ROMANIAN ACADEMY

INSTITUTE OF BIOCHEMISTRY

Ph.D. THESIS SUMMARY

- MELANOMA BIOMARKERS AND MOLECULAR MECHANISMS IN VEMURAFENIB-RESISTANT TUMOR CELLS -

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2023

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1. THESIS STATEMENTS

The work presented is structured in two parts and addresses two important topics in defining the aspects that contribute to the unfavorable prognosis in melanoma. In a first part we tried to define a representative panel of biomarkers for amelanotic melanoma and thus facilitate the early detection of this subtype of cancer. In recent years, there has been an increased interest in defining aspects regarding melanoma heterogeneity. The need to identify new biomarkers with high specificity for different melanoma subtypes is highlighted by the poor prognosis determined by delayed diagnosis in most cases. A particular subtype of melanoma is represented by amelanotic melanoma, characterized by the absence of pigmentation or a very weak pigmentation of the cells. Amelanotic melanoma is often difficult to diagnose, and the delay in establishing a correct diagnosis determines its discovery in advanced stages. In order to identify specific biomarkers that could help in the early diagnosis of amelanotic melanoma, we performed a comparative proteomic analysis based on mass spectrometry. The analysis was conducted on five metastatic melanoma cell lines that differ from each other in the intensity of cell pigmentation. At the proteome level, we have identified a series of defining features for the amelanotic cell lines A375 and SKMEL28, and a series of biomarkers have been proposed both for diagnostic purposes and to assess the ability of cancer cells to migrate. The increased specificity of the proposed set of biomarkers allowed a better differentiation of amelanotic melanoma from the pigmented one, and the comparative proteomic analysis revealed a series of biological processes that give amelanotic melanoma an aggressive character. However, there is no consensus regarding the prognosis of amelanotic melanoma, and the comparison with pigmented melanoma in terms of aggressiveness is intensely debated. In general, the poor clinical prognosis attributed to amelanotic melanoma has been attributed to advanced forms at the time of diagnosis. The results of the analysis carried out in this first part indicate a series of defining characteristics for amelanotic melanoma cells characterized by an increased migration capacity. This aggressive phenotype of non-pigmented melanoma cells can be explained mainly by the identified biomarkers, as well as by the cellular processes identified to be strongly regulated in these cells.

The second part of the work focused on studying the mechanisms involved in the development of melanoma cell resistance to BRAF inhibitors. For this purpose, two melanoma cell lines resistant to vemurafenib were generated and a general characterization was performed in

a first step. In addition, a number of cellular processes and proteins have been highlighted that could play an important role in the development of resistance. A first approach was the comparative proteomic analysis of resistant and sensitive cells to vemurafenib. Also, the role of the transcription factor HIF1α in this mechanism was studied, and a series of dynamic processes regarding extracellular matrix modeling or the production of exosomes were analyzed.

The thesis is structured in 4 main chapters. In the INTRODUCTION, aspects regarding the pathology of melanoma and the molecular mechanisms involved in the malignant transformation of cells and tumor progression are detailed. The main analysis techniques used are presented in the MATERIALS AND METHODS chapter, and the results of the two topics addressed are presented in detail in the RESULTS chapter. The conclusions of the two studies were summarized in the last chapter, CONCLUSIONS, and present the most important observations.

2. INTRODUCTION

2.1 Melanoma – general aspects, causes, risk factors and therapy

Melanoma is the most aggressive type of skin cancer, being considered the leading cause of death among patients diagnosed with skin cancer. In recent years, the increased incidence of this type of cancer has highlighted the complexity of the causative factors and the marked heterogeneity of these tumors. In 2018, a 3 times higher incidence of newly diagnosed melanoma cases was recorded compared to 1975, with approximately 25 melanoma cases reported per 100,000 people (Saginala et al., 2021). However, a relatively high frequency of melanoma cases is restricted to certain geographic areas, being very high in Australia and New Zealand (Sneyd and Cox, 2013). The main risk factor in the development of melanoma is represented by prolonged exposure to the sun, UV radiation being incriminated in the genetic changes that favor the appearance of melanoma. The white-skinned population is mainly affected, and genetic factors play an important role in the development of cancerous lesions. Melanoma is much more common in people with an increased number of nevi and a family history of melanoma (Saginala et al., 2021). Also, the features of nevi can represent another indicator regarding the possibility of these lesions to become malignant (Zalaudek et al., 2009). The starting point is represented by melanocytes, the pigment-producing cells that are distributed mainly in the skin, inner ear, eye and leptomeninges, and mutations in these cells cause the appearance of melanoma (Domingues et al., 2018). The survival rate drops significantly when metastases associated with the primary tumor are confirmed, and wrong or delayed diagnosis is the main reason for the unfavorable prognosis (Grant-Kels, Bason and Grin, 1999; Sandru et al., 2014).

Four types of melanoma have been defined according to histopathological features: superficial, nodular, lentiginous, and acral lentiginous. The most common type of melanoma is represented by the superficial melanoma, being confirmed in approximately 70% of melanoma cases (Longo, Casari and Pellacani, 2012), with nodular melanoma being the second most common (Erkurt et al., 2009).

Superficial melanoma is characterized by radial growth in the early stages, while nodular melanoma shows vertical growth. Another form of melanoma is defined by lentiginous melanoma, represented by cells distributed at the dermis-epidermis interface and skin appendages. Lesions

associated with this type of melanoma are most often determined by exposure to UV radiation, while the acral lentiginous subtype is characteristic of the extremities and is not caused by sun exposure (Bristow et al., 2010; Arisi et al., 2018; Sundararajan et al., 2023).

Diagnosis involves the identification of specific phenotypic characteristics defined by the ABCDE classification system (A-asymmetry, B-irregular border, C-color, D-diameter, E-evolution), as well as tumor staging based on histopathological criteria: Clark index, Breslow index and TNM staging (T- primary tumor extent, N-presence of lymph node-associated metastases, M-presence or absence of distal metastases) (Rotte and Bhandaru, 2016).

2.2 Pigmentation process

The pigmentation process is specific for melanocytes, cells specialized in melanin production that originate in neural crest cells. The differentiation of neural crest cells into melanocytes involves first the formation of bipotent melanoblast-glial progenitor cells SOX10 positive. Next, these cells differentiate into melanoblasts that express the transcription factor MITF, DCT and the KIT protein. These cells are characterized by an increased migratory capacity and an accelerated proliferation. In general, cells that migrate to the dorsolateral region during embryogenesis differentiate into melanocytes (Mull, Zolekar and Wang, 2015). The melanocytes thus formed presented a specific signature, and the specific markers they express are mainly represented by tyrosinase (TYR), tyrosinase-related proteins (TYRP-1 and TYRP-2), Pmel17, MART-1 and MITF. The process of melanogenesis takes place in melanosomes, organelles related to lysosomes, where proteins involved in pigmentation process are located. The maturation process of melanosomes is dependent on the internal pH of this compartment, and values between 5 and 6.8 are required for melanosomes formation (Schallreuter et al., 2008; D'Mello et al., 2016). The biogenesis of melanosomes involves several stages, 4 stages being described during the maturation process of this compartment. The first two stages are characterized by the absence of pigmentation and the formation of the fibrillar matrix, which will constitute the support for melanin deposits in stages III and IV (Raposo and Marks, 2007). Once the process of melanin synthesis is completed, the melanosomes are transferred from the perinuclear area to the periphery of the cell through a transport regulated by motor proteins such as kinesin and dynein. The protein complex represented by melanophilin (MLPH), myosin Va (MYO-5A) and RAB27A facilitates the binding of

melanosomes to actin filaments, this step being essential for the concentration of mature melanosomes at the dendritic endings of melanocytes (Wu et al., 2006). The transfer of melanosomes to keratinocytes is thus determined by the interaction of melanocytes with approximately 40 keratinocytes (Moreiras, Seabra and Barral, 2021).

Proteins with a major role in melanosomes biogenesis can be classified into components of the amyloid-type fibrillar matrix (Pmel17) and enzymes involved in melanin synthesis. Melanosome matrix components are specific of the first two melanosome stages, while melanogenetic enzymes are specific for mature melanosomes (Raposo and Marks, 2007).

2.3 Signaling pathways in melanoma

The main signaling pathways in melanoma are defining for the aggressiveness of these cancer cells, but also for their ability to synthesize melanin. The most important are represented by the mitogen-activated protein kinase (MAPK) signaling pathway, the AKT signaling pathway, signaling pathways involved in cell cycle regulation, and the p53 protein signaling pathway. In addition, the process of melanogenesis is regulated by signaling pathways involving the activity of melanogenic enzymes and is specific for melanocytes (Palmieri et al., 2015).

One of the most important signaling pathways is MAPK pathway. This signaling pathway regulates the response of cancer cells in favor of proliferation and tumorigenesis, but also for adaptation of cells to various environmental factors. In mammalian cells, three families of MAPK proteins have been described: the classical signaling pathway mediated by ERK proteins, the JNK/SAPK signaling pathway, and p38 kinase. The best characterized pathway is the Raf-MEK-ERK pathway (Zhang and Liu, 2002). Activation of this pathway is determined by the binding of growth factors to receptor tyrosine kinases (RTKs) and the sequential activation of Ras, Raf, MEK and ERK proteins. Mutations targeting key proteins such as BRAF, NRAS, KIT and NF1 result in hyperactivation of the MAPK signaling pathway. The most common mutation, BRAF, has several variants, of which the substitution of valine with glutamic acid in position 600 (BRAFV600E) is the most common. Other variants include BRAFV600D, BRAFV600K and BRAFV600R (Ascierto et al., 2012; Guo, Wang and Li, 2021).

BRAF mutation is characteristic of cutaneous melanoma and constitutes an unfavorable prognostic factor (Long et al., 2011; Nassar and Tan, 2020). Another characteristic of melanoma

is the uncontrolled proliferation of cells, which is mainly determined by alterations in cell cycle involving mutations of key proteins. An example is represented by the mutations of the CDKN2A gene, which encodes two proteins, p16Ink4a and p14Arf. Such mutation will alter the normal function of the two proteins, and the uncontrolled cell cycle progression will be the consequence of abnormal function of RB1 and p53 proteins (Guo, Wang and Li, 2021).

2.4 Melanoma biomarkers

A correct diagnosis is of particular importance and involves the need of a classification system with a high degree of specificity. A favorable prognosis is often associated with an early diagnosis, but the marked heterogeneity of melanoma often complicates the diagnostic process. Thus, the need of a panel of specific biomarkers becomes imperative for the different subtypes of melanoma. The histological diagnosis first involves checking the expression of melanocyte differentiation markers and the most important ones are represented by tyrosinase, Melan-A, HMB-45 and MITF (King et al., 1999; Orchard, 2000; Jing, Michael and Theoharis, 2013; Weinstein et al., 2014). Differential diagnosis in melanoma is mainly done with benign formations such as dysplastic nevi, basal cell epithelioma, blue nevus, angiokeratoma, hemangioma and pigmented actinic keratosis (Goldstein and Goldstein, 2001). Biomarkers proposed for this purpose must show an increased sensitivity for malignant melanoma, and some examples are represented by the proteins CSPG4, MLANA, BUB1 and CD63 (Lewis et al., 2005; Weinstein et al., 2014). From the need to anticipate the evolution of melanoma and in order to select the right therapy, a series of biomarkers with a prognostic role have been recommended, and among them are Ki-67 (Frahm et al., 2001), MCAM (Pacifico et al., 2005) , S100B (Gogas et al., 2009), MMP-2, MMP-9 (Department of Radiopharmaceutical and Chemical Biology, Institute of Radiopharmaceutical Cancer Research, Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany et al., 2017), MIA (Stahlecker et al., 2000), PAX3 (Liu et al., 2019) and osteopontin (Rangel et al., 2008). Different strategies have been proposed, and the use of a combination of biomarkers represents an alternative with a specificity and sensitivity superior to that based on the use of a single biomarker in establishing diagnosis or assessing prognosis (Muinao, Deka Boruah and Pal, 2019; Trager et al., 2022).

2.5 BRAF inhibitor resistance- Vemurafenib

The presence of the BRAF mutation, encountered among melanoma patients, requires a therapeutic approach based mainly on the use of specific inhibitors of this oncogene. The mutation is also found in other types of cancer such as thyroid cancer, colorectal cancer, lung cancer, blood cancer, liver cancer or ovarian cancer, but is absent in uveal melanoma (Rimoldi et al., 2003; Owsley et al., 2021). The most common BRAF mutations are represented by substitution at position 600 of valine with glutamic acid (BRAFV600E) or lysine (BRAFV600K) (Lokhandwala et al., 2019). Targeted therapy presents, at least in the case of melanoma, the disadvantage of selecting resistant cell populations, distinct from the point of view of the adaptive mechanisms that these cells develop. A series of studies demonstrate the effectiveness of anti-BRAF therapy by reducing the level of ERK protein phosphorylation after approximately 15 days of treatment. This effect is accompanied by the decrease of cyclin D1 expression level, the activation of some proteins that act as cell cycle inhibitors such as the p27 protein and the decrease in the expression level of cell proliferation marker Ki-67. Different hypotheses have been formulated in the context of vemurafenib resistance, and the existence of secondary mutations in the NRAS and MEK1 oncogenes have been involved in the reactivation of the MAPK pathway (Trunzer et al., 2013). In addition, the sustained proliferation of selected cells is dependent on the presence of the inhibitor in the medium, and the response can be shaped by adjusting the dosage (Das Thakur et al., 2013). Also, the morphology of the resistant cells is modified, the stress fibers being much more pronounced and the cells more elongated (M. H. Kim et al., 2016). The heterogeneity of melanoma cells is also highlighted at the molecular level by the different response that these cells present when the resistance is established, the reactivation of the MAPK pathway, respectively PI3K/AKT pathway being the most frequently reported (Radić et al., 2022).

3. RESULTS

3.1 Biomarkers for differentiating amelanotic from hyperpigmented melanoma

3.1.1 Expression of pigmentation markers in melanoma cell lines

In order to differentiate the cell lines according to their pigmentation state, comparative proteomic analysis was conducted on 2 amelanotic cell lines (A375 and SKMEL28), 2 moderately pigmented cell lines (SKMEL23 and Me290) and one hyperpigmented cell line (MNT1) (**Figure 1A**). A first characterization was represented by the determination of the expression level of melanocyte differentiation markers (**Figure 1B**). An increased expression level of TYRP1, PMEL and TYR proteins can be observed in MNT1 cell line, while the SKMEL28, SKMEL23 and Me290 cell lines show an increased expression of the DCT protein. LC-MS/MS analysis additionally revealed an increased expression in the hyperpigmented cell line of the proteins RAB38, RAB27A, RAB32 and ABCB6, proteins with a role in the process of maturation and transport of melanosomes (**Figure 1C**).

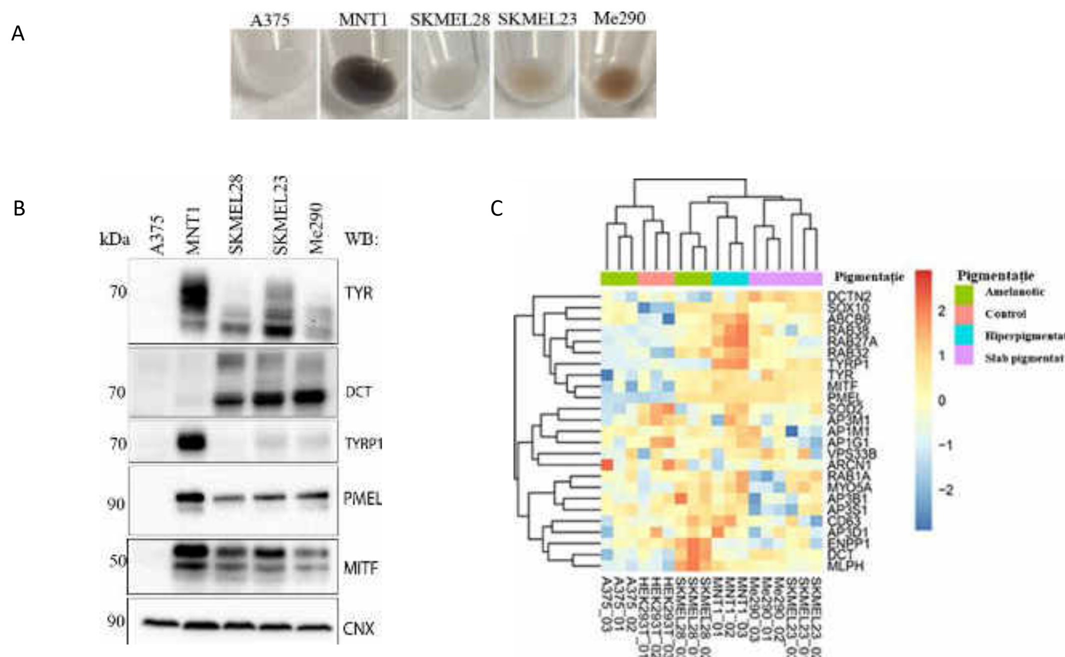


Figure 1. Pigmentation of melanoma cell lines and expression of key proteins involved in the pigmentation process.

(A) Visual examination of the 5 melanoma cell lines in terms of pigmentation intensity. (B) Western blot determination of TYR, DCT, TYRP1, PMEL and MITF protein expression. The internal control is represented by calnexin (CNX). (C) Heatmap representation of the expression of proteins annotated for the pigmentation process (GO:0043473). The LFQ intensity value (z-score) for the identified proteins was used for representation.

3.1.2 Evaluation of cell migration capacity

The ability of the cells to migrate was assessed in order to characterize the cell lines in terms of their aggressiveness. An increased migration capacity was observed for A375 and SKMEL28 cell lines (**Figure 2A-B**). Thus, it can be concluded that there is a direct correlation between the absence of pigmentation and the increased migration capacity of these cells.

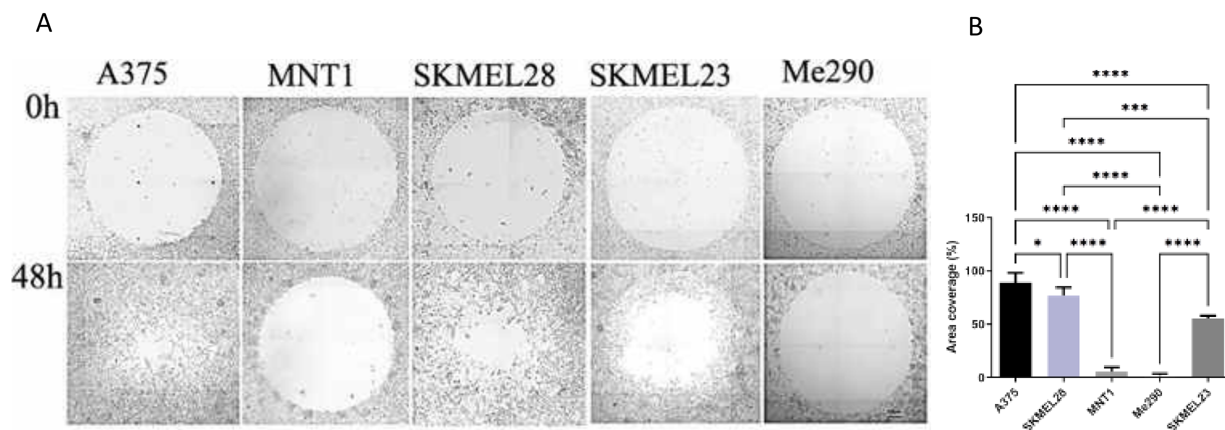


Figure 2. Evaluation of the migration capacity of melanoma cells.
(A) Melanoma cell migration was assessed by determining the surface occupied by the cells at 48h. (B) Statistical analysis was conducted by applying one-way analysis of variance (ANOVA, ****$p < 0.0001$; ***$p < 0.001$, *$p < 0.05$).

3.1.3 Specific biomarkers for amelanotic melanoma

Several biomarkers correlated with cell pigmentation were identified by LC-MS/MS analysis (**Figure 3**). Also, the expression of these proteins can represent an indicator of cell lines aggressiveness, and the identified proteins can be considered biomarkers of poor prognosis. Moderately pigmented cell lines showed an intermediate profile, the proteins identified allowing the differentiation of the three cell subtypes. In addition, the two amelanotic cell lines differ at the proteome level in the expression of melanocyte markers and especially in the expression of the transcription factor MITF. Thus, the common set of proteins was determined by comparing A375 and SKMEL28 with MNT1 cell line. 42 common proteins were identified, and the most important differences were observed for the proteins AHNAK, PLEC, MYOF, ANXA1, ITGA6, CNN2.

Important changes were also observed for the proteins involved in the composition of the cytoskeleton, and recent studies mention the role of the transcription factor MITF in the organization of the cytoskeleton. However, taking into account the "MITF-low" profile of A375 cells and the increased protein expression level of this transcription factor in the SKMEL28 cell line, we can conclude the existence of a phenotype characterized by the absence of pigmentation and the existence of highly regulated biological processes in these cells, independent of MITF protein expression.

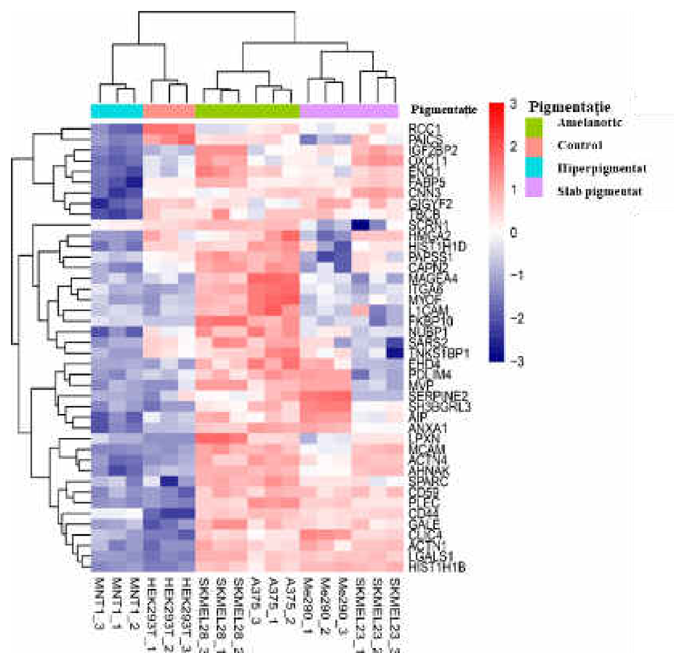


Figure 3. Supervised clustering of the 6 cell lines based on significantly increased proteins common to the two amelanotic melanoma cell lines (A375 and SKMEL28) versus the MNT1 cell line (z-score for LFQ intensity values).

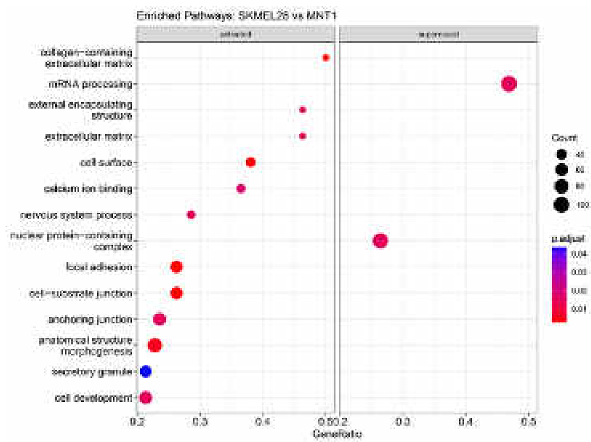
3.1.4 Significantly altered cellular processes in amelanotic cell lines

The most important differences regarding the cellular processes that are strongly regulated in the amelanotic cell lines were represented by the cell adhesion process (Figure 4A-B), with an increased expression of the proteins that are part of the focal adhesion complex: PLEC, TGM2, PXN.

An increased expression of the proteins involved in the epithelial-mesenchymal transition process was also observed, and the most important differences were observed for the proteins HMGA2, ROCK2 and RTN4 (**Figure 4C**). Similarly to the two amelanotic cell lines, SKMEL23 cell line presented an intermediate profile in terms of the expression of epithelial-mesenchymal transition markers, with the enhanced migration capacity of the SKMEL23 cell line being comparable to that of A375 and SKMEL28.

The analysis based on the identification of biological processes that might be significantly regulated in the amelanotic cell lines highlighted the cell adhesion process as being highly regulated in these cells. Thus, the expression level of integrins was determined, and a direct correlation could be observed between the expression of these proteins and the absence of pigmentation (**Figure 4D**). A series of integrins were determined by LC-MS/MS analysis and their expression was increased in A375 and SKMEL28 lines. Thus, ITGA3, ITGA5, ITGA6, ITGB4 and ITGB5 showed an increased expression level in A375 cell line, while the proteins ITGAV and ITGB3 showed specificity for SKMEL28 cell line.

A



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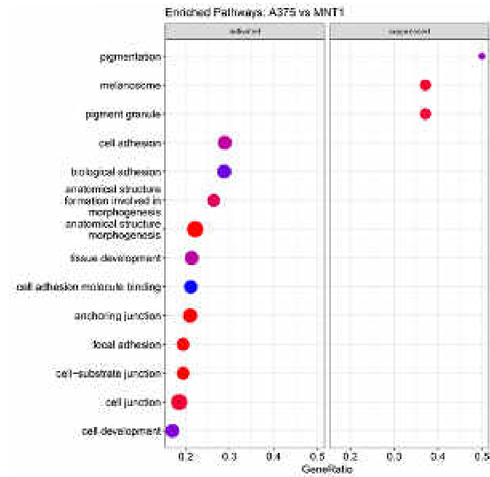

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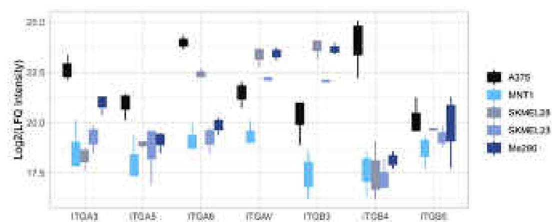

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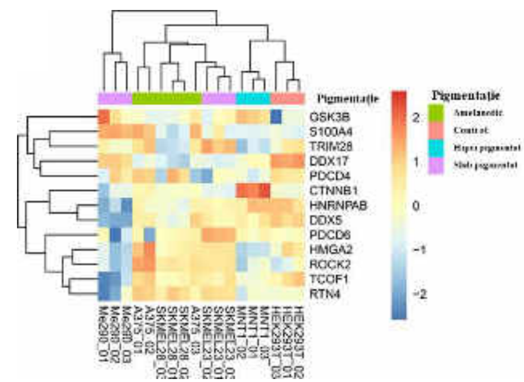

Figure 4. Analysis of biological processes in amelanotic cells.

(A-B) Annotated terms for biological processes (BP), cellular component (CC) and molecular functions (MF) were considered for analysis (adjusted p-value<0.05, correction - Benjamini and Hochberg (BH) method). (C) Boxplot of log2(LFQ intensity) expression for ITGA3, ITGA5, ITGA6, ITGAV, ITGB3, ITGB4 and ITGB5 in the 5 melanoma cell lines. (D) Heatmap representation showing unsupervised clustering of proteins annotated for epithelial-mesenchymal transition (EMT).

3.1.5 Proteomics data validation

Proteomics data were validated using public proteomics and transcriptomics datasets. Thus, the first dataset comprised proteomics data covering 33 melanoma cell lines (Figure 5A), and the unsupervised hierarchical clustering included, in addition to the proposed biomarkers, the proteins TYR, TYRP1, MITF, RAB32, MLANA and DCT. A negative correlation was observed

between the expression level of these markers and the proteins CRIP2, FSCN1, PTRF, PLOD1, ACTN4, ITGA5, ITGA6, CNN3, FN1, THBS1, EPHA2, SCRN1, CAPN2, ASPH, PLEC, AHNAK, TGM2, MYOF, TNS3 (**Figure 5A**). In addition, a number of proteins show specificity for highly pigmented cell lines, and these are represented by PRKD3, ACSL1, NR4A3, CDK2. The second proteomics data set included 10 cell lines isolated from patients, for which cell pigmentation is documented (**Figure 5B**). Increased expression of the proteins AHNAK, PLEC, ITGA5, ITGA3, ITGA6, ITGAV, MYOF, CD44, ANXA1, CAPN2, CSRP1, PDLI4, EPHA2, CLIC4, MVP, LGALS1, ACTN4, CPNS1, VASP, PTRF, ASPH, FSCN1, THBS1, TGM2, PLOD2, FN1 (FINC), APMAP and ACTN1 was observed in amelanotic cell lines. Also, the pigmented cell line, C037, shows an increased expression of LDHB, ACSL1, CTNNB1, CDK2, DUSP3, UAP1L, FKBP4 and COX41 proteins, highlighting the possibility of the existence of a specific signature for hyperpigmented melanoma.

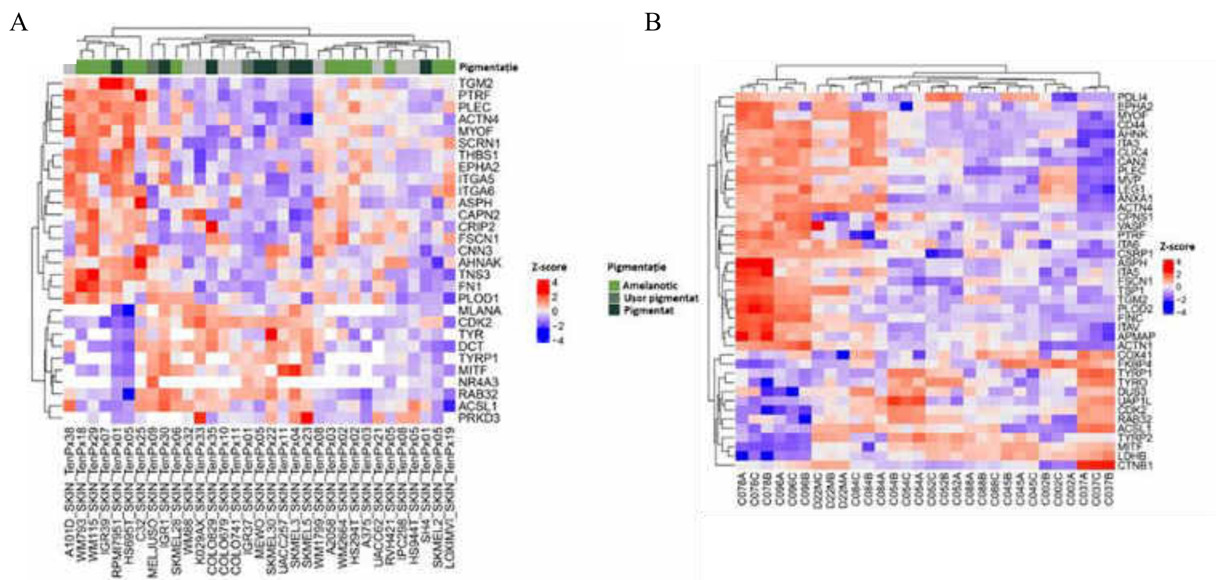

Figure 5. Unsupervised clustering highlights the specificity of the signature that allows the differentiation of the amelanotic phenotype from the pigmented one.

(A) Grouping of the 33 melanoma cell lines into 3 clusters according to the 19 proposed biomarkers and the correlation with the proteins involved in the pigmentation process. (B) Clustering of the 10 melanoma cell lines based on proposed biomarkers reveals the existence of 3 cell subtypes characterized by the absence of pigmentation, hyperpigmented cells and cells with an intermediate profile.

At the transcriptional level, some of the proposed biomarkers were validated, and the 18 proteins identified as specific to amelanotic melanoma are represented by ITGA6, ANXA1, MYOF, AHNAK, SPARC, FN1, CNN3, ASPH, CLIC4, TGM2, ITGA5, THBS1, SCRN1, PDLIM4, PTRF, FSCN1, CRIP2 and EPHA2 (**Figure 6**).

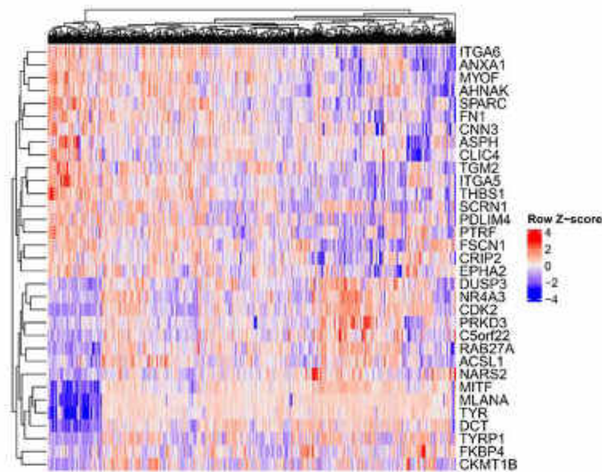

Figure 6. Validation of the molecular signature at the mRNA level in 442 biological samples.
Heatmap representing the unsupervised clustering of 442 biological samples highlights the specificity of the signature for the amelanotic and melanotic phenotype respectively (RSEM z-score).

3.2 Molecular mechanisms in tumor cells resistant to vemurafenib

3.2.1 Generation of resistant cell lines

In order to study melanoma cell resistance to vemurafenib therapy, two resistant cell lines were generated by treating melanoma cells, A375 and SKMEL28, with increasing doses of the drug for a period of 4 months. Installed resistance was verified by determining the IC50 value and the level of ERK1/2 protein phosphorylation (**Figure 7A-B**). Heterogeneity is also observed in cellular mechanisms responsible for therapy resistance and the activation of the signaling pathway mediated by the AKT protein is highlighted in the SKMEL28R cell line (**Figure 7C**). In addition,

a number of phenotypic features were highlighted in the resistant cells and among them was the loss of expression of the melanocyte markers in the SKMEL28R cell line (**Figure 7D**).

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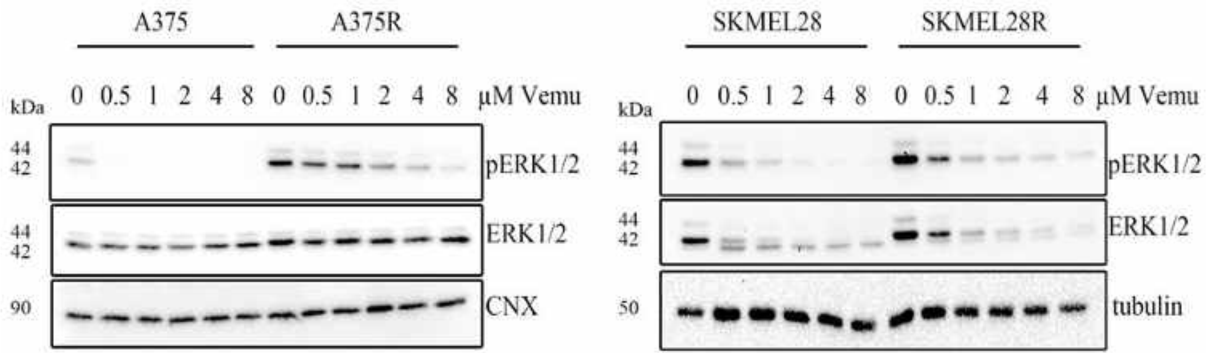

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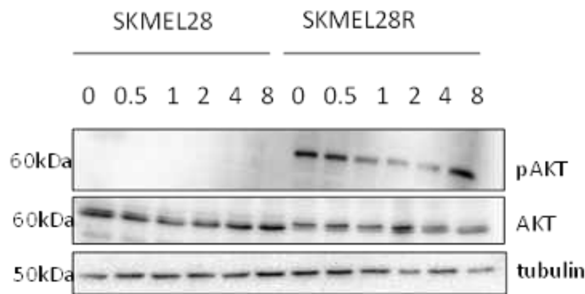

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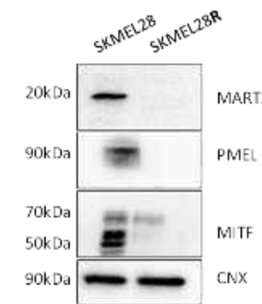

Figure 7. Sensitive and resistant melanoma cells response to vemurafenib.

(A) Cells were harvested, lysed, and pERK1/2 and ERK protein expressions were determined by Western blotting. The internal control was represented by calnexin. (B) IC50 determination for A375R and SKMEL28R cell lines. (C) pAKT and AKT protein expressions were determined in SKMEL28 and SKMEL28R cell lines. The internal control was represented by tubulin. (D) The expression level of MART1, PMEL, MITF, TYR, and DCT proteins was determined by Western blotting. The internal control was represented by calnexin.

3.2.2 Cellular processes in resistant cell lines

Reorganization of the cytoskeleton of resistant cells was evidenced by GSEA analysis in both resistant cell lines (**Figure 8A**). This process is accompanied by the increase expression level of proteins involved in the cell adhesion process. Furthermore, in SKMEL28R cell line both the pigmentation process and aerobic respiration are negatively regulated. The reorganization of the cellular cytoskeleton, the existence of pronounced stress fibers and the identification of an increased expression of the YAP1 protein indicate not only cellular processes regulated in favor of tumor progression, but also the aggressive phenotype of these cells. Also, the expression level of the focal adhesion protein, zyxin, is increased in the resistant lines (**Figure 8B**). For the SKMEL28R cell line, the process that regulates the cell-extracellular matrix interaction is positively regulated and the extracellular matrix in the resistant cells is highlighted by the increased expression level of fibronectin and the formation of a dense matrix (**Figure 8C-E**).

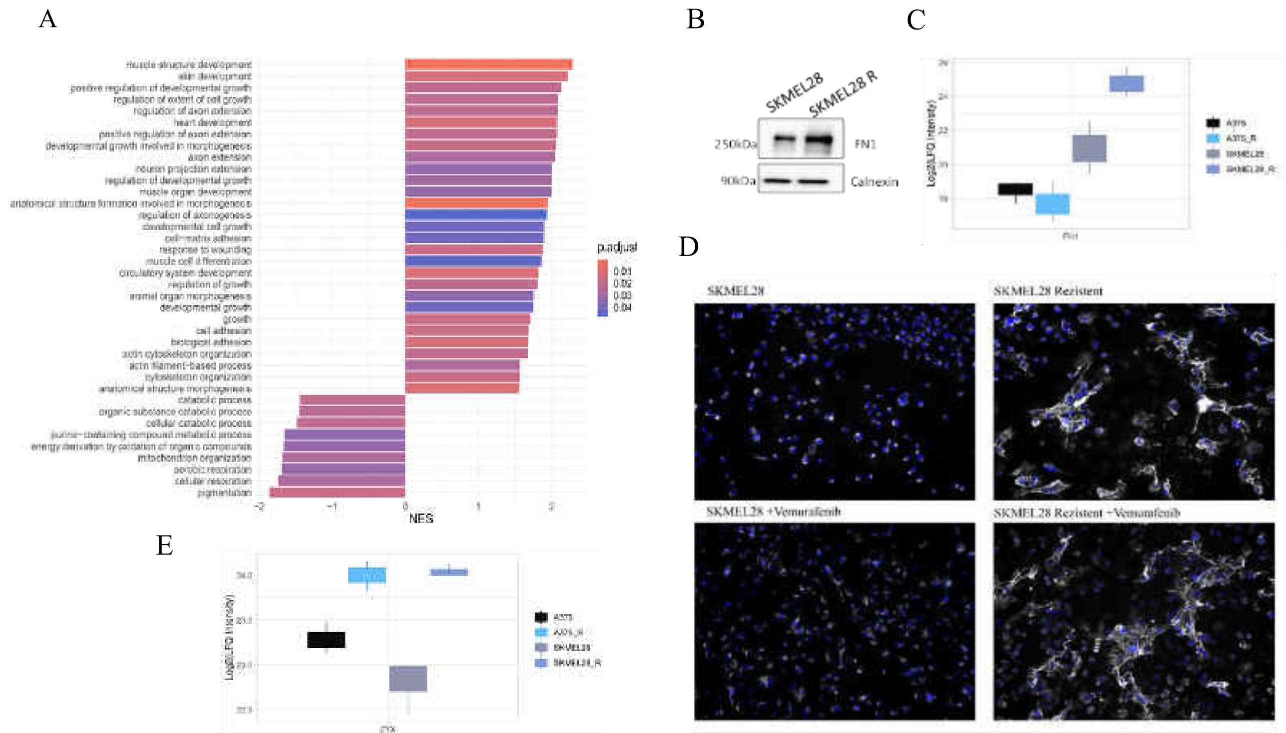

Figure 8. Cellular processes in resistant cells.

(A) Significantly altered biological processes in the resistant cell line SKMEL28R. (B) Western blot analysis of fibronectin expression in SKMEL28 and SKMEL28R cell lines. The internal control was represented by calnexin. (C) Fibronectin expression (log2(LFQ Intensity) determined in the four melanoma cell lines by mass spectrometry. (D) Immunofluorescence analysis of fibronectin in vemurafenib-sensitive and resistant SKMEL28R cells in the presence or absence of drug (24h). (E) Boxplot representation of the quantitative variable LFQ for zyxin protein in the 4 cell lines.

3.2.3 Glucose dependence of resistant cells

Regarding the nutrient dependence of resistant cells, a low concentration of glucose in the environment generates cellular stress. The identification of aerobic respiration process as being negatively regulated in SKMEL28 resistant cells indicates the preference of these cells for aerobic glycolysis over the process of oxidative phosphorylation for energy production. Consequently, these cells will use increased concentrations of glucose to support the energy needs of the cell. Thus, an increased expression of the transcription factor ATF4 was observed in the resistant cells (**Figure 9A-B**). Also, the metabolic activity of resistant cells is strongly influenced by the low availability of glucose (**Figure 9C**).

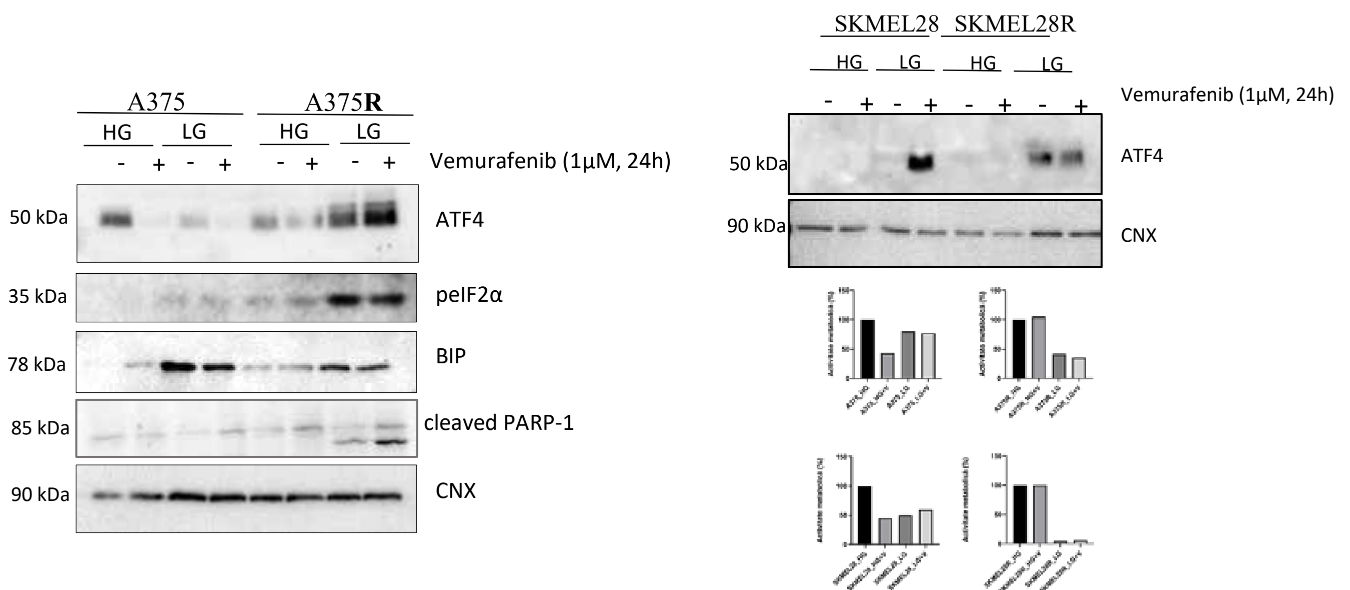

Figure 9. Dependence of resistant melanoma cells for glucose

(A) The expression of proteins (ATF4, peIF2α, BIP, cleaved PARP-1) involved in the response of cells to stress and apoptosis was quantified by Western blot from the cell lysates obtained after culturing the cells for 24h in the presence of 4.5g/L and 1g/L glucose respectively in the culture medium. (B) ATF4 expression was determined by Western blot in SKMEL28 and SKMEL28R cell lines. The internal control was represented by calnexin (CNX). (C) Evaluation of the metabolic activity of vemurafenib-sensitive and -resistant cells in the presence of high (4.5g/L) or low (1g/L) glucose concentrations.

3.2.4 The role of HIF1α protein in the development of resistance to vemurafenib

The role of the transcription factor HIF1a has been studied in the context of resistance to vemurafenib, and the most important observations concern the function of this protein in regulating the expression of extracellular matrix proteins. Thus, the increased expression of fibronectin in the SKMEL28R cell line is regulated under normoxia conditions by the constitutive activation of the transcription factor HIF1α (**Figure 10**).

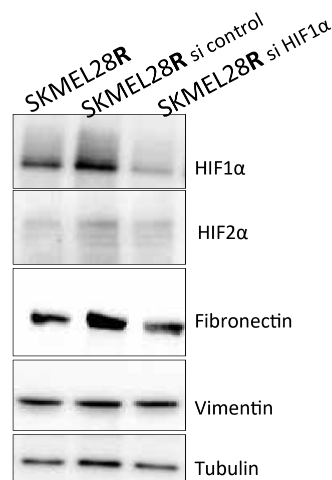

Figure 10. Western blot analysis of HIF1α, HIF2α, fibronectin and vimentin protein expression. The internal control was represented by tubulin.

4. CONCLUSIONS

Defining the aspects that contribute to the marked heterogeneity of melanoma, as well as the discovery of the mechanisms by which these cells adapt to chronic therapy with specific anti-BRAF inhibitors was the aim of this thesis. The presented results introduce a series of characteristics specific for amelanotic melanoma and allow a better characterization of this subtype of cancer. Also, a number of mechanisms involved in the development of resistance to vemurafenib were characterized, and the response of resistant cells was tested under different stress conditions. In the first part of the study, the comparative proteomic analysis allowed us to identify a panel of biomarkers showing specificity for an aggressive subtype of amelanotic melanoma. Beyond the increased specificity of the proposed set of biomarkers, these proteins can also define the increased migration capacity of these cells, being representative for the aggressiveness of the analyzed amelanotic melanoma cell lines.

The proteins proposed as candidates for the diagnosis of amelanotic melanoma are mainly represented by: AHNAK, ANXA1, CAPN2, MYOF, ASPH, THBS1, TGM2, ACTN4, ITGA6, FSCN1, PLEC, EPHA2, FN1. The difficulty of defining the amelanotic character of the cells is demonstrated by SKMEL28 cells profile. The presence of melanocyte differentiation markers does not reflect the absence of pigmentation for this cell line. The absence of pigmentation in the case of the SKMEL28 line is the consequence of defects in the maturation of melanosomes and implicitly in melanin synthesis. Comparative proteomic analysis failed to detect the absence of pigmentation for this cell line. Instead, TYRP1 protein expression was correlated with increased pigmentation of MNT1 cells. The need to define a complex classification system of melanoma cells is demonstrated by the impossibility of assessing the pigmentation of the cells only through the lens of markers known up to now. A direct correlation with the absence of pigmentation has been demonstrated for integrins. Furthermore, proteins involved in the focal adhesion process as well as those involved in cytoskeleton organization are weakly expressed in MNT1 cell line. Thus, cellular processes involving increased expression of actin-interacting proteins or those involved in extracellular matrix modeling are strongly regulated in A375 and SKMEL28 cell lines. In addition, the expression of proteins annotated for the epithelial-mesenchymal transition groups the cell lines according to their migration capacity. The validation of the proteomics data involved the use of public data sets that allowed the differentiation of melanoma lines according to the pigmentation

profile of the cells. Thus, the specificity of the identified biomarkers was tested on three sets of proteomics and transcriptomics data and allowed the highlighting of a direct correlation between the expression of the identified proteins and the absence of pigmentation. In addition, a number of proteins showed increased expression in the hyperpigmented cell line, MNT1, and an increased level of these proteins was attributed to increased pigmentation of the cells. These proteins are represented by DUSP3, NR4A3, CDK2, PRKD3, C5orf22, ACSL1, NARS2, FKBP4 and CKMT1B. The results were published in Frontiers in Oncology, January 26, 2023, Molecular and Cellular Oncology Section, Volume 12 - 2022.

In the second part of this work we studied the mechanisms involved in vemurafenib resistance. The two cell lines used in the first part to define biomarkers specific for amelanotic melanoma were treated with the BRAF inhibitor for 4 months. Installed resistance was verified by determining IC50 and persistence of activation of the MAPK signaling pathway. Comparative proteomic analysis was conducted on drug-sensitive and drug-resistant cell lines. Changes in the expression of proteins involved in the cell adhesion process were observed for both resistant cell lines. Moreover, another important component of vemurafenib resistance is represented by the transcriptional activity of the transcription factor HIF1α. This protein is constitutively expressed in resistant cell lines and thus regulates the adaptive mechanisms of the cells. Furthermore, the decreased sensitivity of melanoma cells to vemurafenib under hypoxic conditions indicates the major role that hypoxia plays in promoting tumor progression and regulating cell response to therapy. Regarding the stress response of the A375R and SKMEL28R cell lines, it is different from that presented by the parental cell lines and is mainly characterized by a reduction in ATF4 expression level. The dependence of resistant cells on an optimal glucose concentration is evidenced by the increased expression of ATF4, the presence of the cleaved form of PARP and the significant reduction of cell proliferation in the presence of a low glucose concentration. Thus, it can be concluded an increased need in resistant cells for nutrients in order to support cell proliferation under conditions of chronic treatment.

The results suggest that in the resistant cells the tumor microenvironment is transformed, and the most important effect observed was represented by a significantly higher amount of exosomes secreted by the two resistant cell lines. The existence of mechanisms that involve an intensified intercellular communication is an indication of the presence of a complex regulation.

The use of paclitaxel may represent a beneficial therapeutic strategy to reduce the risk of developing resistance to vemurafenib. Expression of proteins identified with increased expression in resistant lines is reduced in a dose-dependent manner in resistant cells treated with paclitaxel.

The results of the studies carried out within the thesis demonstrate the heterogeneous nature of melanoma and the complex response of cancer cells to targeted therapy. This highlights the need to consider a personalized strategy based on the detailed characterization of melanoma cells. The specificity of the set of biomarkers proposed in the first part of the thesis can be of great value in order to establish an early diagnosis and in assessing the prognosis in melanoma. Last but not least, the response of cells to therapy may indicate the selection of resistant cell populations and the adaptation of cells by activating compensatory survival mechanisms.

The most important observations supported by the results presented in this study are represented by:

- defining a panel of specific biomarkers for aggressive forms of amelanotic melanoma;
- defining a phenotype characterized by cytoskeleton reorganization in non-pigmented melanoma cells;
-identification of a specific signature of hyperpigmented melanoma;
-differentiation of pigmented cell lines from amelanotic ones in public proteomics datasets;
-highlighting by bioinformatics analysis of the common biological processes of the two amelanotic cell lines compared to the pigmented one;
-generation of cell lines resistant to vemurafenib and evaluation of cellular response to targeted therapy;
- identification by LC-MS/MS analysis of a set of proteins with increased expression in the resistant cell lines;
- highlighting through bioinformatics analysis the processes of adhesion, proliferation and organization of the cytoskeleton as being strongly regulated in resistant cells;
-identification of the transcription factor HIF1α as one of the main promoters of resistance to vemurafenib;
- highlighting an increased expression of fibronectin in the resistant cell line SKMEL28 and the increased secretion of the protein in the extracellular environment;

- the need for an optimal concentration of glucose to support the proliferation of resistant cells and the proapoptotic effect mediated by the transcription factor ATF4 in the presence of a low concentration of glucose.

LIST OF PUBLICATIONS

SCIENTIFIC ARTICLES

1. **Militaru IV**, Rus AA, Munteanu CVA, Manica G, Petrescu SM. New panel of biomarkers to discriminate between amelanotic and melanotic metastatic melanoma. Front Oncol. 2023 Jan 26;12:1061832. doi: 10.3389/fonc.2022.1061832. **IF: 4.7, AIS:1.00**.

2. Rus AA, **Militaru IV**, Popa I, Munteanu CVA, Sima LE, Petrescu SM. NPC1 plays a role in the trafficking of specific cargo to melanosomes. Journal of Biological Chemistry. 2023. doi: 10.1016/j.jbc.2023.105024. **IF: 5.5, AIS: 1.454**.

BOOK CHAPTERS

Albulescu R, Petrescu JA, Sarbu M, Grigore A, Ica R, Munteanu CVA, Albulescu A, **Militaru IV**, Zamfir AD, Petrescu S and Tanase C. Mass Spectrometry for Cancer Biomarkers [Internet]. Proteomics Technologies and Applications. IntechOpen. 2019. doi: 10.5772/intechopen.85609.

POSTERS

1. **Militaru IV**, Munteanu CVA, Petrescu SM. “New Potential Melanoma- Associated Prognostic Biomarkers Before and After Vemurafenib Treatment", 2021, Protein Society 35th Annual Symposium.
2. **Militaru IV**, Munteanu CVA, Petrescu SM. “A proteomic study of the relationship between hypoxia and Braf inhibitors treated melanoma cells”, RSBMB Annual International Meeting, 2020, Romania.
3. **Militaru IV**, Chiritoiu GN, Munteanu CVA, Petrescu SM, Pena F. “PDI proteins contribute to the formation of extracellular matrix”, 2019, RSBMB Annual International Meeting, Romania.
4. **Militaru IV**, Ivan M, Petrescu SM. “Knockdown of hypoxia-inducible factors by lentivirus-mediated shRNA” RSBMB Annual International Meeting, 2018, Romania.

BIBLIOGRAPHY

Arisi, M., Zane, C., Caravello, S., Rovati, C., Zanca, A., Venturini, M., Calzavara-Pinton, P., 2018. Sun Exposure and Melanoma, Certainties and Weaknesses of the Present Knowledge. Front. Med. 5, 235. https://doi.org/10.3389/fmed.2018.00235

Ascierto, P.A., Kirkwood, J.M., Grob, J.-J., Simeone, E., Grimaldi, A.M., Maio, M., Palmieri, G., Testori, A., Marincola, F.M., Mozzillo, N., 2012. The role of BRAF V600 mutation in melanoma. J Transl Med 10, 85. https://doi.org/10.1186/1479-5876-10-85

Bristow, I.R., de Berker, D.A., Acland, K.M., Turner, R.J., Bowling, J., 2010. Clinical guidelines for the recognition of melanoma of the foot and nail unit. J Foot Ankle Res 3, 25. https://doi.org/10.1186/1757-1146-3-25

Das Thakur, M., Salangsang, F., Landman, A.S., Sellers, W.R., Pryer, N.K., Levesque, M.P., Dummer, R., McMahon, M., Stuart, D.D., 2013. Modelling vemurafenib resistance in melanoma reveals a strategy to forestall drug resistance. Nature 494, 251–255. https://doi.org/10.1038/nature11814

Department of Radiopharmaceutical and Chemical Biology, Institute of Radiopharmaceutical Cancer Research, Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany et al. (2017) ‘Biomarkers in Malignant Melanoma: Recent Trends and Critical Perspective’, in Department of Surgical Oncology, Fox Chase Cancer Center, Philadelphia,PA, USA et al. (eds) Cutaneous Melanoma: Etiology and Therapy. Codon Publications, pp. 39–56. Available at: https://doi.org/10.15586/codon.cutaneousmelanoma.2017.ch3.

D’Mello, S., Finlay, G., Baguley, B., Askarian-Amiri, M., 2016. Signaling Pathways in Melanogenesis. IJMS 17, 1144. https://doi.org/10.3390/ijms17071144

Domingues, B., Lopes, J., Soares, P., Populo, H., 2018. Melanoma treatment in review. ITT Volume 7, 35–49. https://doi.org/10.2147/ITT.S134842

Erkurt, M.A., Aydogdu, I., Kuku, I., Kaya, E., Basaran, Y., 2009. Nodular melanoma presenting with rapid progression and widespread metastases: a case report. J Med Case Reports 3, 50. https://doi.org/10.1186/1752-1947-3-50

Frahm, S.-O., Schubert, C., Parwaresch, R., Rudolph, P., 2001. High proliferative activity may predict early metastasis of thin melanomas. Human Pathology 32, 1376–1381. https://doi.org/10.1053/hupa.2001.29658

Gogas, H., Eggermont, A.M.M., Hauschild, A., Hersey, P., Mohr, P., Schadendorf, D., Spatz, A., Dummer, R., 2009. Biomarkers in melanoma. Annals of Oncology 20, vi8–vi13. https://doi.org/10.1093/annonc/mdp251

Goldstein, B.G., Goldstein, A.O., 2001. Diagnosis and management of malignant melanoma. Am Fam Physician 63, 1359–1368, 1374.

Grant-Kels, J.M., Bason, E.T., Grin, C.M., 1999. The misdiagnosis of malignant melanoma. Journal of the American Academy of Dermatology 40, 539–548. https://doi.org/10.1016/S0190-9622(99)70435-4

Guo, W., Wang, H., Li, C., 2021. Signal pathways of melanoma and targeted therapy. Sig Transduct Target Ther 6, 424. https://doi.org/10.1038/s41392-021-00827-6

Jing, X., Michael, C.W., Theoharis, C.G.A., 2013. The use of immunocytochemical study in the cytologic diagnosis of melanoma: Evaluation of three antibodies. Diagn. Cytopathol. 41, 126–130. https://doi.org/10.1002/dc.21791

Kim, M.H., Kim, Jongshin, Hong, H., Lee, S., Lee, J., Jung, E., Kim, Joon, 2016. Actin remodeling confers BRAF inhibitor resistance to melanoma cells through YAP / TAZ activation. EMBO J 35, 462–478. https://doi.org/10.15252/embj.201592081

King, R., Weilbaecher, K.N., McGill, G., Cooley, E., Mihm, M., Fisher, D.E., 1999. Microphthalmia Transcription Factor. The American Journal of Pathology 155, 731–738. https://doi.org/10.1016/S0002-9440(10)65172-3

Lewis, T.B., Robison, J.E., Bastien, R., Milash, B., Boucher, K., Samlowski, W.E., Leachman, S.A., Dirk Noyes, R., Wittwer, C.T., Perreard, L., Bernard, P.S., 2005. Molecular classification of melanoma using real-time quantitative reverse transcriptase-polymerase chain reaction. Cancer 104, 1678–1686. https://doi.org/10.1002/cncr.21372

Liu, Y., Cui, S., Li, W., Zhao, Y., Yan, X., Xu, J., 2019. PAX3 is a biomarker and prognostic factor in melanoma: Database mining. Oncol Lett. https://doi.org/10.3892/ol.2019.10155

Lokhandwala, P.M., Tseng, L.-H., Rodriguez, E., Zheng, G., Pallavajjalla, A., Gocke, C.D., Eshleman, J.R., Lin, M.-T., 2019. Clinical mutational profiling and categorization of BRAF mutations in melanomas using next generation sequencing. BMC Cancer 19, 665. https://doi.org/10.1186/s12885-019-5864-1

Long, G.V., Menzies, A.M., Nagrial, A.M., Haydu, L.E., Hamilton, A.L., Mann, G.J., Hughes, T.M., Thompson, J.F., Scolyer, R.A., Kefford, R.F., 2011. Prognostic and Clinicopathologic Associations of Oncogenic BRAF in Metastatic Melanoma. JCO 29, 1239–1246. https://doi.org/10.1200/JCO.2010.32.4327

Longo, C., Casari, A., Pellacani, G., 2012. Superficial Spreading Melanoma, in: Hofmann-Wellenhof, R., Pellacani, G., Malvehy, J., Soyer, H.P. (Eds.), Reflectance Confocal Microscopy for Skin Diseases. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 151–178. https://doi.org/10.1007/978-3-642-21997-9_13

Moreiras, H., Seabra, M.C., Barral, D.C., 2021. Melanin Transfer in the Epidermis: The Pursuit of Skin Pigmentation Control Mechanisms. IJMS 22, 4466. https://doi.org/10.3390/ijms22094466

Muinao, T., Deka Boruah, H.P., Pal, M., 2019. Multi-biomarker panel signature as the key to diagnosis of ovarian cancer. Heliyon 5, e02826. https://doi.org/10.1016/j.heliyon.2019.e02826

Mull, A., Zolekar, A., Wang, Y.-C., 2015. Understanding Melanocyte Stem Cells for Disease Modeling and Regenerative Medicine Applications. IJMS 16, 30458–30469. https://doi.org/10.3390/ijms161226207

Nassar, K.W., Tan, A.C., 2020. The mutational landscape of mucosal melanoma. Seminars in Cancer Biology 61, 139–148. https://doi.org/10.1016/j.semcancer.2019.09.013

Orchard, G.E., 2000. [No title found]. The Histochemical Journal 32, 475–481. https://doi.org/10.1023/A:1004192232357

Owsley, J., Stein, M.K., Porter, J., In, G.K., Salem, M., O'Day, S., Elliott, A., Poorman, K., Gibney, G., VanderWalde, A., 2021. Prevalence of class I–III BRAF mutations among 114,662 cancer patients in a large genomic database. Exp Biol Med (Maywood) 246, 31–39. https://doi.org/10.1177/1535370220959657

Pacifico, M.D., Grover, R., Richman, P.I., Daley, F.M., Buffa, F., Wilson, G.D., 2005. Development of a Tissue Array for Primary Melanoma with Long-Term Follow-Up: Discovering Melanoma Cell Adhesion Molecule as an Important Prognostic Marker: Plastic and Reconstructive Surgery 115, 367–375. https://doi.org/10.1097/01.PRS.0000148417.86768.C9

Palmieri, G., Ombra, M., Colombino, M., Casula, M., Sini, M., Manca, A., Paliogiannis, P., Ascierto, P.A., Cossu, A., 2015. Multiple Molecular Pathways in Melanomagenesis: Characterization of Therapeutic Targets. Front. Oncol. 5. https://doi.org/10.3389/fonc.2015.00183

Radić, M., Vlašić, I., Jazvinšćak Jembrek, M., Horvat, A., Tadijan, A., Sabol, M., Dužević, M., Herak Bosnar, M., Slade, N., 2022. Characterization of Vemurafenib-Resistant Melanoma Cell Lines Reveals Novel Hallmarks of Targeted Therapy Resistance. IJMS 23, 9910. https://doi.org/10.3390/ijms23179910

Rangel, J., Nosrati, M., Torabian, S., Shaikh, L., Leong, S.P.L., Haqq, C., Miller, J.R., Sagebiel, R.W., Kashani-Sabet, M., 2008. Osteopontin as a molecular prognostic marker for melanoma. Cancer 112, 144–150. https://doi.org/10.1002/cncr.23147

Raposo, G., Marks, M.S., 2007. Melanosomes — dark organelles enlighten endosomal membrane transport. Nat Rev Mol Cell Biol 8, 786–797. https://doi.org/10.1038/nrm2258

Rimoldi, D., Salvi, S., Liénard, D., Lejeune, F.J., Speiser, D., Zografos, L., Cerottini, J.-C., 2003. Lack of BRAF mutations in uveal melanoma. Cancer Res 63, 5712–5715.

Rotte, A., Bhandaru, M., 2016. Melanoma—Diagnosis, Subtypes and AJCC Stages, in: Immunotherapy of Melanoma. Springer International Publishing, Cham, pp. 21–47. https://doi.org/10.1007/978-3-319-48066-4_2

Saginala, K., Barsouk, Adam, Aluru, J.S., Rawla, P., Barsouk, Alexander, 2021. Epidemiology of Melanoma. Medical Sciences 9, 63. https://doi.org/10.3390/medsci9040063

Sandru, A., Voinea, S., Panaitescu, E., Blidaru, A., 2014. Survival rates of patients with metastatic malignant melanoma. J Med Life 7, 572–576.

Schallreuter, K.U., Kothari, S., Chavan, B., Spencer, J.D., 2008. Regulation of melanogenesis - controversies and new concepts. Experimental Dermatology 17, 395–404. https://doi.org/10.1111/j.1600-0625.2007.00675.x

Sneyd, M.J., Cox, B., 2013. A comparison of trends in melanoma mortality in New Zealand and Australia: the two countries with the highest melanoma incidence and mortality in the world. BMC Cancer 13, 372. https://doi.org/10.1186/1471-2407-13-372

Stahlecker, J., Gauger, A., Bosserhoff, A., Büttner, R., Ring, J., Hein, R., 2000. MIA as a reliable tumor marker in the serum of patients with malignant melanoma. Anticancer Res 20, 5041–5044.

Sundararajan, S., Thida, A.M., Yadlapati, S., Koya, S., 2023. Metastatic Melanoma, in: StatPearls. StatPearls Publishing, Treasure Island (FL).

Trager, M.H., Geskin, L.J., Samie, F.H., Liu, L., 2022. Biomarkers in melanoma and non-melanoma skin cancer prevention and risk stratification. Experimental Dermatology 31, 4–12. https://doi.org/10.1111/exd.14114

Trunzer, K., Pavlick, A.C., Schuchter, L., Gonzalez, R., McArthur, G.A., Hutson, T.E., Moschos, S.J., Flaherty, K.T., Kim, K.B., Weber, J.S., Hersey, P., Long, G.V., Lawrence, D., Ott, P.A., Amaravadi, R.K., Lewis, K.D., Puzanov, I., Lo, R.S., Koehler, A., Kockx, M., Spleiss, O., Schell-Steven, A., Gilbert, H.N., Cockey, L., Bollag, G., Lee, R.J., Joe, A.K., Sosman, J.A., Ribas, A., 2013. Pharmacodynamic Effects and Mechanisms of Resistance to Vemurafenib in Patients With Metastatic Melanoma. JCO 31, 1767–1774. https://doi.org/10.1200/JCO.2012.44.7888

Weinstein, D., Leininger, J., Hamby, C., Safai, B., 2014. Diagnostic and prognostic biomarkers in melanoma. J Clin Aesthet Dermatol 7, 13–24.

Wu, X., Sakamoto, T., Zhang, F., Sellers, J.R., Hammer, J.A., 2006. In vitro reconstitution of a transport complex containing Rab27a, melanophilin and myosin Va. FEBS Letters 580, 5863–5868. https://doi.org/10.1016/j.febslet.2006.09.047

Zalaudek, I., Manzo, M., Savarese, I., Docimo, G., Ferrara, G., Argenziano, G., 2009. The Morphologic Universe of Melanocytic Nevi. Seminars in Cutaneous Medicine and Surgery 28, 149–156. https://doi.org/10.1016/j.sder.2009.06.005

Zhang, W., Liu, H.T., 2002. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res 12, 9–18. https://doi.org/10.1038/sj.cr.7290105