The loss of p53 function allows the cells that have suffered DNA damage to survive and divide, propagating pro-cancerous mutations; unlike many other chemoresistant cancers, melanomas harbour a very low frequency of p53 mutations (109-112). Therefore, other components of the p53 pathway, either upstream or downstream of p53 are likely defective in melanoma. It has been shown that aberrant methylation lead to loss of Apaf-1 expression rendering the cells unable to execute the normal apoptotic response following p53 activation (66,104). It has also been reported that decreased levels of Apaf-1 correlate with advanced disease and chemoresistance (66,113).

High levels of Bcl-2 expression have been found in melanoma and melanocytes (100,114). Aberrations in various signalling pathways contribute to elevated Bcl-2 levels in melanoma (66). In 1999, Borner *et al* found that mutant NRAS upregulates the expression of Bcl-2 *in vitro* and in SCID mice (101). MITF may also contribute to survival by the transactivation of Bcl-2 (61,66).

Alterations in other members of the Bcl-2 family were demonstrated to be involved in melanoma progression and

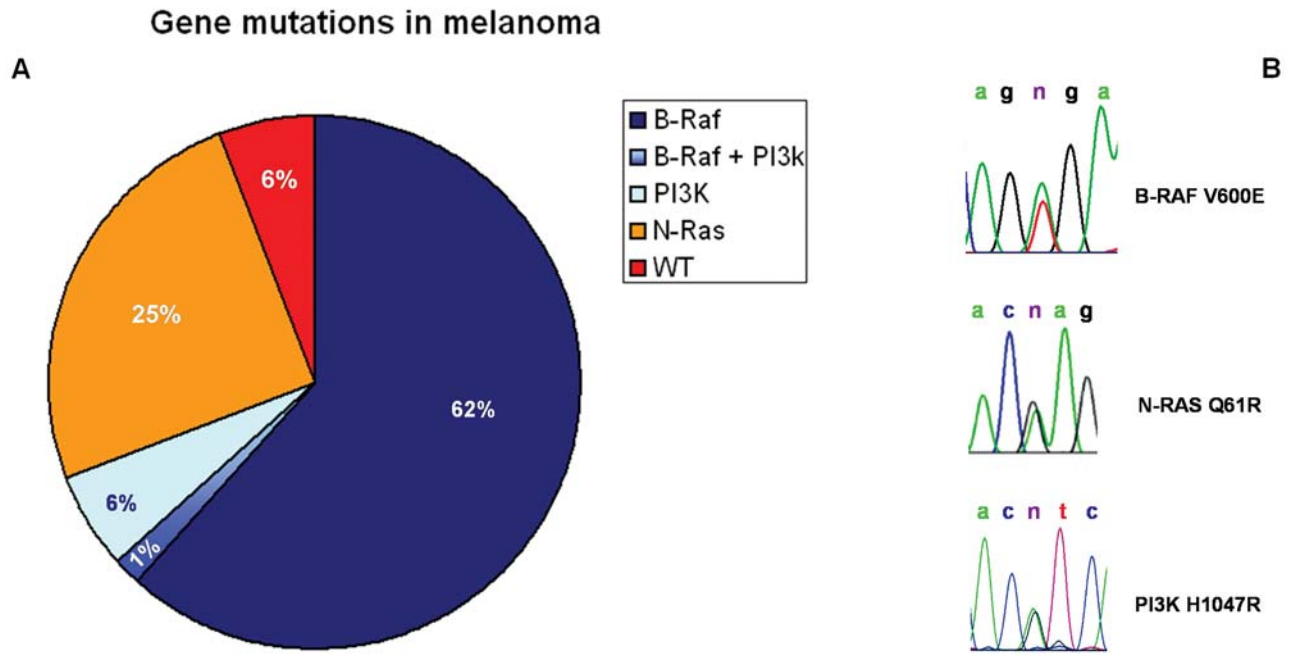

Figure 3. (A), NRAS, BRAF, PI3K mutations in a melanoma case series. (B), Hot-spot mutations of these genes.

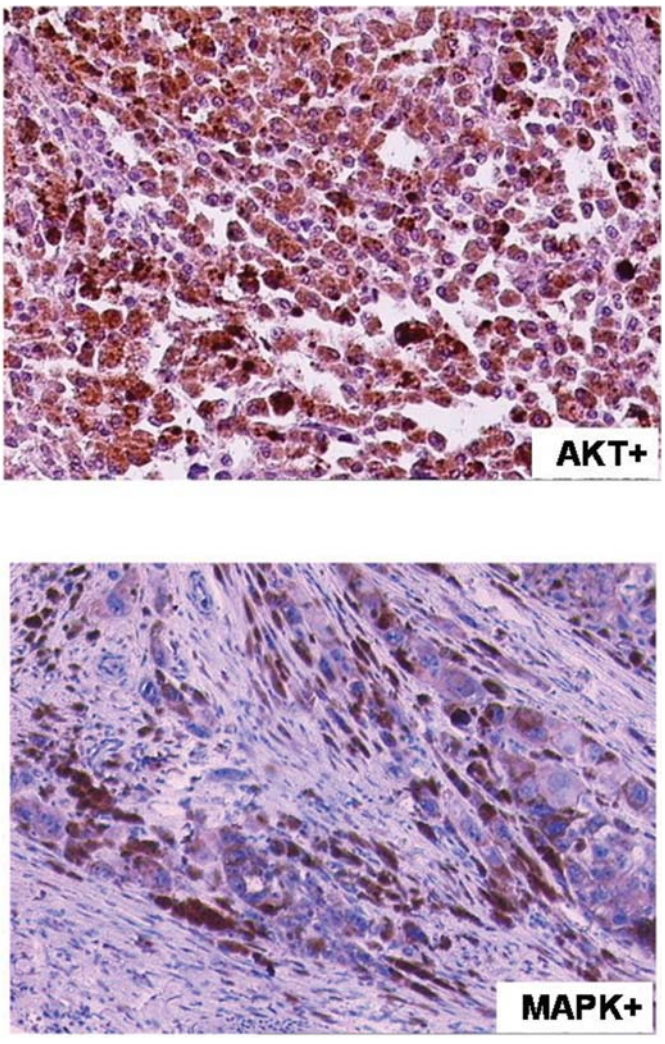

Figure 4. AKT and MAPK activation in a representative melanoma sample with both BRAF and PI3K mutations.

chemoresistance. Several studies have demonstrated that resistance to a variety of traditional and targeted chemotherapeutic agents is largely mediated by Mcl-1 overexpression (66,115-121); unlike other antiapoptotic Bcl-2 family members, Mcl-1 suppresses apoptosis induced by BAK but not BAX (122); Mcl-1 also has the unique property of rapid steady-state turnover due to proteasomal degradation (66,123); thus, in the presence of chemotherapeutics that inhibit proteasome function, such as bortezomib, Mcl-1 can accumulate and result in decreased sensitivity to these agents (66,118).

5. Emerging target therapies

Recent progress in understanding the signalling pathways involved in melanomagenesis has led researchers to develop targeted therapies for this disease. These include selective inhibitors of the RAF and MEK kinases, inhibitors of the PI3K pathway and the Hsp90 chaperone protein.

Inhibitors of the RAF kinases. Sorafenib (BAY43-9006) is an oral multi-kinase inhibitor that decreases activity of RAF, VEGF receptor 1, 2 and 3, PDGFR, Flt-3, p38, c-kit, and FGFR-1 (124), so inhibiting both tumor cell growth and angiogenesis (46,125,126). It has been shown that sorafenib inhibits the growth of melanoma xenografts in mice (46), while it has little or no antitumor activity in advanced melanoma patients as a single agent (127). The reasons why sorafenib failed in clinical trials are not clear; perhaps it is unable to reach a concentration sufficient to inhibit B-Raf or it is possible that proliferation of melanoma cells is driven by alternative signalling pathways after signalling through RAF/MEK/ERK has been blocked (127). To improve the efficacy of sorafenib in the therapy of melanoma, it is being combined with standard chemotherapeutic drugs; preliminary results combining sorafenib with carboplatin and paclitaxel were

encouraging (128). However, phase III trials have shown that this combination failed to improve progression-free survival of patients with advanced melanoma (128). Recently, it has been seen that sorafenib activates glycogen synthase kinase-3ß (GSK-3ß) in melanoma cell lines (129,130); constitutive activation of this kinase correlates with a marked increase in basal levels of Bcl-2, Bcl-x(L) and decreased antitumor efficacy of sorafenib. Therefore, sorafenib given in conjunction with targeted therapies against glycogen synthase kinase-3ß or the antiapoptotic Bcl-2 family members may prove useful (66,130).

The limited activity of sorafenib in tumors with oncogene BRAF prompted the evaluation of the efficacy of more specific BRAF inhibitors, such as RAF-265 (CHIR-265) and PLX-4032 (Plexikkon), in a phase I study for stage III/IV melanoma (http://www.clinicaltrial.gov/ct2/show/NCT00304525?term=NCT00304525&rank=1) and advanced solid tumors, respectively (Tsai J, *et al*, Proc Am Assoc Cancer Res 47: abs. 571, 2006).

Inhibitors of the MEK kinases. Recently, it has been shown that melanoma cell lines with mutant BRAF are more sensitive to MEK inhibition than lines harboring oncogene RAS (131). In BRAF mutant tumors, MEK inhibition results in down-regulation of cyclin D1, upregulation of p27, hypophosphorylation of RB and growth arrest in G1. MEK inhibition also induces differentiation and senescence of BRAF mutant cells and apoptosis in some but not all V600E BRAF mutant models (53,131,132). Two MEK inhibitors are currently being tested in clinical trials: PD0325901 (Pfizer Oncology) and ARRY-142886 (AZD6244).

Inhibitors of the PI3K pathway. CCI-779 (Temsirolimus) and RAD001 (Everolimus), are the most advanced agents in the attack on the PI3K pathway (133). They target mTOR, a serine/threonine kinase downstream of Akt that modulates protein synthesis, cell-cycle progression, and angiogenesis (134). Since mTOR is a cytosolic protein expressed by all tissues, these inhibitors do not have high specificity in targeting melanoma tumor cells (66). Furthermore, it has been determined that the mTOR pathway has a complicated feedback loop that involves suppression of Akt, hence mTOR inhibitors would potentially activate Akt in some cells (36).

The MAPK and the PI3K signalling pathways both play a key role in melanoma cell proliferation and survival (77) suggesting that parallel inhibition of targets in both pathways may result in synergistic inhibition of growth in melanomas (135).

Inhibitors of the Hsp90 chaperone protein. The molecular chaperone heat-shock protein 90 (Hsp90) is required for the folding, conformational maturation, and stability of a subset of signalling molecules, including CRAF, mutant BRAF, HER2 and AKT. Exposure of melanoma cells to the Hsp90 inhibitor benzoquinone anisomycin 17AAG results in the proteasomal degradation of mutant BRAF, inhibition of mitogen-activated protein kinase activation and cell proliferation, induction of apoptosis, and antitumor activity (136,137). Furthermore, clinical activity has been shown with 17-AAG in patients with HER2 amplified breast cancer and multiple myeloma (53,138,139). Though promising, 17-AAG has limited oral bioavailability and is poorly soluble. This has necessitated the use of intermittent intravenous dosing (once or twice weekly) likely limiting its efficacy in cancer patients. Novel small molecule Hsp90 inhibitors with improved oral bioavailability have recently entered phase I clinical testing and clinical evaluation of these compounds in tumors with a high frequency of mutated BRAF is warranted (53,140-142).

6. Conclusions

The recent identification of several key molecular pathways implicated in the pathogenesis of melanoma and induction of chemotherapeutic drug resistance has led to the development of new targeted therapies for this devastating disease. Targeting various effectors of these pathways with pharmacologic inhibitors may inhibit melanoma cell growth and angiogenesis; the specific action of these new molecular targeted agents minimizes unexpected toxicity that is typical of systemic chemotherapy. Ongoing clinical trials provide hope to improve progression-free survival of patients with advanced melanoma.

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