

# SIGNALLING PATHWAYS IN MALIGNANT SKIN MELANOMA

## THE PROGNOSTIC AND THERAPEUTIC IMPLICATIONS

## SEMNALE CELULARE ÎN MELANOMA IMPLICAȚII PROGNOSTICE ȘI TERAPEUTICE

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## Abstract

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Melanoma, Prognostic, Treatment, BRAF, MEK.

Malignant melanoma remains one of the most aggressive forms of cancer, being responsible for 1.7 % of all cancer deaths in the USA. To improve the treatment efficacy, molecular targeted therapies have been developed and gradually introduced in clinical practice. Some of these molecules act on the signal transduction pathway of cell proliferation, pathway named Ras/Raf/MEK/ERK cascade or MAPK/ERK pathway.

The Mitogen - Activated Protein Kinases (MAPKs) are serine-threonine kinases that control fundamental cellular processes such as growth, proliferation, survival or apoptosis and cell transformation, and, in abnormal situations, play a critical role in the development and progression of cancer. Today, the BRAF inhibitors (Vemurafenib and Dabrafenib) and MEK-inhibitors (Trametinib and Cobimetinib) demonstrated effectiveness in the treatment of BRAF V600 mutation-positive, unresectable, locally advanced or metastatic melanoma and therefore can be used in these situations. Regarding RAS and ERT directed therapies, there is no clinical evidence to prove any benefit for Farnesyl Transferase Inhibitors (FTIs) and RNA interference (RNAi) neither for the specific and selective, ATP-competitive, inhibitor of ERK1/2, SCH772984.

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## Rezumat

### Cuvinte-cheie:

Melanom, Prognostic, Tratament, BRAF, MEK.

Melanomul malign ramâne una dintre cele mai agresive forme de cancer, responsabil de 1.7% dintre decesele prin cancer în Statele Unite. Pentru a îmbunătăți eficacitatea terapeutică, tratamente moleculare țintite au fost dezvoltate și introduse treptat în practica clinică. Unele dintre aceste molecule acționează la nivelul căii de transmitere a semnalului responsabil de proliferarea celulară, cale numită Ras/Ras/MEK/ERK sau MAPK/ERK. Proteinele efectoare ale căii Mitogen -Activated Protein Kinases (MAPKs) sunt kinaze tip serin-treonina, care controlează procese fundamentale celulare precum creșterea, proliferarea, supraviețuirea sau apoptoza, precum și transformarea celulară, iar în situațiile anormale joacă un rol esențial în apariția și progresia cancerului.

Astăzi, inhibitorii de BRAF (Vemurafenib și Dabrafenib) și inhibitorii de MEK (Trametinib și Cobimetinib) s-au dovedit eficace în tratamentul melanomului nonrezecabil avansat sau metastatic purtător al mutației BRAF-V600 și prin urmare au fost validați pentru aceste situații. În ceea ce privește tratamentele anti RAS și anti ERK, nu există la momentul actual evidențe clinice privind eficacitatea terapeutică pentru Inhibitorii de Farnesil Transferază (FTIi) sau interferență RNA (RNA interference, RNAi), nici pentru inhibitorul specific și selectiv al ERK 1/ERK2, numit SCH772984.

The incidence of malignant skin melanoma, the most aggressive form of skin cancer, continues to rise in recent years, new 73870 cases have been estimated in 2015 in the United State by the Surveillance, Epidemiology and End Results (SEER) database, and represents 4.5% of all new cancer cases. Regarding the mortality rate, the skin melanoma is responsible for 9940 deaths in USA, representing 1.7 % of all cancer deaths.<sup>(1)</sup>

Melanoma originates from melanocyte, a specialized skin cell derived from neural-crest cells (like neurons and glial cells, adrenal medulla, cardiac cells and craniofacial tissue), whose major function is to produce the melanin.<sup>(2)</sup>

Melanin is a complex mixture of tyrosine-derived biopolymers produced in response to UVR in specialised organelles called melanosomes, and is responsible for the pigmentation of the skin, hair and eyes.<sup>(3)</sup>

Although malignant melanoma is mainly a sporadic disease, about 5 to 10% of the cases are hereditary and show familial aggregation (Familial Malignant Melanoma), defined as the occurrence of melanoma in at least two first-degree relatives or families with at least two melanomas irrespective of the degree of relationship.<sup>(4)</sup> The most common abnormality responsible for tumorigenesis and progression of malignant melanomas concerns the Ras/Raf/MEK/ERK cascade, known as MAPK / ERK pathway<sup>(5)</sup>.

### Mitogen -Activated Protein Kinases (MAPKs)

These are serine-threonine kinases that couple intracellular signals initiated by extracellular or intracellular stimuli to transcription factors which control fundamental cellular processes such as growth, proliferation, differentiation, migration,

survival or apoptosis and transformation<sup>(6)</sup>. Abnormalities in MAPK signalling impinge on most, if not all these processes, and play a critical role in the development and progression of cancer.<sup>(7)</sup>

### RAS (Rat Sarcoma) genes and proteins

Among the 3 RAS known oncogenes (H-RAS, K-RAS, and N-RAS), the NRAS (Neuroblastoma RAS viral oncogene homolog), located on the short (p) arm of chromosome 1, is particularly involved in the induction and progression of malignant melanoma.<sup>(8)</sup>

NRAS mutations are found in all melanoma subtypes, but may be slightly more common in melanomas derived from chronic sun-damaged<sup>(9)</sup>. Normal p21<sup>ras</sup> protein is a small (21 kDa) G-protein (Guanosine-nucleotide-binding protein), that binds GDP (Guanosine Diphosphate) or GTP (Guanosine Triphosphate) and acts as a binary molecular switch ("on" and "off" states) that controls intracellular signalling networks.<sup>(10)</sup> In the inactive ("off") state p21<sup>ras</sup> is bound to the GDP, while in the active growth-promoting signal («on») state, it is bound to GTP.<sup>(11)</sup> Therefore, the ratio of p21<sup>ras</sup>-GDP-bound ("off" state) to p21<sup>ras</sup>-GTP-bound ("on" state) is crucial for the control of cell growth and proliferation, and this ratio must be controlled precisely. In the activation phase GTP triggers a cascade of mitogen-activated protein kinases which ultimately phosphorylate the target, such as a transcription factor, delivering the message to its final destination.<sup>(12)</sup> In normal situations (wild-type p21<sup>ras</sup>) this signal is necessarily self-limiting, the deactivation (GTP to GDP conversion) being achieved by hydrolysis, but mutations of p21<sup>ras</sup> sends a continuously growth-promoting signal by the active GTP-bound form.<sup>(13)</sup>

### **Incidence of NRAS mutation in malignant melanoma**

NRAS mutations, present in 20% all melanomas, are mainly represented by 2 oncogenic changes, the position 61 (a point mutation representing more than 80% of all NRAS mutations) and position 12-13 mutations. Position 61 mutation is an activating mutation which locks the p21<sup>ras</sup> into its activated (GTP-associated) conformation, while mutations at positions 12 and 13 render p21<sup>ras</sup> insensitive to the physiological mechanisms of inactivation.<sup>(14, 15)</sup>

### **Clinical and therapeutically significance**

In terms of morphological and phenotypic diagnosis, NRAS mutated melanomas do not exhibit any particular characteristics, but the presence of a NRAS mutation has a prognostic significance. NRAS mutant melanomas are thicker tumours, typically located at the extremities with greater rates of mitosis compared to BRAF mutant melanomas. Regarding the overall survival, the NRAS mutation is an unfavourable prognostic factor for melanoma patients compared with non mutated patients<sup>(16)</sup>

### **Ras-directed therapies**

There are 2 distinct anti-Ras strategies: actions at the p21<sup>ras</sup> proteins level and actions at the RAS gene expression level.

**At the p21<sup>ras</sup> level**, the main directed therapy is represented by Farnesyl Transferase Inhibitors (FTIs), a class of drugs designed to inhibit the post-translational farnesylation of a number of target proteins, including p21<sup>ras</sup>, preventing its membrane attachment and signalling function.<sup>(17)</sup> Despite potent target inhibition in tumour tissue (85-98%), FTIs showed no clinical benefit (no clinical responses).<sup>(18)</sup>

**At the RAS gene expression level** the idea is to prevent the Ras expression by RNA interference (RNAi), a biological process in which RNA molecules inhibit gene expression, causing the mRNA cleavage, effects dependent on the doubling time of the cells.<sup>(19, 20)</sup>

### **B-RAF (Rapidly Accelerated Fibrosarcoma) genes and proteins**

Located on the long (q) arm of chromosome 7, the BRAF gene provides instructions for making a protein that helps transmit chemical signals from outside the cell to the cell's nucleus.<sup>(21)</sup>

The RAF family proteins (there are three known mammalian RAF isoforms: A, B and C-Raf) are serine/threonine kinases which play a role in various normal physiological processes as cellular metabolism, cell cycle progression, cell death and neurological function.<sup>(22)</sup> Most RAF kinase protein located in the cytosol where the enzymes lie, are in their dormant state. Being activated by RAS-GTP complex, the RAF kinases participate in the RAS-RAF-MEK-ERK signal transduction cascade.<sup>(23)</sup> On

the contrary, by inhibiting RAF, the MAPK pathway is being blocked and therefore attenuates cell cycle progression by arresting cells at the Go/G1 boundary.<sup>(24)</sup> Mutations in the BRAF gene leads to a mutant protein whose kinase activity is greatly elevated, which constitutively stimulates ERK activity independent of RAS. As a result, mutated BRAF causes overactive downstream signalling via MEK and ERK, leading to excessive and uncontrolled cell proliferation, independent of growth factors and resistance to apoptosis (programmed cell death).<sup>(25)</sup>

### **Incidence of BRAF mutation in malignant melanoma**

Approximately 40-50% of melanomas harbour activating BRAF mutations without correlation between BRAF mutational status and age, sun exposure, and Clark's level.<sup>(26)</sup> Most commonly (90-95% of BRAF mutant melanoma), the valine at amino acid 600 is replaced by glutamate (V600E) through mutation of a single nucleotide (GTG to GAG), which leads to a 5000 fold increase in its kinase activity.<sup>(27)</sup>

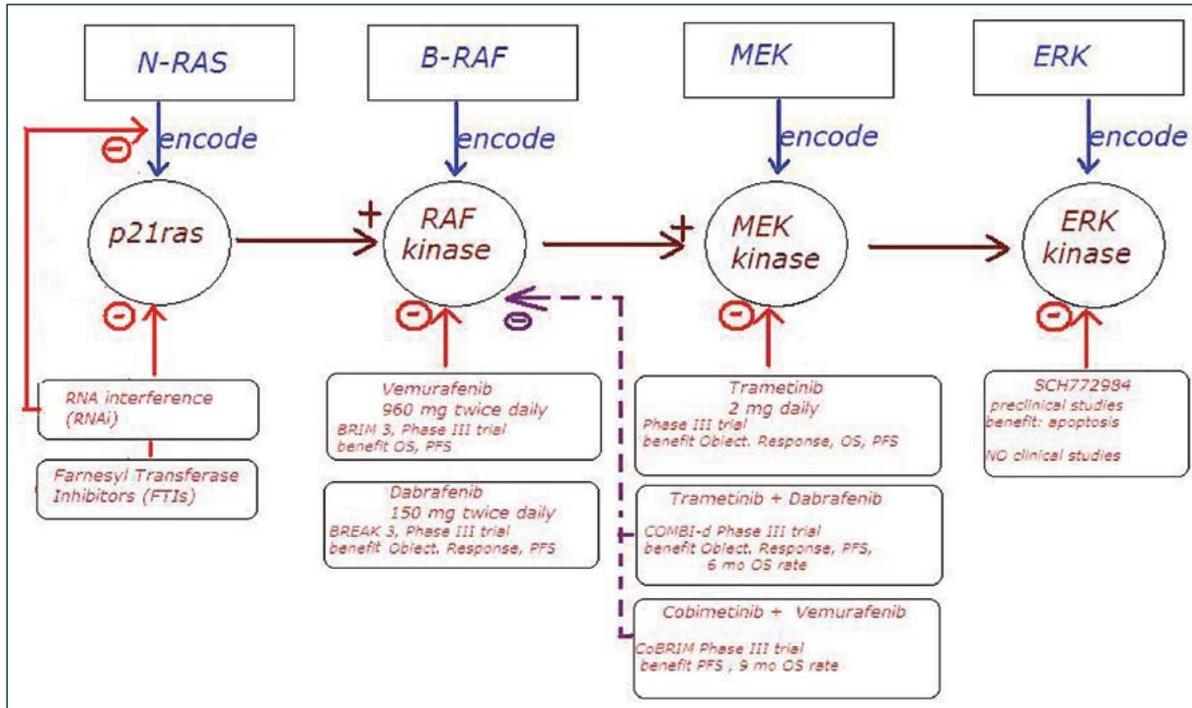
### **Clinical and prognostic significance**

For localised skin melanoma BRAF-mutation is correlated with age (younger patient, ≤ 50 years), location (truncal location), histopathology subtype (superficial spreading or nodular melanoma) and lack of cumulative sun-induced damage (CSD) at the primary site<sup>(28)</sup>. In metastatic cases B-RAF mutation, identified in more than 70% of cases is principally correlated with age (younger patients). The interval from diagnosis of melanoma to first distant metastasis (including first unresectable locoregional recurrence) was not statistically different between BRAF-mutant and BRAF wild-type melanoma. B-RAF mutation is a survival unfavourable prognostic factor, the overall survival time from diagnosis of first distant metastases being 4 time shorter for the BRAF-mutated patients compared with wild-type BRAF patients (11.1 months vs. 46.1 months).<sup>(29)</sup>

### **BRAF mutation detection methods**

**A) Tumor biopsy methods.** Several methods can be used to detect BRAF mutations, variations in reported BRAF mutation rates being attributable to differences in the detection methods used. The Cobas 4800 BRAF V600 Mutation Test is a Real-Time Polymerase Chain Reaction (RT-PCR) on a formalin-fixed, paraffin-embedded (FFPE) tissue in order to amplify and detect the target DNA. It presents cross-reactivity with non-BRAF p.V600E mutations (eg, p.V600K, p.V600D, and p.V600E2')<sup>(30)</sup> Sanger sequencing is a method of DNA sequencing (determine the precise order of nucleotides within a DNA molecule) used to identify mutations in DNA. However, sequencing has relatively low analytical sensitivity, meaning that a mutation must be present in >15% to 20% of tumor content to be detected.<sup>(31)</sup> Immunohistochemistry (IHC), a rapid

**Table 1**



and inexpensive test, particularly useful for specimens not suitable for molecular analysis, uses a mouse monoclonal antibody directed against the BRAF V600E mutant epitope, that only recognizes the V600E epitope and not others (eg, BRAF wild type, V600K, V600D, V600R, K601E, etc).<sup>(32)</sup>

A comparative molecular study shows that BRAF immunohistochemistry (IHC) has excellent specificity for detecting BRAF V600 mutations and confers comparable results with cobas test, but concludes that IHC should not be used alone and the cobas test remains the FDA-approved diagnostic standard.<sup>(33)</sup>

**B) 'Liquid biopsies' methods.** Liquid biopsies are non-invasive methods to detect Circulating Tumour Cells (CTCs) and Circulating Nucleic Acids (CNA), especially circulating tumour DNA (Cell-free or circulating tumour DNA, ctDNA) which are fragments shed into the blood from the primary tumour and from metastatic sites.<sup>(34)</sup> The circulating mutated DNA detection method must be extremely sensitive, because tumor-specific DNA can greatly vary related to individual-specific and tumor-specific factors and, most important, the most cfDNA present in the serum are wild-types and the tumor-derived mutant DNA fraction could be <0.01%.<sup>(35)</sup> Using a Droplet digital PCR (ddPCR), an analytically sensitive technique for quantifying small concentrations of DNA, BRAF-V600E mutation in circulating DNA (cfDNA) has been detected in >75% of late-stage melanoma patients with BRAF mutation-positive tumors, with the correspondence between tumor tissue BRAF(V600E) and plasma cfBRAF (V600E) of 84.3%.<sup>(36)</sup>

**B-RAF-V600E mutation and target therapy**

There are 2 drugs against BRAF protein, Vemu-

rafenib (Zelboraf) and Dabrafenib (Tafinlar), approved for the treatment of metastatic or unresectable melanoma for patients with BRAF V600E mutation (not indicated in patients with wild-type BRAF melanoma)<sup>(37)</sup>

**A) Metastatic melanoma.**

BRIM-3, a phase III randomized clinical trial on 675 treatment-naive patients with BRAF V600E-mutated stage IIIC/IV metastatic melanoma, compares Vemurafenib (960 mg orally twice daily) with Dacarbazine (1,000 mg/m<sup>2</sup> intravenously every 3 weeks). Vemurafenib produced improved rates of overall survival (84% vs. 64% at 6 months) and progression-free survival (5.3 against 1.6 months) than classical chemotherapy with Dacarbazine.<sup>(38)</sup>

**BREAK-3** is a phase III trial on 250 patients with stage IV or inoperable stage IIIC BRAFV600E melanoma, with no prior therapy, that compares Dabrafenib (150 mg twice daily) with Dacarbazine (1000 mg/m<sup>2</sup> every 3 weeks, IV), with a crossover to Dabrafenib for the patients that progresses in Dacarbazine group. Dabrafenib is superior to classical Dacarbazine regarding objective response rate (52% vs 17%) and progression free survival (5.1 vs 2.7 months). Dabrafenib's toxicity is mainly directed to the skin (hyperkeratosis, papillomas, palmar-plantar erythrodysesthesia), followed by pyrexia, fatigue, headache and arthralgia.<sup>(39)</sup>

**B) Nonmetastatic malignant melanoma.**

Currently neoadjuvant treatment for resectable/unresectable stage III melanoma remains an investigational approach, but for inoperable bulky melanoma with positive BRAF V600E mutation, neoadjuvant therapy can be recommended to reduce the tumour burden and facilitate tumor resection.<sup>(40)</sup> A study on 11 patients with advanced locoregional BRAF V600E mutant melanoma treated with

6.0 months ( 1.2-29.4 months) Vemurefenib demonstrate objective responses in 50% of patients without major complications attributable to BRAF-targeted therapy <sup>(41)</sup>

### **MEK (MAP kinase / ERK Kinase ) genes and proteins**

The MEK family of genes, also known as MAPKK (Mitogen-Activated Protein Kinase Kinase) or MAP2K, located on the long (q) arm of chromosome 15 (15q) for MEK 1 and on the long (q) arm of chromosome 19 (19q) for MEK2 respectively.<sup>(42)</sup>

MEK genes provide instructions for making MEK protein kinases (molecular weight about 45-50 kDa), dual-specificity kinases, kinases that can act as both tyrosine kinase and serine/threonine kinase.

The most potent activator MEK kinase is the BRAF. The RAF kinase causes the phosphorylation and activation of MEK1/2, which in turn phosphorylates and activates Extracellular signal-regulated kinase (ERK). Constitutive activation of MEK1 (caMEK) can induce the oncogenic transformation.<sup>(43)</sup>

### **MEK mutants actions**

Constitutively active MEK mutants promote long-term events such as cell differentiation, proliferation and transformation, inducing the formation of aggressive tumors that progress up to the metastatic stage.<sup>(44)</sup> Conversely, dominant negative MEK mutants prevent cells proliferation and can revert RAF, RAS transformed cells.<sup>(45)</sup>

### **Incidence of MEK mutation in malignant melanoma and therapeutic implications**

The incidence of MEK mutation in malignant melanoma is relatively low (3 to 8 %), only 7 MEK1 and one MEK 2 mutation being identified in the analysis of tumor samples of 127 melanoma patients.<sup>(46)</sup> The MEK mutation downstream of BRAF causes the reactivation of kinase activity and confers resistance of BRAF- inhibitors ( resistance-associated MEK1 mutations).<sup>(47)</sup> MEK inhibitors are able to target MAPK-dependent tumors and exhibit distinct efficacies against BRAF- and KRAS-mutant melanomas.

Since 1995 highly selective MEK1/2 inhibitors have been tested clinically or are currently undergoing clinical trial evaluation.<sup>(48, 49)</sup>

### **Patients selection.**

Pre-clinical studies suggest that patients harboring activating mutations in RAS or BRAF genes are better candidates for treatment with these kinase inhibitors.<sup>(50)</sup>

### **Therapeutic scheme selection : Monotherapy versus combination.**

Two highly selective allosteric inhibitors of MEK1 and MEK2, Trametinib and Cobimetinib , are currently used for the treatment of metastatic melanoma patients carrying the BRAF V600 mutation.

### **MEK inhibitors as monotherapy**

Trametinib was studied in a Phase III randomized, open-label, multisite study on 322 patients with metastatic BRAF V600E/K mutation-positive melanoma. Trametinib 2 mg once daily compared to chemotherapy increases median Progression-free Survival (PFS) from 1.5 months to 4.8 months (P<0.001) and Overall Survival (OS) at 6 months from 67% to 81 % (P=0.01). The objective response rates according to Response Evaluation Criteria In Solid Tumors (RECIST) were 22% for trametinib against 8% for chemotherapy (P=0.001). The results of this study led to approval of trametinib as a single agent for treatment of patients with unresectable or metastatic melanoma with BRAF V600E or V600K mutations.<sup>(51)</sup>

### **MEK inhibitors associated with BRAF inhibitors**

The BRAF-MEK inhibitor combination tries to realise multistage inhibition of the MAPK pathway to avoid the development of acquired resistance due to intrinsic resistance and MAPK-reactivation.<sup>(52)</sup> There are 3 Phase III clinical trials that compare the combination of Trametinib or Cobimetinib and Dabrafenib versus Dabrafenib or Vemurafenib alone on patients with unresectable stage IIIC or metastatic stage IV melanoma harboring a BRAF V600E or V600K mutation.

**The Phase III trial (COMBI-d)** conducted on 423 previously untreated patients, demonstrates that association of Trametinib (2 mg orally once daily) and Dabrafenib (150 mg orally twice daily) is superior to Dabrafenib alone in terms of overall response rate (67% vs 51%) , median progression-free survival ( 9.3 vs 8.8 months, p=0.03) and interim 6-month overall survival rate (93% vs 85%, P=0.02). Toxicity was similar in the two groups except for pyrexia, which occurred more often in the combination arm than in the monotherapy arm (51% versus 28%).<sup>(53)</sup>

**The Phase III open-label trial (COMBI-v)**, on 704 treatment-naïve patients, shows that combination of Trametinib-Dabrafenib (same dosage as in COMBI-d) are superior to Vemurafenib (960 mg twice daily) alone in terms of overall survival rate at 12 months (72% vs 65%; p=0.005), median progression-free survival (11.4 vs 7.3 months; p<0.001) and objective response rate (64% vs 51 , p<0.001). Rates of severe adverse events and study-drug discontinuations were similar in the two groups. Cutaneous squamous-cell carcinoma and keratoacanthoma occurred in 1% of patients in the combination-therapy group and 18% of those in the vemurafenib group.<sup>(54)</sup> The results of phase III CoBRIM trial, adding Cobimetinib to Vemurafenib in 495 patients with BRAF V600 mutation-positive, unresectable, locally advanced or metastatic melanoma show an increase in the.. median progression-free survival (from 7.2 to 12.3 months) and overall survival rate at 9 months (from 73% to 81%) compared to Vemurafenib alone.<sup>(55)</sup> As a result of these excellent findings The US Food

and Drug Administration - FDA has approved the combination of Dabrafenib (150 mg orally twice daily) and Trametinib (2 mg orally once daily) for the treatment of patients with BRAF V600E/K-mutant metastatic melanoma, and their use seem to be currently the best approach. (56)

### ERK (Extracellular signal-Regulated Kinases) genes and proteins

ERK gene (also known as Mitogen Activated Protein Kinase 3), located on the short (p) arm of chromosome 16 (16p), is responsible for the coding of ERK proteins, a 44 kDa (ERK1) and 42-kDa (ERK2) serine/threonine kinase.<sup>(57)</sup> ERK1 /2 (MAPK3/1) is regulated by the dual-specificity kinases MEK1/2 through phosphorylation, then activated ERK1/2 translocates to the nucleus, where it phosphorylates many different substrates involved in various cellular responses from cytoskeletal changes to gene transcription.<sup>(58)</sup>

It has been shown that activation of ERK1/2 (MAPK3/1) is crucial for cyclin D1 induction, providing a molecular link between ERK signalling and cell cycle control as cyclin D1 gene is essential for G1 to S-phase progression.<sup>(59)</sup> The kinetics and duration of ERK activation may play an important role in influencing its effect on cell fate, in the sense of cell survival and proliferation (transient activation) or, to the contrary, in cell apoptosis (prolonged ERK activation).<sup>(60)</sup> In normal state, melanocytes do not have detectable ERK activity (phosphorylated ERK1/2 levels were low in normal human melanocytes ) and require a number of growth factors, secreted by nearby keratinocytes, to survive. There are at least 2 pathways whereby growth factors can activate ERK in melanoma. The first "classical" pathway involves direct activation of the Ras/Raf pathway, while the other pathway involves the activation of Ras through a novel cAMP-driven exchange factor.<sup>(61)</sup>

### Incidence of ERK activation in malignant melanoma and therapeutic implications

In malignant melanoma, activation of ERK (phosphorylation of ERK, p-ERK) is present in 54% of primary and 33% of metastatic tumors respectively, but no correlation was observed between p-ERK expression and melanoma-specific survival.<sup>(62, 63)</sup>

From treatment point of view, a successful therapy needs a significant reduction in ERK1/2 phosphorylation. Studies on Cisplatin, one of the most potent antitumor agents, showed that it not decrease ERK1/2 phosphorylation in vitro; on the contrary, it increased the activation of ERK1/2 and consequently enhanced chemoresistance.<sup>(64)</sup>

ERK activation can also represent one of the principal mechanisms of resistance to BRAF/MEK inhibitors. To block these potentially activating pathways, a novel molecule, SCH772984 a specific and selective, ATP-competitive, inhibitor of ERK1/2, activity has been tested on resistant cell lines.<sup>(65)</sup>

In preclinical studies, SCH772984 inhibits cellular proliferation and causes selective apoptosis in RAS or BRAF mutated tumour cell lines, being effective even in tumor cells that were resistant to either BRAF or MEK inhibitors and in cells that became resistant to the dual combination of these inhibitors. No clinical studies are available.<sup>(66)</sup>

In conclusion, the development of novel treatments that selectively inhibit the RAS-RAF-MAPK pathway represents a milestone in the malignant melanoma therapeutic strategy that improves the outcome, compared with classical cytotoxic chemotherapy.

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