UNIVERSITA' DEGLI STUDI DI CATANIA
DEPARTMENT OF BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES
International Ph.D. in Basic and Applied Biomedical Sciences
XXXIV Cycle

Ph.D. Thesis

LUCA FALZONE

Identification and Validation of DNA Methylation Hotspots as Biomarkers for Cutaneous Melanoma

Ph.D. Tutor:
CHIAR.MO PROF. MASSIMO LIBRA

Ph.D. Coordinator:
CHIAR.MA PROF. STEFANIA STEFANI

ACADEMIC YEAR 2020-2021

ABSTRACT

Cutaneous melanoma represents the most aggressive skin cancer due to its high invasive and metastatic behavior. Despite the adoption of novel screening programs and the development of new pharmacological treatments, the incidence and mortality rates of cutaneous melanoma are constantly increasing highlighting the need for novel diagnostic and prognostic biomarkers as well as new therapeutic targets for the management of this pathology. Recently, several studies have demonstrated that the development of cutaneous melanoma is not only prompted by gene mutations affecting key oncogenes but is also associated with several epigenetic modifications responsible for the alterations of different cellular and molecular processes underlying the neoplastic transformation of melanocytes.

Among the epigenetic alterations, DNA methylation represents the non-structural modification of DNA mainly involved in the alteration of the expression levels of genes potentially responsible for the loss of apoptotic processes and uncontrolled cell proliferation responsible for the onset and progression of tumors. It was also hypothesized that DNA methylation could represent an early event of neoplastic transformation and that the identification of DNA methylation hotspots could be used as a promising strategy for the early diagnosis of cutaneous melanoma.

On these bases, the aim of the present study was to evaluate the overall DNA methylation status in cutaneous melanoma in order to identify DNA methylation hotspots involved in the development and progression of this tumor. Particular attention was paid to the study of DNA methylation phenomena affecting genes involved in melanocyte differentiation and epithelial to mesenchymal transition. For these purposes, a bioinformatics analysis was first performed using the EpiMethEx R-package to evaluate the correlation between DNA methylation and gene expression data contained in The Cancer Genome Atlas (TCGA) and GTEx databases thus identifying genes whose methylation status correlated positively or negatively with gene expression. Subsequently, Gene Ontology analyses were performed to establish the functional role of these genes and to identify a subgroup of transcription factors involved in embryonic development and neural crest formation as well as in the melanocytic differentiation potentially involved in the development of melanoma. Through these computational analyses, it was possible

to identify a set of DNA methylation hotspots affecting dysregulated genes involved in the development and progression of cutaneous melanoma. Among these genes, DNA methylation hotspots affecting RARB and ISL1 were selected for the validation analyses performed on melanoma cell lines and melanoma FFPE samples.

In particular, the expression and methylation levels of these two transcription factors were evaluated in five melanoma cell lines, A375, A2058, M14, MeWo and SK-23-MEL. The expression levels of both RARB and ISL1 were evaluated by using droplet digital PCR (ddPCR) while the methylation levels of the DNA methylation hotspots computationally identified were assessed using a custom protocol developed by the Laboratory of Experimental Oncology of the University of Catania defined Methylation Sensitive Restriction Enzyme droplet digital PCR (MSRE-ddPCR). The *in vitro* findings were further validated in a pilot cohort of melanoma patients through the analysis of the methylation levels of the DNA methylation hotspots of RARB and ISL1 detected in FFPE melanoma tissues and normal controls.

The *in vitro* and clinical evaluations of RARB and ISL1 methylation levels confirmed the bioinformatic results obtained through EpiMethEx demonstrating a negative correlation between RARB promoter methylation and gene expression and a positive correlation between ISL1 intragenic methylation and gene expression. Therefore, the bioinformatics and experimental data obtained in this study demonstrate the high predictive value of the analyzes performed with the EpiMethEx tool as well as the reproducibility of the results obtained by the highly sensitive MSRE-ddPCR methylation analysis protocol here developed.

Overall, the findings of this study pave the way for the development of novel strategies for the identification of diagnostic and prognostic melanoma biomarkers. The results obtained so far need to be clinically validated in a series of patients with cutaneous melanoma, healthy individuals and individuals at risk for this pathology.

TABLE OF CONTENTS

1. INTRODUCTION

Cutaneous melanoma is a malignant tumor originating from the neoplastic transformation of melanocytes present in the skin layer. Due to its aggressiveness and high metastatic power, cutaneous melanoma represents the leading cause of death related to skin tumors. Different risk factors are associated with the development of cutaneous melanoma; among these, solar UV exposure, family history, age and artificial tanning represent the most important ones (Fechete O et al, 2019; Bourneuf E, 2017).

Of note, the development of cutaneous melanoma is associated with the accumulation of different somatic mutations as a consequence of DNA damages occurring in melanocytes (Testa U et al, 2017).

Despite melanoma is one of the cancers with the highest mortality rates, a diagnosis in the early stages of the disease is associated with a good prognosis in 80% of patients who have undergone surgical treatment. On the other hand, in patients with a diagnosis of advanced or metastatic melanoma, the prognosis is often poor (Pejkova S et al, 2016). In particular, patients with a diagnosis of advanced melanoma often develop drug resistance with low overall survival. However, in recent years, the development of new targeted therapy strategies and the application of new immunotherapy treatments has significantly improved patients' life expectancy (Leonardi GC et al, 2020; Retseck J et al, 2018).

From a pathogenetic point of view, cutaneous melanoma originates from the abnormal proliferation of melanocytes in the basal layer of the epidermis which normally produce melatonin with a protective function towards UV-mediated DNA damages (Emri G et al, 2018). In particular, several studies have shown how UV rays are able to induce important modifications of the structure of different proteins and of the DNA double helix through direct mechanisms or indirectly through the production of Reactive Oxygen Species (ROS) (Candido S et al. 2014; Zhang M et al. 2012; Hodis E et al. 2012).

The malignant transformation of melanocytes and the metastatic potential of melanoma are related to their embryogenic origins. Indeed, melanocytes

originate from the cells of the neural crest. These are multipotent cells that, following a cellular process called Epithelium-Mesenchymal Transition (EMT), move away from the neural tube to invade the embryo along the vertebral axis and migrate to various anatomical sites (Kulesa PM and Gammill LS, 2010). EMT is an embryonic mechanism according to which epithelial cells lose most of their characteristics and acquire the typical phenotype of mesenchymal cells. This process is also involved in the growth and proliferation of tumors (Lambert AW et al, 2017; Roche J, 2018). A fundamental hallmark of the EMT process is represented by the alteration of the expression levels of transcription factors involved in the regulation of genes associated with cell cycle progression, proliferation, cell survival (apoptosis).

Besides genetic alterations, EMT is prompted by several epigenetic modifications, of which DNA methylation represents the most studied and involved in tumor progression. Therefore, the over-expression of transcriptional factors involved in EMT can be also due to DNA methylation phenomena affecting these genes. On these bases, the cellular plasticity driven by embryonic transcription factors that are reactivated in advanced tumors can be related to both genetic and epigenetic alterations and is responsible for the high metastatic power of cutaneous melanoma. In this context, DNA methylation is the most studied epigenetic modification characterized by the addition of methyl groups at the level of the cytosines adjacent to the guanines, the so-called CG sites also called CpG islands (Pastushenko I et al, 2018; Nieto MA et al, 2016). As widely explained in the following paragraphs, DNA methylation is strongly involved in the development and progression of cutaneous melanoma, therefore, studying the global DNA methylation status of this tumor and identifying key genes affected by DNA methylation could give important diagnostic and prognostic information for a better management of this tumor.

1.1 Cutaneous Melanoma

Cutaneous melanoma is a malignant tumor that mainly originates from the melanocytes of the skin, of the mucous membranes, from those placed in extracutaneous areas (such as meninges, inner ear, etc.). In 85% of cases, cutaneous melanoma affects the skin. It accounts for 3-5% of all skin cancer,

however, it represents the most aggressive tumor among all skin cancers due to its invasiveness and high rate of metastasis (Russo AE et al, 2009). According to the Globocan cancer observatory of the International Agency for Research on Cancer (IARC), in 2020 the number of new melanoma diagnoses for both sexes and all ages was about 324,500 with over 57,000 deaths (Sung H et al, 2021). In Italy, cutaneous melanoma is the third most common malignancy in young people of both sexes. There are several risk factors associated with the development of cutaneous melanoma; these are divided into two categories, i.e. endogenous risk factors related to intrinsic characteristics of individuals, and exogenous factors, mainly related to the environmental and modifiable risk factors.

Except for rarer amelanotic forms (achromic melanoma), cutaneous melanoma appears as a pigmented neoformation, expanding on the surface or in-depth of skin with a tendency to late ulceration and metastasis formation by lymphatic and blood routes. It can arise on healthy skin or on an acquired or congenital melanocytic nevus; in rare cases, the onset is metastatic without any evident primitive lesions.

1.1.1 Epidemiology

As already mentioned, melanoma is a tumor characterized by high mortality rates. The incidence of malignant melanoma has increased worldwide during the last years as a consequence of different bad habits including excessive tanning and the use of tanning beds. Therefore, cutaneous melanoma now represents a socio-economic health problem of great importance in different Countries (Meyle KD and Guldberg P, 2009). Substantial differences in the incidence rates worldwide depend on the geographical area considered as well as taking into account ethnicity, sex and age of individuals. In particular, several studies have shown that latitude represents a risk factor among individuals of the same ethnic group. Indeed, the incidence of cutaneous melanoma increase in decreasing latitude due to a difference in atmospheric incidence and absorption of solar UV rays (Liu-Smith F et al, 2017). The presence of a “latitude gradient” of cutaneous melanoma incidence was already observed in 1956 where higher incidence rates for skin melanoma were observed near the equator (Lancaster HO, 1956). This

gradient was also evident in the geographical areas adjacent to the equator (Aitken JF et al, 2018; Curchin DJ et al, 2018). At present, the "latitude gradient" theory was revised as it was observed a reverse gradient in the Countries belonging to the temperate zone areas such as Europe, where high incidence rates are recorded in Scandinavian countries, while in southern Europe the melanoma incidence is lower (Ferlay J et al, 2015). Since the 1960s, the incidence of skin cancer has increased in Caucasian populations, so it is clear that melanoma has become one of the most common cancers in populations with fair skin phototypes (Caini S et al, 2009). Indeed, skin pigmentation plays a protective role in the non-Caucasian population (Fajuyigbe D and Young AR, 2016). In the United States, melanoma is the fifth most common cancer in men and the sixth most frequent in women (Markovic SN et al, 2007). From the U.S. Surveillance, Epidemiology and Result Program (SEER), it has been estimated that there are approximately 793,283 men and women in the United States with a history of invasive melanoma (Rigel DS, 2010). About 80% of cutaneous melanomas that arise annually in the world affect the populations of Oceania, North America and Europe. As already mentioned, cutaneous melanoma is the third most frequent tumor in both sexes in young individuals in Italy (Table 1). As better discussed in the following chapter, the risk of onset is linked to genetic, environmental and phenotypic factors.

Table 1. Cutaneous melanoma epidemiological data in Italy (AIRTUM 2020)

Incidence	In 2020 were provided about 14.900 new diagnosis of skin melanoma. (Men = 8.100; Women = 6.700)
Mortality	2.065 deaths, 1193 in men and 872 in women
Five-year survival rate	85% in men, 89% in women
Ten-year survival rate	90% in men, 91% in women
Prevalence	There are 169.900 people living in Italy after a diagnosis of skin melanoma. (men = 80.100; women = 89.800)

1.1.2 risk factors

Several studies have demonstrated that several individual and environmental factors are associated with a higher risk of the development of cutaneous melanoma (Kulichová D et al, 2014). Among these risk factors, excessive sun

exposure can cause DNA damage due to the damaging effects of UV rays able to directly or indirectly induce genetic alterations. In particular, UV rays play a fundamental role in the etiology of melanoma determining the formation of free radicals that in turn induce the modification of the DNA and protein structure (Candido et al, 2014; Zhang et al, 2012; Hodis et al, 2012).

Melanoma risk factors can be distinguished into two different categories that are "Acquired and Environmental risk factors" and "Host-related risk factors". These latter can be also divided into "Demographic and Phenotype risk factors" and "Familial, genetic and epigenetic risk factors" (Figure 1) (Azoury SC and Lange JR, 2014).

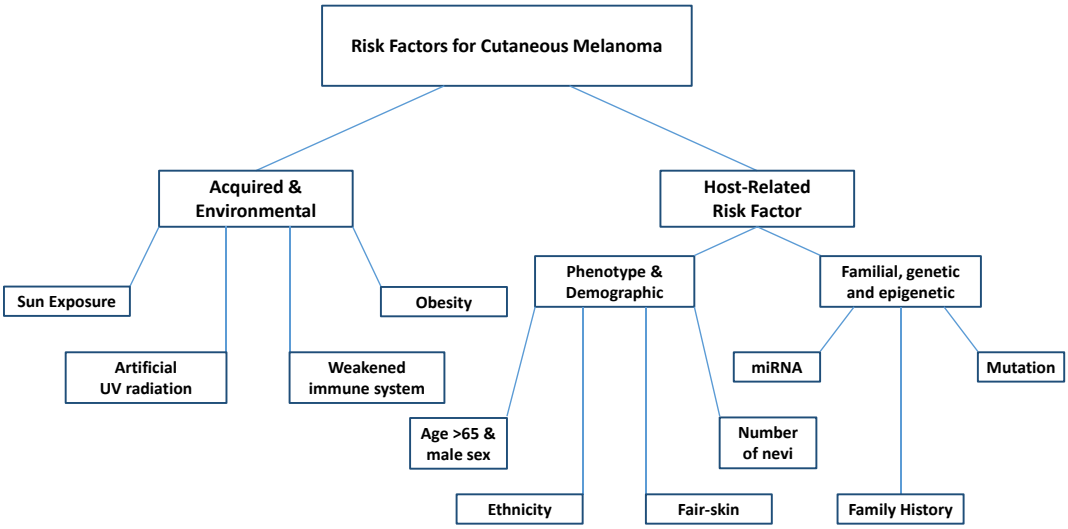

Figure 1. Schematic representation of melanoma risk factors (From Azoury SC and Lange JR, 2014).

Among the host-related individual risk factors, the most important are represented by the number of melanocytic nevi, fair phototype, genetic susceptibility and family history. It was widely demonstrated that the number of nevi is the most important predisposing factor for melanoma development independently from their size and anatomical distribution. Indeed, a considerable percentage of cutaneous melanoma (about 20-25%) origins from preexisting nevus (Alendar T and Kittler H, 2018). In particular, the risk of melanoma increases proportionally to the increment of the number of nevi as well as their the size and shape (İyidal AY et al, 2016; Pergoli L et al, 2014; Goldstein AM and Tucker MA, 2013). A broad review of the literature revealed that the presence of more than 100 melanocytic nevi or 5 atypical nevi is associated with

a 7-fold and 6-fold increment of melanoma risk. A similar increment in the risk of melanoma development is associated with the presence of a single atypical nevus or larger and giant nevi (>5 mm and >20 mm) (Rastrelli M et al, 2014).

Another individual risk factor is represented by the family history, i.e. the presence of a close relative with a past diagnosis of melanoma. Although the reasons behind a higher risk of melanoma development in individuals with a family history are not fully understood, recent studies showed that melanoma inheritance may be linked to a clear pattern of autosomal dominant mutation and polymorphisms. Among the germline mutations mostly associated with the development of cutaneous melanoma, inherited mutations affecting the CDKN2A, CDK4 and MC1R were frequently found in melanoma (Udayakumar D et al, 2010). Other mutations affect genes involved in the DNA repair mechanisms and in the regulation of the cell cycle including the breast cancer 1 (BRCA1), BRCA1-associated protein 1 (BAP1), and telomerase reverse transcriptase (TERT) (De Simone P et al, 2017). Overall, the inherited cutaneous melanoma accounts for about the 10% of all melanomas. Individuals with a relative with a previous diagnosis of melanoma have a 2-fold higher risk of developing the same tumor (Soura E et al, 2016). Familiar melanoma generally occurs at a younger age with multiple primary lesions.

Inherited melanoma can be also associated with different genetic syndromes such as Lynch syndrome, RB1 defects, and Li-Fraumeni syndrome (Bruno W et al, 2016; Curiel-Lewandrowski C et al, 2011). Finally, a melanoma-specific syndrome is the Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome characterized by the onset of numerous atypical moles. Individuals with FAMMM are a 15-fold increased risk of developing melanoma, especially in presence of more than ten atypical nevi (Lynch HT and Shaw TG, 2016).

Other individual risk factors are phenotypic features including red and blond hair, blue or green eyes, fair skin, freckles and sun sensitivity. All these characteristics determine the so-called lower skin phototype associated with an increased risk of melanoma (Grigore M et al, 2018). In particular, a sensitive skin is more susceptible to sunburn and genotoxic damages induced by UV rays (Pinault L and Fioletov V, 2017). In these individuals frequent dermatological

screenings are needed in order to early detect atypical nevi thus preventing the neoplastic transformation of nevi.

Other risk factors are represented by sex, age and ethnicity. In particular, male Caucasian individuals over the age of 65 and of Caucasians are the most predisposed to the development of melanoma (Ward-Peterson M et al, 2016; Kosary CL et al, 2014).

Interestingly, several studies have demonstrated how epigenetic alterations can be responsible for the development of melanoma. In particular, it was demonstrated how some miRNA and DNA methylation phenomena may be used as indicators for the assessment of melanoma risk (Shen J et al, 2017; Varamo C et al, 2017; Gomez-Lira M et al, 2015). Finally, another risk factor of fundamental importance for melanoma development is represented by somatic mutations, which will be extensively discussed in the next section.

Besides all these host-related unmodifiable risk factors, the development of melanoma is strongly associated with a plethora of environmental risk factors that if recognized could be corrected thus reducing the risk of developing melanoma. Among the environmental risk factors, solar UV exposure is the most important risk factor and the first recognized for the development of melanoma (Elwood JM and Lee JA, 1974).

A growing body of evidence has demonstrated the close relationship between UV exposure and melanoma development due to the genotoxic effects induced by these radiations (Martens MC et al, 2018; Reichrath J and Rass K, 2014). More recently, it was demonstrated that intermittent sun exposure is more harmful compared to chronic sun exposure as tanning protects the skin from UV rays. On the contrary, sunburns due to intermittent and acute sun exposure are able to induce several genetic damages to exposed cells (Pinault L and Fioletov V, 2017; Behrens CL et al, 2013).

Besides solar UV exposure, since the 1970s another unhealthy habit has worsened the damages induced by UV rays. Indeed, due to the use of tanning beds, which use artificial sources of UV rays to tan the unprotected skin, the incidence of cutaneous melanoma increased significantly in this population (Boniol M et al, 2012). At present, the UV rays of tanning beds are considered a

recognized risk factor for the development of melanoma and exposed individuals have a 75% increased risk of contracting melanoma (IARC, 2007). It has also been shown that the risk of developing cutaneous melanoma increases with the increase in the number of sunbed tanning sessions (Boniol M et al, 2012).

As environmental factors, chronic and/or intermittent solar UV exposure and the use of tanning beds are modifiable risk factors, therefore primary prevention campaigns could reduce these risk factors especially in young individuals with 15-50 years old. In this context, the use of protective creams represents an effective strategy to reduce sunburns and in turn the risk of the development of melanoma.

UV rays are also able to compromise the immune system through indirect mechanisms. Primary or secondary immune deficiencies are also considered risk factors for tumors, including cutaneous melanoma; in patients treated with chemotherapy or who experienced organ transplant, excessive sun exposure or infectious or chronic diseases (HIV/AIDS or lymphoma), the risk of melanoma is increased (Robbins HA et al, 2015). Behind this increased risk, immunosuppression induces a lower cancer control towards dysplastic cells, therefore, aberrating cells are not eliminated and accumulate genomic mutations acquiring a neoplastic behavior. Some studies have demonstrated that immunosuppression due to organ transplantation leads to an increased risk of development of cutaneous melanoma many years later the transplantation (Ascha M et al, 2017; Fattouh K et al, 2017).

Other modifiable risk factors are represented by obesity and body mass index (BMI) already associated with the development of different tumors (Stone TW et al, 2018). As regards cutaneous melanoma, the role of obesity has not been fully clarified yet. In a well-detailed meta-analysis, Sergentanis et al. revealed that high BMI increases the risk of melanoma in both sexes especially in male individuals (Sergentanis TN et al, 2013). Mor recently, another study refused the results obtained by Sergentanis and colleagues demonstrating that obesity and high BMI correlates with positive outcomes in male patients with metastatic melanoma receiving immunotherapy and targeted therapy, while patients treated with standard chemotherapy have a worse prognosis when obese. For female

patients the BMI was irrelevant in predicting the therapeutic efficacy of targeted therapies and immunotherapy (Hayes AJ and Larkin J, 2018; Fang S et al, 2017).

1.2 Clinical and Molecular Features of Cutaneous Melanoma

The classification of cutaneous melanoma is based on the observation of different morphological and clinical characteristics. From a macroscopic and morphological point of view, cutaneous melanoma can be divided into flat or nodular melanoma. Flat melanoma is a pigmented lesion with an irregular shape and well-defined but irregular margins. The color is often inhomogeneous color ranging from pink to dark brown or blue color and dimensions over 6 mm. The spreading of this form of melanoma is often horizontal maintaining the flat thickness and invading only the epidermis. These tumors are often in the limbs of women and in the trunk of men, although melanoma can be found in both sexes in each anatomical site. At present, flat melanoma is the most diagnosed in the population thanks to the screening programs and the informative campaigns against skin cancers (Miller AJ et al, 2006; Russo A et al, 2014).

As regards nodular melanoma, also defined as raised melanoma, they form nodular lesions raised above the skin layer. This kind of tumor could be pigmented or achromatic with a smooth surface that often can be ulcerated or present squamous crust. It is often diagnosed in male individuals (Russo A et al, 2014).

Considering the histological and clinical features, cutaneous melanoma can be divided into 4 categories: superficial spreading melanoma, lentigo maligna melanoma, acral melanoma and nodular melanoma (Shain AH and Bastian BC, 2016):

- Superficial Spreading Melanoma (SSM) represents about 70% of all melanoma diagnoses and it is often diagnosed at an early stage. This form of melanoma is directly associated with acute and intermittent sun exposure. The clinical presentation of SSM is a flat lesion of different colors (brown, black, violet, gray and in some cases blue). It is often diagnosed in the legs of women and or backs of men (Greenwald HS et al, 2012). In general, the prognosis of SSM is good when the lesion is diagnosed in a non-advanced stage as the surgical resection

followed by pharmacological treatment is curative in the majority of cases (Carrera C et al, 2017).

- Lentigo Maligna Melanoma (LMM) is a rare form of melanoma accounting for about 7-15% of cutaneous melanomas. This lesion is often diagnosed in elderly patients as a result of chronic and prolonged sun exposure. From a clinical point of view, this cancer shows a variety of colors including black or brown on a tan background and it is the result of the neoplastic transformation of pigmented cells within the papillary dermis occurring in long periods of 20-30 years. LMM is characterized also by large dimensions and irregular margins which can extend on the whole face or neck of the patients. The malignant proliferation is often localized in the basal layers of the epidermis (Markovic SN et al, 2007).

- Acral Lentiginous Melanoma (ALM) is less frequent compared to SSM, however, it is often diagnosed in Asian, Hispanic and African patients compared to those of Caucasian ethnic group who often develops SSM or nodular melanoma. ALM is often diagnosed in elderly patients, especially in women. It is mainly found in the extremities of upper and lower limbs, in the skin adjacent digits, palms and soles; it usually involves the nail bed of the great toe or thumb (Nakamura Y and Fujisawa Y, 2018; Myles ZM et al, 2012).

- Nodular Melanoma (NMM) is the most aggressive form of melanoma. It accounts for 5% of all melanomas frequently affecting the trunk and limbs of patients aged between 50 and 60 years of male individuals. It affects preferentially males than women. When diagnosed in an advanced stage, NMM is often and could invade the basal layers of skin and is characterized by a vertical growth phase (Ciarletta P et al, 2011). From a morphological point of view, NMM is a nodular formation of dark brown or blue-black color. The morphological features vary from a smooth surface to ulcerated polyps o with irregular margins. Similar to SSM, also nodular melanomas are associated with acute intermittent UV sun exposure (Green AC et al, 2018). This form of tumor is characterized by an intraepidermal growth through the invasion of the skin layers until the blood and lymphatic vessels which facilitate the metastatic dissemination of cutaneous melanoma (Erkurt MA et al, 2009).

Another uncommon form of melanoma is represented by balloon cell melanoma, myxoid melanoma, osteogenic melanoma, rhabdoid melanoma and desmoplastic melanoma (Rongioletti F and Smoller BR, 2005). This latter form is diagnosed in elderly patients> 65 years old and it is often diagnosed on the head and neck or mucosal sites. The main characteristic of desmoplastic melanoma is the amelanotic structure. This form of melanoma can also invade nerves and it is characterized by a high relapse rate due to its perineural invasion (Khan F et al, 2017).

1.2.1 Staging and classification of cutaneous melanoma

At present, the American Joint Committee on Cancer (AJCC) staging system is the most used classification method worldwide. This system is constantly updated adding novel molecular and clinical findings obtained in recent years, such as the presence of activating mutations affecting the BRAF gene. The AJCC system classifies cutaneous melanoma according to the TNM scoring parameters that are tumor size (T), lymph node involvement (N) and presence of metastases (M) (Keung EZ and Gershenwald JE, 2018). This system classifies melanoma into four groups according to tumor dimensions and. More recently, the AJCC classification added two important parameters useful to assess the invasiveness of melanoma that are the Clark's level and the Breslow depth index (Keung EZ and Gershenwald JE, 2018).

More in detail, the Clark's Level is a staging system that is used to measure the epidermal invasion of melanoma determining the skin thickness invaded. This index is used together with the Breslow's depth index. Clark's system was formulated in the early 1960s by Wallace H. Clark Jr. and is currently adopted (Clark WH Jr. et al, 1969). According to this staging system, there are 5 levels of invasion where the higher levels correlate with a worse prognosis:

Level 1. Melanoma in situ limited to the epidermis surface;

Level 2. Invasion of the papillary dermis;

Level 3. Invasion to the junction of the papillary and reticular dermis

Level 4. Invasion into the reticular dermis

Level 5. Invasion into the subcutaneous fat.

As regards the Breslow's depth index, it was developed by Alexander Breslow in 1970 (Breslow A, 1970). The Breslow's depth measures the tumor thickness by evaluating the entire melanoma bulk after an excisional biopsy. According to the tumor thickness, there are 5 different stages of Breslow's depth:

Stage I. less or equal to 0.75mm;

Stage II. 0.76 mm - 1.50mm;

Stage III. 1.51 mm - 2.25mm;

Stage IV. 2.26 mm - 3.0mm;

Stage V. greater than 3.0 mm.

As described for the Clark's levels, a higher stage is related to higher tumor size and a worse prognosis due to the infiltration of the subcutaneous tissues as well as vessels. Therefore, higher Breslow's depth index correlates with a worse prognosis for patients and could be predictive of the development of metastasis (Stiegel E et al, 2018; Breslow A, 1970).

1.2.2 Molecular alterations in cutaneous melanoma

For the correct classification of cutaneous melanoma, it is also important to evaluate the molecular alterations harbored by neoplastic cells that could be useful to clinicians to establish the prognosis of patients and to define the therapeutic schedule.

Of note, the malignant transformation of melanocytes is sustained by the accumulation of several genetic alterations affecting different cellular and molecular pathways involved in the regulation of cell cycle and apoptosis (Jackett LA and Scolyer RA, 2019; Palmieri G et al, 2015).

As already mentioned, the DNA damages accumulated by melanocytes are often driven by UV rays exposure able to alter the double-strand structure of DNA (Schuch AP et al, 2013; Petersen B et al, 2014). Such damages can be direct or indirect. In particular, UVA and UVB radiations are able to induce the formation of pyrimidine dimers or the methylation of cytosine residues that lead to the fragmentation of the DNA molecule. Such mutations affect often TP53 gene involved in the maintenance of cellular homeostasis and DNA repair mechanisms (Choi YS and Fisher DE, 2014).

Solar UV radiations are also responsible for the development of single-strand breaks (Cadet J et al, 2015), DNA oxidative damages with the formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) (Ravanat JL et al, 2014) and protein-DNA crosslinks (Nakano T et al, 2016).

All these UV-induced mutations can alter the structure of several genes involved in melanoma development. Among the most frequently mutated genes there are those belonging to the MAPK and PI3K/Akt pathways as well as genes involved in the Kit receptor signaling pathway (Leonardi GC et al, 2018; Hodis E et al, 2012).

Numerous studies on the genomic 'landscape' of cancer (Hodis E et al, 2012; Zhang T et al, 2016) and more recent data from The Cancer Genome Atlas (TCGA) project have revealed that cutaneous melanoma has a very high frequency of somatic mutations produced by UV radiation's mutagenic effects.

As regards activating mutations affecting the MAPK pathway, the most common mutation is that affecting BRAF gene. About 60% of cutaneous melanoma harbor an activating mutation affecting the BRAF gene leading to the constitutive activation of the MAPK pathway and consequently to the abnormal proliferation of pre-malignant melanocytes (Wellbrock C, 2014; Fowles JS et al, 2015). More in detail, BRAF is a serine/threonine kinase regulated by RAS, which is involved in the regulation of cell proliferation. Mutations affecting BRAF are responsible for the constant activation of RAS and RAF proteins and in turn to the activation of different transcription factors including MEK and ERK (Russo AE et al, 2015). BRAF mutations are involved in the development, and progression of tumors and represents early events of neoplastic transformation, however, BRAF mutations alone are not sufficient for the development of tumors as also benign nevi often harbor this kind of mutations (Kanitakis J et al, 2010). For the development of melanoma other driver and passenger mutations are needed to have a malignant phenotype (Dhomen N and Marais R, 2007).

As mentioned above, about 60% of melanomas have an activating mutation of BRAF mutation (Ruiz-Garcia E et al, 2018), More than 90% of these mutations affect the 600 codon codifying for valine, this mutation is known as

BRAFV600E mutation (1799 T>A; codon GTG>GAG) leads to a Valine-Glutamic Acid change. Another common mutation affecting BRAF is the BRAFV600K, accounting for 5-6% of BRAF mutations, where Valine is substituted with Lysine. This mutation represents about 5-6 % (GTG > AAG) of all BRAF mutations (Long GV et al, 2011; Ascierto PA et al, 2012) (Figure 2).

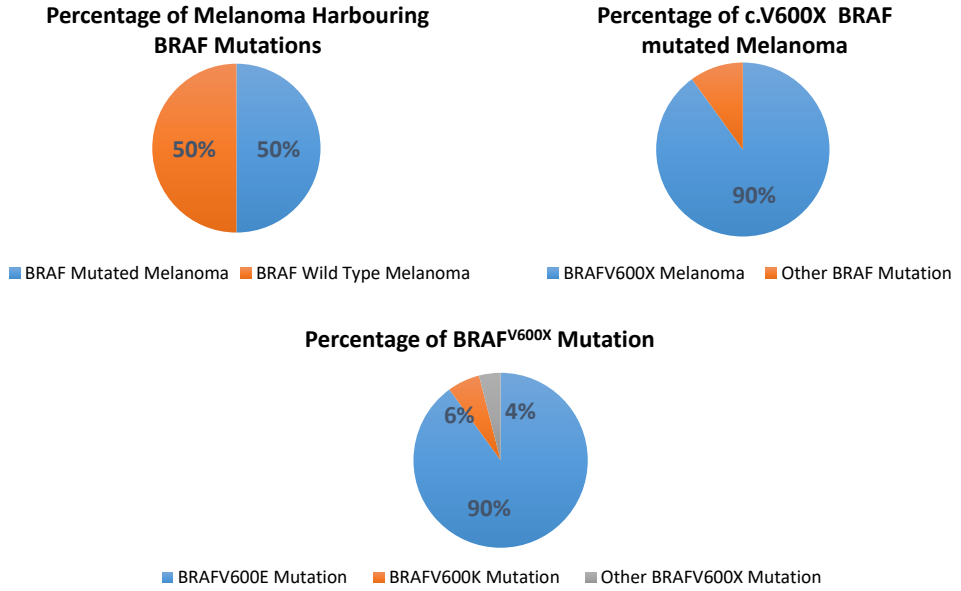

Figure 2. Frequency of BRAF mutations in cutaneous melanoma.

The mechanisms behind the development of BRAF mutations are not clearly understood. Indeed, different studies hypothesized a possible involvement of solar UV rays, however, recently it was demonstrated that such mutations are mostly observed in patients with intermittent sun exposure and not in individuals with chronic UV exposure (Candido S et al, 2014). Other studies demonstrated that BRAF positive melanoma was highly represented in patients with a history of high UV exposure (Thomas NE et al, 2007) while no BRAF mutation was detected in UV-protected mucosal melanoma (Edwards RH et al, 2004). All these studies highlighted a potential correlation between chronic and intermittent sun exposure and mutations in the BRAF gene, however, further studies are needed to elucidate the precise molecular mechanisms.

Based on the pivotal role of BRAF mutations in the development of melanoma, several targeted therapies have been developed to target overactivated BRAF. The currently adopted targeted therapies are that of Dabrafenib and Vemurafenib

that are effective in the treatment of advanced melanoma, however, a significant fraction of tumors often develops drug resistance mechanisms that lead to therapeutic failure. These drug resistance mechanisms are related to the accumulation of other driver mutations affecting key genes involved in the PI3K/Akt pathway responsible for the alteration of the cell cycle and the further proliferation of cancer cells (Figure 3). These different routes of cancer progression represent important molecular targets for the development of combined targeted therapies to be used for the treatment of different neoplasms (Deken MA et al, 2016; McCubrey JA et al, 2012).

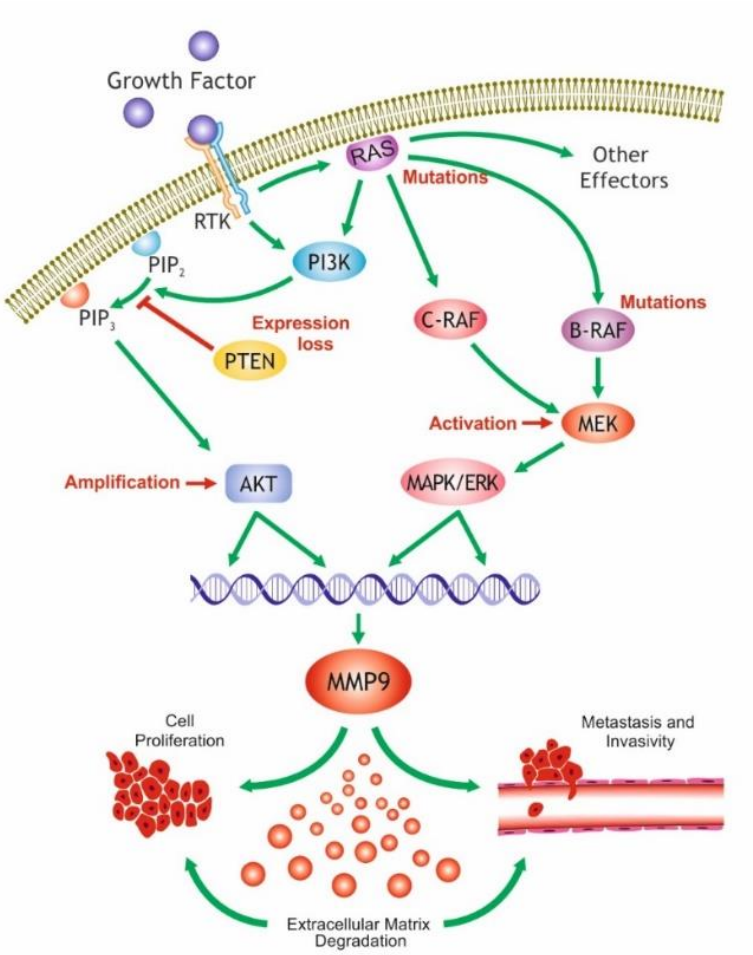

Figure 3. Ras-Raf-MEK-ERK and PI3K/PTEN/AKT/mTOR signal transduction pathway involved in melanoma.

Another gene frequently mutated in cutaneous melanoma is NRAS. This gene is involved in different signaling pathways including that of MAPK and PI3K/Akt (Leonardi GC et al, 2018). Sun exposure is recognized as a well-established risk

factor for NRAS mutation at codon 61 through the fusion of two residues of thymine in dipyrimidine sites after the exposure to direct UV rays (Curtin JA et al, 2005; Keijzer W et al, 1989). Often NRAS mutations are found in patients with xeroderma pigmentosum both at dipyrimidine sites or in neighboring regions always after UV sun exposure further highlighting the pathogenetic role of chronic and intermittent sun exposure for skin diseases (Laughery MF et al, 2020).

As mentioned, RAS proteins are involved in the regulation of the MAPK pathway through the regulation of the Ras-Raf-MEK-ERK axis. However, RAS proteins are also involved in the indirect regulation of collateral signaling pathways like the PI3K/Akt pathway. In particular, RAS could activate the catalytic subunit of PI3Ks inducing their activation (McCubrey JA et al, 2012). Phosphatidylinositol-3-kinase (PI3K) is a heterodimeric protein with an 85-kDa regulatory subunit and a 110-kDa catalytic subunit (PIK3CA) (McCubrey JA et al, 2011; Steelman LS et al, 2011). Notably, PI3K is involved in several cell regulatory mechanisms through PIP2>PIP3 phosphorylation leading to a signal transduction cascade that activates several proteins, such as protein kinase AKT. PI3Ks are divided into three classes of which the most studied and expressed in humans is the class I PI3K which is often altered in different pathologies including that of cancer. Alterations affecting class I PI3K are responsible for the constitutive activation of AKT and in turn to abnormal proliferation of cancer cells and loss of apoptosis (Hafsi S et al, 2012; Cui W et al, 2014). More in detail, AKT act as a transcription factor for genes involved in the proliferation, survival and cell growth. Among the PI3K mutations, these affecting the PIK3CA catalytic subunit are the most widely associated with cancer development or with patients' worse prognosis (Fang WL et al, 2016; Davis NM et al, 2014). The most frequent mutations are the PIK3CA H1047R, E542K and E545K, all mutations involved in the onset of drug resistance mechanisms in cutaneous melanoma (Russo A et al, 2014). In particular, such mutations are associated with the development of BRAF inhibitor drug resistance, therefore combined treatments using both BRAF and PIK3CA inhibitors are currently under investigation (Sweetlove M et al, 2015; Van Allen EM et al, 2014). Contrary to the previously

described mutations, there are no studies demonstrating the correlation between sun exposure and the development of PIK3CA mutations, therefore, these mutations could be the results of adaptative survival mechanisms played by tumor cells under treatments. Another mutation within the PI3K/Akt pathway is that of PTEN. In this case, PTEN mutations lead to a loss of function and consequently to the inactivation of this protein. In 62% of cases, PTEN dysfunction can be due to epigenetic alterations like promoter hypermethylation (Cabrita R et al, 2020). In general, mutation affecting BRAF are mutually exclusive with other mutations affecting master genes like PTEN or NRAS.

Other mutations can be found in the KIT gene. It encodes for a tyrosine kinase transmembrane receptor able to recognize stem cell growth factor SCF. The constitutive activation of c-KIT due to gene mutation is responsible for the constitutive activation of this intracellular signaling pathway which induces cell proliferation and cell growth as well as promotes tumor invasion, metastasis and the loss of the apoptotic processes. This signaling pathway is normally active during embryogenesis and in particular during the migration of the embryonic folds and the migration of cells. In tumors, including cutaneous melanoma, c-KIT is reactivated in more than half of early-stage melanomas when the tumor cells are ready to migrate and form distant metastases. After the formation of metastases, mutations of c-KIT are lost. Finally, c-KIT is involved in the activation of several signaling pathways and in particular the PI3K/Akt pathway (Pham DDM et al, 2020).

Besides all these somatic mutations occurring during the life of individuals, there are also several germline mutations predisposing for the development of cutaneous melanoma. Among these mutations, those affecting the CDKN2A and CDK4 are of particular importance as these two genes are strongly involved in cell proliferation, therefore, they are found in dysplastic nevi or melanoma occurring in young people (Udayakumar D. et al, 2010). More in detail, CDKN2A favors the expression of several proteins, including INK4/p16, which prevents RB phosphorylation by CDK4 and CDK6, thereby preventing promotion of the G1-S transition (Karim RZ et al, 2009; Soto JL et al, 2005). CDKN2A induces also the overexpression of ARF/p14, which in turn binds

MDM2 preventing ubiquitination of p53 by MDM2 and its subsequent degradation (Lewis JM et al, 2002). Therefore, such germline mutations inactivate the INK4/p16 tumor suppressor axis predisposing cells to neoplastic transformation. In 20-40% of melanoma cases, these mutations are germline mutations, however, in the majority of cases, CDKN2A mutations can be accumulated during the lifetime by somatic cells. More in detail, the activation of p53 is the first step in the process of melanin production following sun exposure. The over-expression of TP53 exerts protective mechanisms towards cells. Therefore, a decrement of p53 protein levels due to the inactivation of CDKN2A and the consequent over-expression of MDM2 is often observed in melanoma. In addition, also TP53 inactivation due to genetic mutations or epigenetic deregulation is observed in melanoma (Lu M et al, 2014; de Polo A et al, 2017).

Other germline mutations affect genes involved in DNA repair mechanisms. Among these genes, BAP1 mutations are associated with higher rates of BRCA1 ubiquitination; therefore, degraded BRCA1 leads to a dysregulation of the DNA repair mechanisms and consequently to the accumulation of different mutations (Jensen DE et al, 1998). BAP1 mutations are frequently observed in cutaneous-ocular melanoma (Njauw CN et al, 2012).

In some melanomas, it was demonstrated the presence of somatic activating mutations affecting TERT gene, which encodes the catalytic subunit of telomerase that is involved in the immortalization of cancer cells and in the reduction of cell senescence due to telomerase loss. Often TERT mutations are found in individuals with chronic or intermittent sun exposure (Demenais F et al, 2010).

In addition, many germline mutations occur in melanin biosynthetic genes. MCR1 encodes a G-protein receptor that activates adenyl cyclase, which in turn binds a melanocyte-stimulating hormone and increases MITF expression (Microphthalmia-associated Transcription Factor) (Sturm RA et al, 2014; Davies JR et al, 2012). In particular, MITF is involved in the transcription of different genes responsible for the correct differentiation of melanocytes protecting melanoblasts from UV-mediated DNA damages and regulating the migration of

cells during embryogenesis (Yokoyama S. et al, 2011; Bertolotto C. et al, 2011). In the pathogenesis of melanoma, MITF may be mutated at the germline and somatic levels.

Finally, other molecular alterations frequently associated with melanoma aggressiveness are related to the alteration of the expression levels of several factors, and in particular of matrix metalloproteases (MMPs), i.e., zinc-dependent endopeptidases primarily involved in extracellular matrix remodeling (Napoli S et al, 2020). Among MMPs, the most involved in melanoma progression are MMP-2 and MMP-9 whose expression correlates positively with higher invasion rates and metastatic spread and in turn to lower survival (Salemi R et al, 2018). In particular, MMP-9 expression is increased during the horizontal growth phase of melanoma therefore it is one of the first dysregulated factors identified in early melanoma. In addition, MMP-9 overexpression is often correlated with the presence of activating mutations affecting the PI3K/Akt pathway (Bertolotto C, 2013; Garg M., 2013). More recently, it was also demonstrated how epigenetic phenomena are able to alter the expression levels of MMPs and in particular MMP-9. Among these alterations the dysregulation of several miRNAs can lead to the dysregulation of MMPs (Napoli S et al, 2020); similarly, hypermethylation phenomena occurring in the intragenic portion of MMP9 are responsible for its overexpression and a more aggressive tumor phenotype (Falzone L et al, 2016). Based on these mutational patterns, cutaneous melanoma could be divided into three main molecular subgroups (Table 2).

Table 2. Molecular subtypes of cutaneous melanoma.

Subtype	Most frequently mutated genes (≥10% of cases)	Less frequently mutated genes (<10% of cases)
Mutated BRAF	TP53, CDKN2A, PTEN, ARID2	PPP6C, NF1, MAP2k1, RAC1, IDH1, DDX3X, SNX31, TACC1, CTNNB1, PREX2, PIK3CA, STK19, EZH2, FBXW7, RB1, WT1
Mutated RAS	CDKN2A, TP53, ARID2, NF1, PPP6C	DDX3X, RAC1, IDH1, PTEN, MAP2K1, RB1, TACC1, PREX2, CTNNB1, FBXW7, PIK3CA, STK19, WT1
Non mutated BRAF/RAS	NF1, TP53, ARID2, RAC1	KIT, CDKN2A, PTEN, IDH1, MAP2K1, RB1, SNX31, PPP6C, PIK3CA, STK19, EZH2, WT1, PREX2

1.2.3 Diagnosis of cutaneous melanoma

The survival rates of melanoma patients are strongly correlated with the time of the diagnosis and the dimension of tumors. The early identification of precancerous lesions or stage I melanoma is associated with a good prognosis as the surgical resection of the lesion is curative in the majority of cases. In the case of early diagnosis, the surgical and pharmacological treatments are able to significantly improve the 5-year survival of patients. The diagnosis of cutaneous melanoma is relatively easy due to the exterior presentation of the lesions in the external skin layers. Therefore, dysplastic nevi or early melanoma can be easily detected by using non-invasive or less-invasive approaches. Thanks to the surveillance strategies and several preventive campaigns against melanoma, especially in summer, at present there is a better awareness of this disease thus people started to skin self-examination and use protective systems like sun creams. Although skin self-examination is of particular importance, the clinical examination performed by a dermatologist still represents the gold standard for the diagnosis of melanoma.

As regards skin self-examination, it represents the most simple and convenient screening method for the early identification of melanoma (Rigel DS et al, 2010). Self-examination is of particular importance as before the prevention campaigns cutaneous melanoma was often diagnosed in an advanced stage or when metastases were already present. However, in the last years, a growing number of individuals are able to recognize morphological signs suspicious for the development of cutaneous melanoma thus requiring a clinical visit made by a dermatologist (Rigel DS et al, 2010). At the basis of the self-examination there is the so-called ABCDE self-diagnosis system developed in 1985. This system is useful to evaluate five different morphological or clinical signs typical of precancerous lesions. In particular, “A” means asymmetry, “B” means the shape of the border, “C” means color, “D” means diameter and finally “E” stands for evolution of the lesion (Tsao H et al, 2015; Abbas Q et al, 2013). The ABCDE self-diagnosis system is particularly useful for people or clinicians specialized in non-dermatological areas in order to discriminate benign nevi from potentially

malignant lesions and to send patients to specialists to begin the most appropriate therapeutic strategy in a timely manner.

Another self-diagnosis method is the Glasgow 7-point checklist including three main criteria about the size, shape and color of nevi plus four additional minor criteria about the diameter of 7 mm or greater, the presence of inflammation, sensory change and the presence of crust or blood (Glazer AM et al, 2017). However, this system is not widely used by the general population as it is most difficult to learn compared to the ABCDE method.

Besides these self-examination strategies, the gold-standard method to correctly diagnose a cutaneous melanoma is represented by a dermatological examination performed by a trained dermatologist. Melanoma diagnosis is mainly based on the use of light beams and optical instruments to better display the features of nevi. In particular, dermoscopy is a non-invasive diagnostic technique for the external observation of the skin. This technique is based on the use of dermatoscope or epiluminescent microscopes used for the direct observation of the skin lesions to magnify and better observe the details of the lesion for a correct and timely diagnosis of cutaneous melanoma (Mataca E et al, 2018). Extending the use of dermatoscopy to the whole body it is possible to obtain a total-body map of nevi collecting different images. This strategy is particularly useful to monitor the evolution of suspicious lesions during the time thus early identifying normal nevi that progress to precancerous lesions or melanomas. Another recent strategy used for the early diagnosis of melanoma is represented by Reflectance Confocal Microscopy (RCM) which is used to evaluate the progression of melanocytic nevi over the time. This strategy uses a laser and gives more accurate images of the structure of the nevi. Overall, the advantages obtained thanks to these non-invasive imaging tools are the improvement of diagnostic accuracy, the selection of lesions that should be biopsied *in vivo* and the assessment of the surgical area (Mataca E et al, 2018).

Besides these non-invasive diagnostic strategies, the correct diagnosis of cutaneous melanoma is performed also by the pathologist who evaluates the histological features of melanocytes thus establishing their malignant potential. However, no effective humoral biomarkers are available for the effective

diagnosis of this tumor or to define the prognosis of the patients. Some studies have tried to identify biomarkers for this pathology, however, inconclusive results were obtained. Among the currently used biomarkers there is the S100B protein, used in immunohistochemistry to highlight malignant melanoma cells. S100B, together with LDH, has been recently used as prognostic serum biomarkers (Wagner NB et al, 2018). Other studies have tried to evaluate the diagnostic and prognostic significance of circulating tumor DNA and circulating mutations, however, these studies need further validation (McEvoy AC et al, 2019; Salemi R et al, 2018).

1.2.4 Therapeutic approaches for cutaneous melanoma

The therapeutic strategies adopted for the treatment of cutaneous melanoma depend on different clinical-pathological features of patients including age and health status as well as the type of tumor, the site of origin and the stage of the disease. The surgical treatment represents the most effective treatment and the first therapeutic choice in almost all cases. However, for advanced or metastatic melanoma often it is not possible to remove the tumor bulk. Anyway. After surgical removal of melanoma the patients often undergo photodynamic therapy, chemotherapy, radiotherapy or targeted therapy and immunotherapeutic protocols to ensure complete removal of tumor cells (Figure 4).

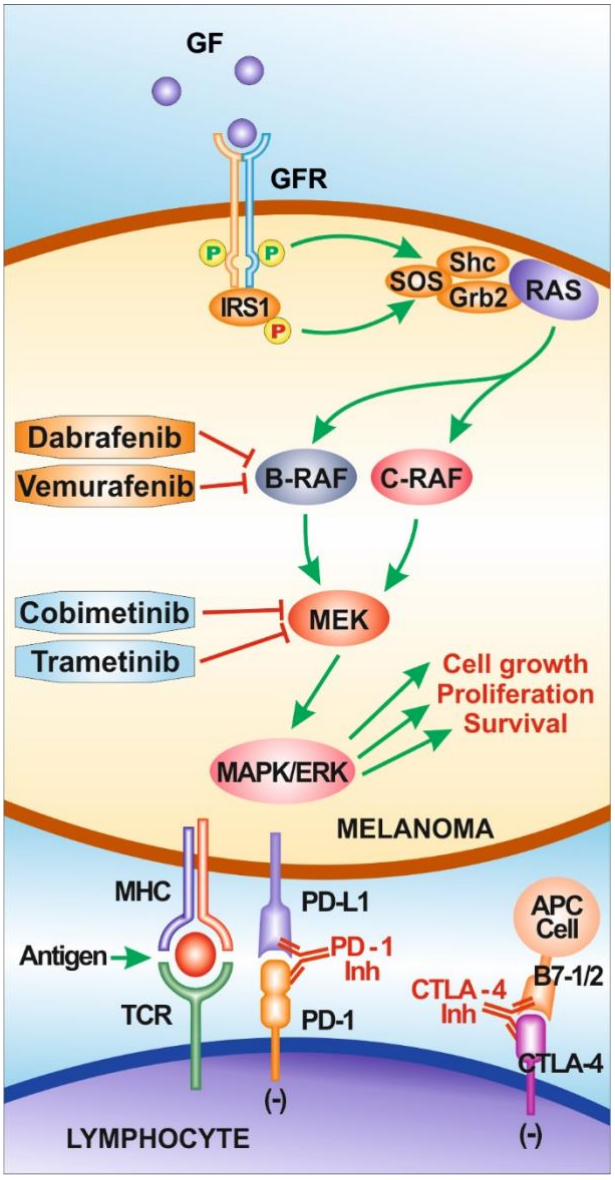

Figure 4. Targeted therapies and immunotherapy for the treatment of cutaneous melanoma.

As already stated, surgical excision represents the gold-standard treatment for the cure of skin cancer. Dermatological excision is frequently used also to remove suspected lesions performing excisional or incisional biopsies that are subsequently evaluated through histopathological analyses. In case of large skin resection, plastic surgery can be also used to reconstruct the wound with skin flaps or grafts. Plastic surgery is also used in case of surgery of the eyelids, lips or nose in order to repair these tissues for both aesthetic and functional purposes (Campagnari M et al, 2017).

Major surgery is mainly performed for the treatment of melanoma with big dimensions or that have invaded the subcutaneous tissues. In these cases, the primary tumor and the sentinel lymph nodes are removed and, when indicated, also the distal metastases that limit vital functions are removed. In presence of neoplastic cells within the sentinel lymph node all the other loco-regional lymph nodes are removed. The presence of positive lymph nodes or distant metastases represents a negative prognostic factor for patients and further pharmacological treatments are needed (Ciarrocchi A et al, 2017).

The pharmacological strategies for the treatment of cutaneous melanoma are based on the use of different drugs that can be used depending on the type of tumor, its biological and molecular features the involvement or absence of lymph nodes and/or metastases in other organs (Leonardi GC et al, 2018).

The use of standard antiblastic chemotherapeutic agents was considered the standard pharmacological approach until 2010. Chemotherapy is based on the use of substances with cytotoxic effects towards cancer cells, and to a lesser extent towards normal cells. These are very toxic agents acting mainly on with a high turn-over including that of the bone marrow and gastric and intestinal mucosa. At present, standard chemotherapy is rarely used for the treatment of melanoma because the low chemosensitivity of malignant melanocytes and only local-regional treatments are used to treat particular neoplasms (e.g. limbs or extremities of limbs) to increase the concentration of the drugs in these sites (Falzone L et al, 2018).

At present, standard chemotherapy is widely substituted by targeted therapy which represents the gold-standard pharmacological method for the treatment of cutaneous melanoma. Targeted therapy is used for the treatment of melanoma harboring specific molecular alterations used as pharmacological molecular targets. The use of targeted drugs towards these mutated proteins is very effective in reducing the growth and proliferation of cancer cells with lower side effects compared to standard chemotherapy (Long GV et al, 2017). Notably, targeted therapy can be used only if the tumor is positive for certain gene mutations, therefore, before starting the treatment it is fundamental to perform biomolecular investigationstoo establish which mutations are present in the tumor bulk.

The use of novel targeted therapies for the treatment of advanced melanoma has significantly improved the prognosis of patients with melanoma as well as their quality of life (Long GV et al, 2017). As regards the currently approved targeted therapies for the treatment of melanoma, these selective inhibitors towards the mutated forms of BRAF and MEK. In particular, two selective inhibitors of BRAF have revolutionized the effectiveness of melanoma anticancerr treatments, i.e. Dabrafenib, Vemurafenib and Encorafenib, used for the treatment of patients harboring the BRAFV600E mutation (Leonardi GC et al, 2018; Long GV et al, 2017). In addition to these two drugs, in cases of advanced melanoma combined treatments with both BRAF and MEK inhibitors are used. Among these latter, the most used are Trametinib, Cobimetinib and Binimetinib which have shown an increment of the percentage of patients who respond positively to therapies (Leonardi GC et al, 2018; Long GV et al, 2017). In addition, currently ongoing clinical trials are trying to evaluate the efficacy of novel combined therapies using BRAF and PIK3CA inhibitors or MEK and PIK3CA inhibitors (Falzone L et al, 2018).

In the last 5-10 years, immunotherapy, another type of treatment, has been used for cutaneous melanoma. In particular, immunotherapy can stimulate the immune system to react against cancer cells through the binding and inhibition of immune-suppressive molecules. The most used immunotherapies are those based on the administration of monoclonal antibodies defined immune checkpoint inhibitors (ICIs) able to bind several molecules which inhibit the immune system thus favoring the tumor development and progression. Among the currently approved ICIs for the treatment of cutaneous melanoma, there are ICIs against the inhibitory checkpoint receptors PD-1 and CTLA-4 (Christofi T et al, 2019; Flynn M et al, 2018).

The first approved ICI was the anti-CTLA-4 antibody Ipilimumab which gained approval for the treatment of melanoma in 2011 (Cameron F et al, 2011). Ipilimumab acts by blocking the immuimmune-suppressiveecule CTLA-4 expressed by tumor cells which inhibits the cross-activation of lymphocytes through the inhibition of the CD80/86 receptors. Ipilimumab was effective in reducing the risk of recurrence of melanoma as well as prolonging the overall

survival of melanoma patients, lung cancer patients and renal cell carcinoma patients (Chen L et al, 2018). Ipilimumab can be used alone or in combination with other ICIs demonstrating a good efficacy for different cancers (Christofi T et al, 2019, Larkin J et al, 2015).

Other ICIs are the PD-1 inhibitors Nivolumab and Pembrolizumab that were recently approved for the treatment of melanoma, lung cancer (NSCLC), kidney cancer, bladder, etc. (Christofi T et al, 2019). Both these ICIs are able to block the binding between programmed death-ligand 1 (PD-L1) and programmed cell death protein 1 (PD-1) responsible for the suppression of the immune system and the progression of cancer due to immune surveillance escape. Several clinical trials showed the benefits of the PD-1 inhibitors and encourage the use of these treatments also for other cancer types (Leonardi GC et al, 2020; Spain L et al, 2017).

Besides all these pharmacological treatments, radiation therapy represents an important supportive care especially for the treatment of brain metastases and against local recurrence. Radiotherapy uses x-rays or photons to induce genetic damages in cancer cells inducing their death. Radiotherapy is characterized by fewer side-effects as directed towards tumor cells only; therefore, it is a non-invasive and painless intervention administered daily for 4-8 weeks depending on the size of tumor and its anatomical site (Shi W, 2015). As already mentioned, radiotherapy is often used for the treatment of bone and brain metastases (Chicas-Sett R et al, 2017).

Another non-invasive treatment is photodynamic therapy, a treatment based on photochemical reactions. Photodynamic therapy can be used only on some types of skin tumors and is less effective in melanoma. It consists on the application of a non-active substance on the skin lesion. This substance is metabolized by tumor cells into a photoactive substance that when stimulated with light exerts toxic effects killing the tumor cells (Kawczyk-Krupka A et al, 2013).

1.3 Melanocyte Differentiation and Melanomagenesis

The clinical characteristics and the aggressive behavior of cutaneous melanoma can be explained by taking into account the embryogenic origins of melanocytes and the mechanisms leading to the neoplastic transformation of these cells.

Melanocytes take their origin from the migration of neural crest cells during the formation of the embryo. Neural stem cells are a population of cells located in the outer part of the neural folds between the neuroepithelium and the ectoderm (Bronner ME and LeDouarin NM, 2012). During the development of the embryo, these transient cells migrate from the dorsal neural tube to distant sites after the stimulation mediated by some glycoproteins of the WNT pathway, which interact with the c-Kit receptor tyrosine kinase in melanocytes and melanoblasts and the growth factors BMP, whose signal transduction leads to the mobilization of members of the SMAD proteins family (Costin GE and Hearing VJ, 2007) (Figure 5).

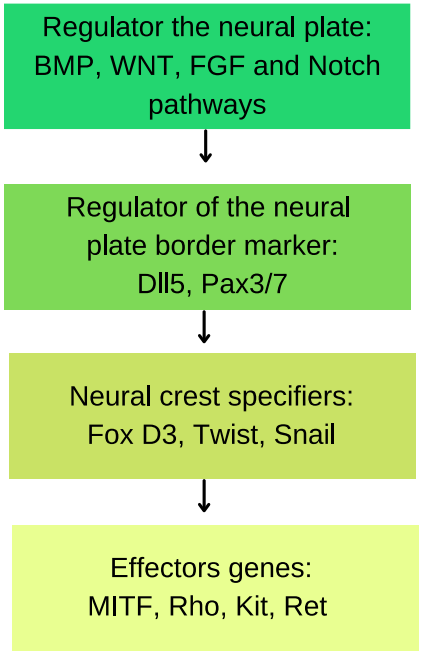

Figure 5. Molecular pathways involved in the differentiation of melanocytes and in melanogenesis.

Both WNT and BMP stimulate the over-expression of different transcription factors called neural plate border specifiers (Pax3/7), which in turn, regulate different genes involved in the so-called epithelial to mesenchymal transition (EMT) process. In particular, Pax3/7 are able to induce the expression of Snail, Sox9, FoxD3 and Sox10, which regulate the migration and differentiation of cells as well as their survival in distant sites. All these EMT genes are also responsible for the regulation of other transcription factors involved in the differentiation of cells of which MITF is the main player of the cytoskeletal

modifications occurring in melanocyte progenitors. Another transcription factor involved in the migration of neural cells and in the formation of melanocytes is the Rho GTPase which is responsible for the switch of N-cadherin to II cadherin or E- to N-cadherin type (Bertolotto C, 2013; Molina-Vila MA et al, 2015). Also metalloproteinases act an important role in this process as these proteinases regulate the adhesion and proliferation of neural cells favoring their migration through the degradation of the extracellular matrix (Napoli S et al, 2020).

The EMT processes occurring during the maturation of the neural tube allow neural crest cells to acquire a mesenchymal migratory phenotype and migrate to distant sites. Subsequently, neural crest cells invade the space between the epidermal and mesodermal layers and subsequently split into different pathways. Cells leading to the ventromedial pathway become spinal ganglia, sympathetic ganglia and ganglia surrounding the aorta, while cells leading to the dorsolateral pathway begin to express the melanocyte specification factor MITF, which regulates the expression of genes for melanin synthesis.

The EMT processes are indispensable for the correct development of individuals during embryogenesis, however, the same processes are reactivated during the neoplastic transformation of cells and are responsible for the de-differentiation of cells and their invasion and migration in other tissues. Indeed, the acquisition of a mesenchymal phenotype is associated with a greater ability of cancer cells to invade and migrate to sites far from the site of origin, thus initiating the process of metastasis (Tang Y et al, 2020). Melanoma is particularly efficient in this process, as transformed melanocytes have been shown to have antigens similar to those of endothelial cells (Braeuer RR et al, 2014).

As mentioned above, the EMT processes are sustained by different stimulating factors. Besides these factors, other mechanisms are responsible for the development of EMT in melanocytes and consequently their malignant transformation. Among these mechanisms, gene mutations affecting EMT genes can be associated with a most aggressive tumor phenotype. However, gene mutations affecting these genes are rare (Li FZ et al, 2015).

More frequently, EMT processes are prompted by epigenetic modifications responsible for the reactivation of EMT genes and the acquisition of a metastatic

phenotype. In particular, it was demonstrated that hypoxia is able to alter the acetylation status of histones through the activation of the TGF-β signaling pathway. In particular, hypoxia-induced EMT is able to alter the expression levels of chromatin modifiers histone deacetylase 3 (HDAC3) which in turn modify the histone structure of EMT genes like CDH1 and VIM (Lin YT et al, 2020).

Another study revealed that malignant melanoma metastases are sustained by EMT processes induced by the down-regulation of ten-eleven translocation (TET) enzymes involved in the conversion of 5-methylcytosine (5mC) to 5-hydroxymethyl cytosine (5-hmC). In particular, the silencing of TET2 and TET3 by DNA methylation phenomena is associated with the EMT-like phenotype in melanoma cells. On the contrary, the over-expression of TET2 after demethylation phenomena is associated with the reduction of tumor growth and cell migration *in vitro* and *in vivo* through the TGF-β pathway (Gong F et al, 2017).

Therefore, it is evident how epigenetic modifications are important processes underlying not only the development of cutaneous melanoma but also the progression of tumors and the formation of metastasis. In this context, it is of fundamental importance to widely investigate the epigenetic alterations inducing an increased risk of melanoma development through the study of omics data collected in the last years about the expression levels of non-coding RNAs, the methylation of DNA and the alteration of histone.

1.4 Epigenetic Alterations in Oncology

Epigenetics (from the Greek ἐπί, epì, "above" and γεννητικός, gennetikòs, "relating to family inheritance") is a branch of genetics that investigate the mechanisms responsible for the modification of the phenotype without any changes in the DNA sequence or the genotype of individuals. There are several mechanisms of epigenetic modification including modifications of DNA (methylation) and histone proteins that can be subjected to methylation, phosphorylation, acetylation and ubiquitination phenomena (etc.). These alterations are also called “epimutations” and originate during the lifetime as a consequence of exposure to several environmental factors. In addition, such

epigenetic modifications can be transmitted to the other cells through cell division (Bird A, 2002). If epigenetic alterations affect germinal cells (ova or spermatozoa), such modifications can be inherited by the next generation (Chandler VL, 2007).

Several studies have demonstrated that epigenetic alterations are involved in both physiological and pathological processes (Esteller M, 2007). From a functional point of view, epigenetic modifications related to the alterations of histone proteins contribute to condensation or decondensation of chromatin that in turn alter the transcription of those genes with histone modifications. Another important epigenetic modification is mediated by the modification of the methylation status of DNA. In particular, different enzymes are able to induce hyper-methylation or hypomethylation in specific DNA sites adding a methyl group to the cytosine nucleotide of a 5'-cytosine-guanine-3 ' dinucleotide (CpG) forming 5'-methylcytosine (5-MeC) (Levenson VV, 2010). The first association between DNA methylation and gene silencing in cancer was observed in 1989 where it was observed that p16INK4a, a tumor suppressor gene, was down-regulated in retinoblastoma as a consequence of methylation phenomena occurring at the promoter level. At present, it was widely demonstrated that methylation occurring in specific genomic portions is associated with the dysregulation of gene expression and consequently to human pathologies (centromeric instability, immunodeficiency and dimorphisms) (Maunakea AK et al, 2010). Therefore, it is evident how gene expression is strongly influenced by the covalent modifications of histone proteins, by DNA methylation, and the alteration of the expression levels of non-coding RNAs, including microRNAs (miRNAs), by the insertion of histone variants and by the remodeling of nucleosomes able to modify the structural organization of chromatin (Jaenisch R and Bird A, 2003).

1.4.1 Histone modifications and tumors

In eukaryotes, DNA is complexed with basic proteins called histones, which form repeated units called nucleosomes. Specifically, these latter consist of a protein core composed of eight histone proteins wrapped in a double strand of DNA of about 146 bp; each nucleosome consists of two H2A histones, two H2B

histones, two H3 histones and two H4 histones (Luger et al., 1997). Nucleosomes have the function of condensing the DNA to allow cells to store it in a restricted volume and to regulate the interaction between transcription factors and regulatory sequences of DNA (Jiang C and Pugh BF, 2009). Genome-wide studies allowed to define a map of the distribution of the nucleosomes to easily identify their localization around the promoters of the genes. In general, the position of the nucleosome is fundamental for the regulation of gene transcription; when altered, its localization in the site of initiation of transcription regulation implies the repression of gene expression (Schones DE et al, 2008), while histone modifications upstream the site of transcription is strongly correlated to the activation of gene transcription (Shivaswamy S et al, 2008; Lin JC et al, 2007). Some regions at the 5 'and 3' ends of the genes, called nucleosome-free regions (NFRs), are modulated by ATP-dependent protein complexes capable of moving nucleosomes in order to determine chromatin remodeling and in turn alteration of gene transcription (Smith CL and Peterson CL, 2005).

Different histone variants such as H3.3 and H2A.Z, are able to influence the localization of nucleosomes, protect genes from methylation and influence the stability of the nucleosome itself (Santenard A and Torres-Padilla ME, 2009; Sarma K and Reinberg D., 2005). Normal histones can undergo post-translational modifications such as methylation, SUMOylation, ubiquitination, citrullination, phosphorylation, acetylation and ADP-ribosylation which influence critical cellular processes such as transcription, replication and repair of genetic material (Azevedo H et al, 2020). Several enzymes are responsible for adding or removing covalent groups; the addition of methyl groups is mediated by the enzyme histone methyltransferase (HMT), while the addition of acetyl groups occurs through the action of histone acetyltransferase (HAT); on the contrary, histone demethylase (HDM) and histone deacetylase (HDA) are respectively responsible for the removal of methyl or acetyl groups (Haberland M et al, 2009; Shi Y et al, 2007).

The alteration of histones is associated with the development of different diseases, including tumors, when the aforementioned modifications occur in

genes coding for oncogenes or tumor suppressor genes (Shanmugam MK et al, 2017; Audia JE and Campbell RM, 2016). For example, histone hypoacetylation is associated with the silencing of proapoptotic proteins, belonging to the Bcl-2 family and regulators of the PI3K/AKT pathway. Similarly, euchromatic histone lysine methyltransferase 2 (EHMT2) can drive melanoma growth and promote an immunosuppressive microenvironment by activating the WNT pathway (Kato S et al, 2020). Deregulation of histone demethylases leading to abnormal histone methylation patterns has also been linked to melanomagenesis (Roesch A et al, 2010).

1.4.2 non-coding RNAs (ncRNAs) and microRNAs

Gene expression is profoundly modulated by non-coding RNAs (ncRNAs), i.e. RNA molecules that are transcribed but not translated into proteins. Of note, ncRNA molecules include long non-coding RNAs (lncRNA from 200 nucleotides to 100 kb), short interfering RNAs (siRNA less than 25 bp), circular RNA (circRNA) in length and microRNAs (miRNA) of 20-25 nucleotides in length (Wapinski O and Chang HY, 2011; Costa FF, 2006; Bartel DP, 2009).

Among ncRNAs, miRNAs are the most studied and their dysregulation was associated with different diseases including tumors. In particular, miRNAs are able to bind specific messenger RNA (mRNA) through a homolog sequence of 4-6 nucleotides called seed region. In case of a perfect match between miRNA and the targeted mRNA the RNA-induced silencing complex (RISC) complex binding the miRNA is able to induce the complete degradation of the mRNA, while if a partial match between miRNA and mRNA exists the RISC complex operates a transient block of targeted mRNA translation (He L and Hannon GJ, 2004). miRNAs are able to influence other mechanisms of epigenetic regulation through the modulation of mRNA coding for different methyltransferases (DNMTs) responsible for DNA methylation or other enzymes such as those responsible for histone modifications (Fabbri M et al., 2007; Friedman JM et al, 2009). Notably, some miRNAs located in the proximity of methylated genes are over-expressed or silenced as a consequence of DNA methylation phenomena, therefore the expression of miRNAs could be regulated by other epigenetic mechanisms (Saito Y et al, 2006).

In cancer, several alterations in the expression levels of specific miRNAs have been observed; in particular, miRNAs are differentially expressed in tumors compared to normal tissues thus representing good diagnostic and prognostic biomarkers (Falzone L et al, 2019, Falzone L et al, 2020, Giambò F et al, 2021). miRNAs can be also distinguished into tumor suppressor miRNAs directed against oncogenic factors and oncogenic miRNAs directed against mRNAs with tumor suppressor functions. In 2006 it was shown that an increase in methylation is related to a decrease in miRNA levels. Some examples are represented by miR-15 and miR-16, both tumor suppressors miRNAs able to target BCL2, which appear to be down-regulated- in chronic lymphatic leukemia due to hypermethylation of the regulatory regions at 5' (Lujambio A et al, 2008; Toyota M et al, 2008; Zhang B et al, 2007; Ventura A and Jacks T, 2009). However, other studies revealed that other miRNAs, such as miR-7a, are decreased in melanoma cells compared to healthy melanocytes; this leads to an increase in its targets, such as the RAS oncogene and the β3 integrin, causing an increase in the invasive capacity of neoplastic cells (Varamo C et al, 2017). On the contrary, miRNAs with an oncogenic function, have an opposite trend; indeed these miRNAs, like miR-221 and miR-222 able to target the p27 protein, appear to be increased in different types of cancer (Galardi S et al, 2007; Mercatelli N et al, 2008) including melanoma, inducing abnormal cell proliferation (Varamo C et al, 2017). As regards cutaneous melanoma, miR-200c is significantly down-regulated in both primary and metastatic melanoma (Liu S et al, 2012). Other studies demonstrated how miR-149 and miR-21 are associated with inhibition of apoptosis and are over-expressed in melanoma (Satzger I et al, 2012; Jin L et al, 2011). Therefore, it is essential to know the molecular mechanisms and specific targets of the different miRNAs in order to develop personalized anticancer therapies or evaluate the expression levels of miRNAs for both diagnostic and prognostic purposes.

1.4.3 DNA methylation in cancer

Among the epigenetic modifications, DNA methylation is the most studied. DNA methylation phenomena are operated by a family of enzymes, called DNA methyl-transferases (DNMT), consisting of 5 classes: DNMT1, DNMT2,

DNMT3A, DNMT3B and DNMT3L (Goll MG and Bestor TH, 2005). These enzymes operate the transfer of a methyl group from the S-adenosyl-L-methionine (SAM) to the carbon in the 5' position of cytosine that precedes guanine thus forming a 5-methyl-cytosine (5me) (Moor LD et al, 2012). In particular, DNMT3A and DNMT3B, activated by DNMT3L, are responsible for the *de novo* methylation occurring during embryonic development (Kaneda M et al, 2004; Okano M et al, 1999). These enzymes have an equal affinity for both hemimethylated DNA strands and non-methylated ones. On the other hand, DNMT1 is responsible for the maintenance of the methylation status, a fundamental process for the preservation of the epigenetic pattern during the replication of DNA. In particular, DNMT1 binds the hemimethylated double strand of the DNA thus re-establishing the correct methylation pattern in the newly replicated strand of the DNA (Figure 6).

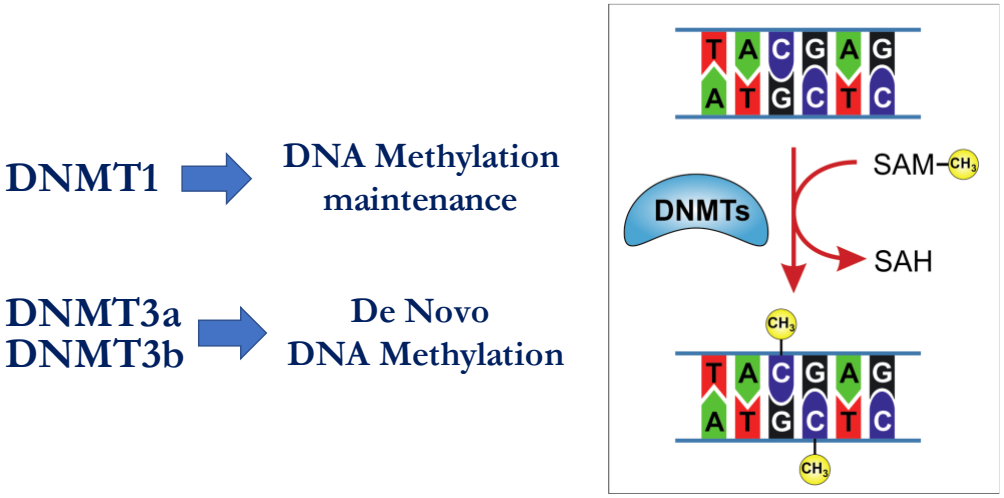

Figure 6. Enzymes involved in the methylation of DNA through the addition of a CH3 methyl group.

As already mentioned, the addition of methyl groups occurs at the level of the so-called CG dinucleotides or CpG sites. These sites are not uniformly distributed throughout the genome but are concentrated in specific regions of the genome containing highly repeated sequences such as retrotransposons or centromeres and in small areas of DNA of at least 200 bp called CpG islands (Bird A et al, 2002; Takai D and Jones PA, 2002). Commonly, CpG islands are found at the 5' end of genes and represent about 60% of the promoters of tissue-specific and housekeeping genes (Wang Y and Leung FC, 2004). Methylation

phenomena occurring in CpG sites are able to inhibit or induce gene transcription. As regards methylation-dependent transcriptional inhibition, it can occur in two different ways; in the first case the methyl group represents an obstacle for the binding of transcription factors to the DNA sequence, such as c-Myc and MLTF (Prendergast GC and Ziff EB., 1991; Watt F and Molloy PL, 1988); in the second case, specific proteins called mCpG binding proteins (MBPs) recognize methylated CpG sites and recruit other protein complexes capable of repressing DNA transcription, such as histone deacetylase (Figure 7).

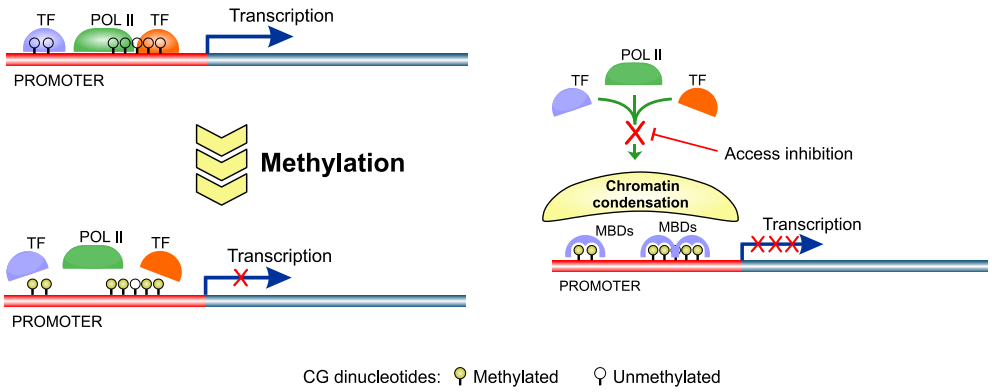

Figure 7. Gene regulatory mechanisms mediated by DNA methylation phenomena affecting the gene promoter.

As regards the over-expression of genes, it was demonstrated that intragenic methylation phenomena could be responsible for increased transcription of methylated regions. In particular, intragenic methylation induces an over-expression of the methylated gene through the activation of sequence enhancers that lead to greater expression of that gene (Falzone L et al, 2016; Singer M et al, 2015) (Figure 8).

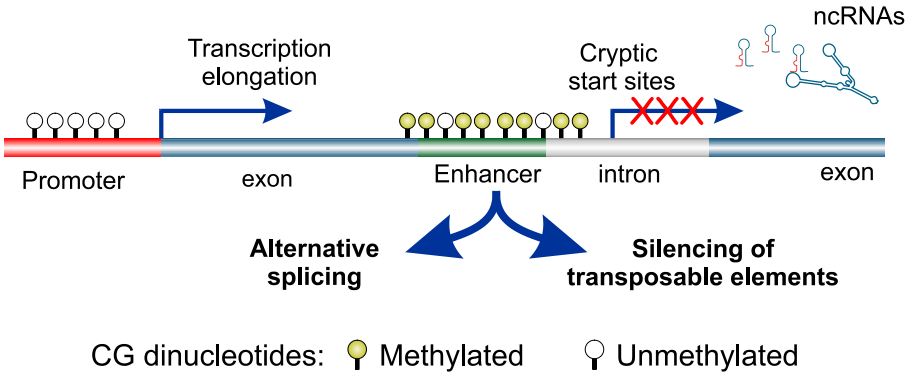

Figure 8. Gene regulatory mechanisms mediated by intragenic DNA methylation.

As mentioned in the previous chapters (*1.4.2 non-coding RNAs (ncRNAs) and microRNAs)* DNA methylation affects large regions of non-coding DNA, this results in a late replication of a large part of the genome of the most evolved organisms, with the formation of inactive chromatin (Antequera F and Bird A, 1993). This process could represent a protective mechanism against cellular damage resulting from the transcription of elements such as inserted viral sequences, repeated sequences and transposons that may alter the structure of genes within the genome. In 2010, a map of the main DNA methylation sites was created in order to investigate the influence of intragenic methylation in tumor progression and the onset of pathologies. From the further analysis it was possible to divide the CpG islands into four different classes:

1. promoter islands: starting 1000 bp upstream from the transcription starting site and ending 300 bp downstream from the transcription start site of a gene;
2. intragenic islands: starting 300 bp downstream from the transcription start site of a gene and ending 300 bp upstream from the transcription end site of the gene;
3. islands in the transcription region 3': starting 300 bp upstream from the end-of-transcription site and ending 300 bp downstream from the end-of-transcription site;
4. intergenic islands: starting 300 bp downstream from the transcription end site of a gene and ending 1000 bp upstream of the contiguous gene start site (Maunakea et al. 2010).

The omics data collected in recent years about the methylations status in tumors have demonstrated that some genes are more susceptible to epigenetic phenomena of methylation. In these genes, rich CpG portions have been identified and called "hotspots". These DNA methylation hotspots are often detected in cancer cells and not in normal cells, therefore, these hotspots could be used for the diagnosis of tumors. It has emerged from several studies that the intragenic portions have a greater number of CpG islands than the promoters and that these sequences are highly conserved in plants and other animals. In particular, intragenic methylation is "positively correlated" to the expression of the same gene and "negatively correlated" to the majority of histone modifications in the hypermethylated region, therefore associated with non-

condensed chromatin regions (Falzone L et al, 2016; Singer M et al, 2015). The methylation pattern of tumor cells is largely modified by hypomethylation which mainly affects gene promoters with low CpG density (Rodriguez J et al, 2006). Furthermore, in tumor cells, site-specific hypermethylation phenomena can also be observed, which determines the silencing of tumor suppressor genes involved in processes such as: apoptosis, cell adhesion, angiogenesis, DNA repair mechanisms and cell cycle which, once altered, facilitate tumorigenesis (Jones PA and Baylin SB, 1999). As DNA methylation represents the most studied epigenetic alteration, the involvement of this modification in cutaneous melanoma will be treated separately in the following chapter.

1.5 DNA Methylation in Cutaneous Melanoma

As already mentioned, the genesis of tumors is favored by the hypermethylation of some CpG islands present in the promoter sites which prevents the transcription of tumor suppressor genes (Sigalotti L et al, 2010). In particular, hypermethylated tumor suppressor genes in melanoma are those involved in transcription, apoptosis, cell cycle regulation, DNA repair, etc. (Muthusamy V et al, 2006; Koga Y et al, 2009). Recent studies have shown that intragenic methylation correlates positively with gene expression and negatively with most of the histone modifications. Specifically, it is believed that methylation in the body of the gene could allow intragenic activation, alternative splicing and transcriptional elongation (Singer M et al, 2015). Melanoma is a tumor characterized by its great ability to metastasize and be invasive. Its diffusion is associated with the degradation of the extracellular matrix mediated by the overexpression of specific metalloproteinases including MMP-9 (Napoli S et al, 2020). In this context, previous studies conducted by the research group of the experimental oncology laboratory of the University of Catania have highlighted an active role of DNA methylation in the over-expression of the MMP-9 gene, underlining how intragenic methylation of the CpG2 island of MMP-9 plays a key role in the over-expression of this tumor-promoting protein which is involved in melanoma invasiveness and metastasis (Candido S et al, 2019; Falzone L et al, 2016).

Starting from these preliminary observations, the interest of several researchers about the role of intragenic and promoter methylation in melanoma, and in tumors in general, was increased. For this purpose, several consortia collected DNA methylation as well as gene expression data of tumors and related control samples in order to identify potential methylation hotspots involved in the dysregulation of oncogenes and tumor suppression genes thus representing reliable diagnostic or prognostic biomarkers for melanoma patients.

Although several studies have investigated the effects of DNA methylation in both gene promoter or intragenic regions towards the expression of the methylated genes, the precise patterns of correlation between hyper- and hypo-methylation in gene regions with the expression of genes have not been fully clarified yet. In this context, the use of high-throughput technologies and bioinformatics analyses provided novel useful information to clarify the functional effects of DNA methylation in tumors (Kagohara LT et al, 2017). Overall, it was established that that methylation in certain CpG regions may have effects on transcriptional elongation, enhancement of intragenic activation and alternative splicing (Mendizabal I et al, 2017; Li S et al, 2017; Zlotorynski E, 2017). Therefore the understanding and identification of the mechanisms underlying the alteration of gene expression after methylation phenomena can lead to the identification of novel biomarkers. In this regard, several bioinformatics tools have been developed in order to easily analyze the huge amount of bioinformatics omics data collected and to correlate DNA methylation levels to gene expression. Among these tools, there are FEM and MethylMix as well as EpiMethEx developed during the research activities of this Ph.D. program (Candido S et al, 2019; Gevaert O, 2015; Jiao Y et al, 2014).

2. AIM OF THE STUDY

The epidemiological data and clinical characteristics of cutaneous melanoma described in the previous paragraphs highlight how the management of this tumor is particularly complex from a diagnostic, prognostic and therapeutic point of view. Indeed, the diagnosis of cutaneous melanoma is still often formulated when the tumor has already invaded the subcutaneous layer and is at an advanced stage thus limiting the survival rates of the patients. An explanation of the late diagnosis of cutaneous melanoma is related to the lack of effective diagnostic and prognostic biomarkers. Therefore, the identification of novel factors capable of predicting the risk of the onset of cutaneous melanoma and its aggressiveness is essential to better manage this tumor.

Based on these premises, the aim of the present project was to evaluate the global status of DNA methylation in cutaneous melanoma in order to identify potential DNA methylation hotspots associated with the alteration of gene expression responsible for the development of cutaneous melanoma, associated with the therapeutic response of patients as well as their prognosis. Particular attention was paid to the study of methylation phenomena and consequent alteration of gene expression affecting transcription factors involved in embryonic development, tumor cell proliferation and development of metastases.

For these purposes, a bioinformatics analysis was first conducted using the EpiMethEx bioinformatics tool developed during the first year of the Ph.D. program by the research group of the Experimental Oncology Laboratory. This tool was used to computationally identify a panel of DNA methylation hotspots affecting key genes potentially associated with the development and progression of cutaneous melanoma. The bioinformatic results highlighted a potential diagnostic role of two transcription factors, RARB and ISL1, whose methylation and expression levels were validated on a panel of five melanoma cell lines by using a novel DNA methylation assay (MSRE-ddPCR) and droplet digital PCR, respectively. In addition, the methylation levels of RARB and ISL1 DNA methylation hotspots were also validated in a case series of FFPE melanoma and control samples by using the innovative technique MSRE-ddPCR developed during the Ph.D. program.

The identification and validation of the methylation hotspots identified in this study through the computational and experimental approaches described in the following chapter will represent a promising strategy for the identification of new diagnostic and prognostic biomarkers for a better management of cutaneous melanoma patients.

3. MATERIALS AND METHODS

3.1 Computational Identification of DNA Methylation Hotspot in Cutaneous Melanoma: EpiMethEx

EpiMethEx is a bioinformatics tool developed by the Experimental Oncology laboratory of the University of Catania together with the Bioinformatics unit of the same University. The tool was developed using the R language in order to create a script capable of processing both expression data and methylation data stored in public databases such as The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus DataSets (GEO DataSets). Through cyclical correlation analyses performed between the expression data and methylation data of the same gene, EpiMethEx is able to identify single portions of the DNA (hotspot) where methylation phenomena are present, but also to study large genomic regions whose methylation is associated with the modulation of the expression levels of the codifying genes by combining the methylation data of multiple contiguous hotspots obtained through the analysis of multiple adjacent CG probsets. The processing of expression and methylation data carried out by EpiMethEx takes place following three main steps: i) Gene expression analysis, ii) CG probsets pre-processing and iii) CG probsets grouping and analysis. A further additional data filtering step is added to these three analytical phases in order to select only statistically significant data. For the correct execution of the EpiMethEx analysis, the data have to be previously ordered into pre-established columns containing, respectively, the levels of gene expression, the levels of methylation, the genomic region of the probset CG for each sample. This will allow EpiMethEx to understand which CG probset maps the specific gene in order to compare gene expression data and methylation data in a gene-specific manner (Figure 9).

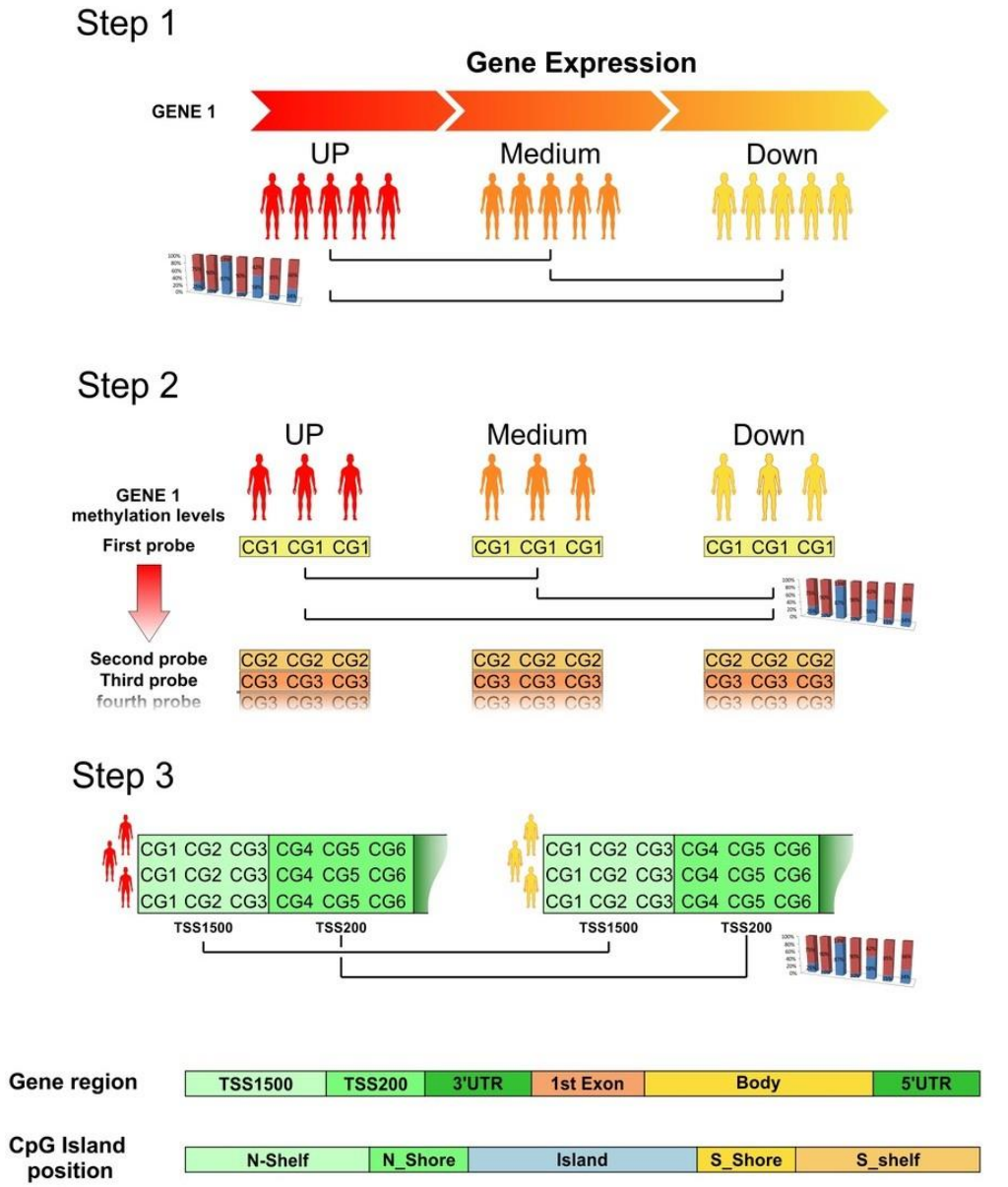

Figure 9. Analytical workflow of EpiMethEx. Step 1 is represented by gene expression analysis; step 2 consists of CG methylation probsets preprocessing and step 3 deals with CG probsets grouping and analysis (From Candido S. et al, BMC Bioinformatics 2019).

The first step of the EpiMethEx processing of data consists in the analysis of gene expression data. For this step, the data is stored and filtered to eliminate those genes that have null or incorrectly annotated values. Next, the expression levels of the various genes for each sample are sorted in descending order (for each gene a different sequence of samples could be obtained) and the samples are stratified into three groups based on the expression levels (up, medium and down). At this point, EpiMethEx performs the differential analysis between two of the three different expression groups (Up vs Medium, Up vs Down and

Medium vs Down), expressing the differential expression as Fold Change (FC) value, i.e. how many times the expression levels have increased or decreased between the groups. This step is useful to identify the difference in the average expression levels between two different groups. Furthermore, this step takes into account the nature of the data, if expressed in linear or logarithmic form, by applying a normalization factor. In particular, if the data are expressed on logarithmic scale, the FC values are converted into linear values. After that, the Student T-Test (T-test) is applied by grouping the matrices in pairs (Up vs Medium, Up vs Down, Medium vs Down).

In the second step (CG probset pre-processing) EpiMethEx assigns each CG probset to the corresponding gene, identifying also the position within the assigned gene region and its position within the CpG islands, including the proximal regions (Shore and Shelf) according to the Infinium HumanMethylation450 BeadChip (440k) platform annotation (GPL13534). The data is first processed to have a unique "location-ID-gene-CG" match as different probes can map to various regions of the same gene and overlapping genes can have identical CG probes. The methylation levels of the various probsets for each gene analyzed in the first step are obtained from the obtained CG probe matrix thanks to the SKCM DNA methylation (Methylation450k) dataset (https://genome-cancer.ucsc.edu/). The probsets are grouped to the corresponding gene taking into account the order of genes obtained from the expression analysis performed in step 1.

In the third step, defined CG probset grouping and analysis, the previously obtained data are used to calculate the median values of the methylation levels for each of the Up, Medium and Down gene expression groups, the value of β-difference (i.e. the variation of methylation levels in the three different expression groups Up, Medium and Down) and p-value for each level of methylation of the CG probset analyzed between the groups compared two by two (Up vs Medium, Up vs Down and Medium vs Down) and the Pearson correlation and p-value between gene expression levels and relative methylation levels of probset CG. EpiMethEx also allows to perform the analysis according

to the CG methylation groups grouped according to the levels of gene expression in Up, Medium and Down following their position according to certain criteria:
- All CG probsets that are part of the same gene region, for each gene, are grouped with the respective level of methylation and classified according to the levels of gene expression in order to create a CG probset matrix;
- All the CG probset existing in the same methylation island and in the adjacent Shore and Shelf regions of a single gene are grouped together; they are also sorted together with their methylation level, according to gene expression levels in order to create a CG probset matrix;
- All probsets that identify the same gene are grouped in order to determine the effects that global methylation has on the regulation of gene expression.

Also in this case, for each gene, the CG probset methylation data are divided into three groups based on their levels (Up, Medium and Down); the β-difference is calculated as the difference between the medians of each group towards the others. After that, Pearson's correlation analysis is carried out to statistically validate the relationship between the methylation state of a specific gene portion and the variation in the gene expression of that gene.

Six different filters can be applied to the data obtained by EpiMethEx in order to evaluate the biological value of the methylation hotspots involved in the mechanisms of gene expression regulation:
- With the first filter, the median values of the methylation levels can be ordered in an increasing or decreasing manner;
- With the second filter it is possible to extract those data that have β-difference values greater than or equal to a value established by the user between the methylation groups;
- With the third filter it is possible to extract those data with a p-value lower than or equal to a threshold value calculated between the various methylation groups (Up vs Medium, Up vs Down, etc.);
- The fourth filter is applied to display only the data relating to genes with FC values greater than or equal to a threshold value, displaying the corresponding methylation values;

- The fifth filter allows the user to select the data based according to the p-values obtained by T-test analyses between the gene expression levels (Up vs Down, Up vs Medium or Medium vs Down) in order to select only those presenting a statistically significant difference (for example $p < 0.01$);
- The sixth filter uses the result obtained from the Pearson correlation. A threshold value is set in order to identify all data that have a statistically significant correlation (positive or negative) between gene expression and methylation levels (Candido S et al, 2019).

To identify methylation probsets and areas of possible biological significance, all filters are run sequentially. The correlation analysis performed with EpiMethEx allows us to obtain four different data matrices (.csv format) named "CG_by_position", "CG_Individually", "CG_of_genes" and "CG_Island" containing r-correlation coefficient, p-value, β-difference and fold change values obtained by different statistical tests.

In this study, EpiMethEx was used to analyze the bioinformatics data contained in the TGCA SKCM database which collects methylation and gene expression data of cutaneous melanoma patients and the data contained in the GTEx database which collects methylation and gene expression data related to normal skin or normal nevi.

The application of EpiMethEx to these bioinformatics data allowed the identification of a set of DNA methylation hotspots affecting different genes and genomic regions whose expression levels were altered as a consequence of the DNA methylation phenomena. The data obtained through EpiMethEx were further analyzed using other bioinformatics software such as DAVID Functional Annotation Bioinformatics Microarray Analysis (https://david.ncifcrf.gov) in order to establish the gene ontology of the genes whose methylation status was correlated with the change of the expression levels.

3.2 Cell Culture

In order to validate the bioinformatics findings obtained through EpiMethEx and confirm the negative correlation existing between promoter methylation and gene expression and the positive correlation existing between intragenic methylation and gene expression, the methylation of four selected DNA

methylation hotspots affecting RARB and ISL1 genes as well as the expression levels of these two genes were evaluated on five different melanoma cell lines available at the cell biobank of the Laboratory of Experimental Oncology of the University of Catania. In particular, the melanoma cell lines A375, A2058, M14, SK23MEL were cultured in RPMI 1640 medium (Cat no. 10-040 - Corning® Life Sciences) while the MeWo cell line was cultured in EMEM medium (Cat no. 15-010 - Corning® Life Sciences) at a constant temperature of 37°C and 5% CO2. Both culture media were supplemented with 2 mM L-glutamine, 100 IU penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) (Cat no. 25-005, Cat no. 30-001, Cat no.35079 - Corning® Life Sciences). The media of the flasks containing cells were checked every two days to remove the exhausted medium and replace it with fresh medium as needed. All cell lines were cultured until 80% confluency in order to collect $5x10^6$ cells useful for the subsequent phase of genomic DNA and RNA extraction.

3.3 Collection of Melanoma FFPE Samples and Normal Controls

The analysis of the methylation levels of the computationally identified RARB and ISL1 DNA methylation hotspots was performed on 15 Formalin-Fixed Paraffin-Embedded (FFPE) melanoma samples and 15 FFPE benign nevi provided by the Pathology Unit of the Ospedale Policlinico Vittorio Emanuele of Catania. For each melanoma or benign nevus sample, ten FFPE sections of 5-8 µm were collected to obtain nucleic acids for the molecular analyses.

The socio-demographics and clinical-pathological features of the melanoma patients enrolled in the study are reported in Table 3.

Table 3. Socio-demographic and clinical features of 15 patients with melanoma.

	All		Cancer progression				Vital status			
			No		Yes		Alive		Dead	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Sex										
Man	8	(53.3)	2	(40.0)	6	(60.0)	2	(25.0)	6	(75.0)
Woman	7	(46.7)	3	(60.0)	4	(40.0)	2	(28.6)	5	(71.4)
Fisher test				p=1.000				p=1.000		
Age at treatment initiation (years)										
<45	4	(26.7)	2	(40.0)	2	(20.0)	1	(55.6)	3	(27.3.)
45-59	5	(33.3)	1	(20.0)	4	(40.0)	2	(11.1)	3	(27.3)
≥60	6	(40.0)	2	(40.0)	4	(40.0)	1	(33.3)	5	(45.4)
Fisher test				p=0.824				p=0.195		
Stage										
M1a	5	(33.3)	2	(40.0)	3	(30.0)	1	(55.6)	4	(36.4)
M1b	3	(20.0)	1	(20.0)	2	(20.0)	2	(11.1)	1	(9.1)
M1c	7	(46.7)	2	(40.0)	5	(30.0)	1	(33.3)	6	(54.5)
Fisher test				p=0.502				p=0.726		

3.4 Nucleic Acid Extraction

Genomic DNA and total RNA were extracted from both melanoma cell lines and FFPE samples by using the following protocols.

For the extraction of genomic DNA from melanoma cell lines the PureLinkTM Genomic DNA Mini Kit (Thermo Fisher Scientific, Cat no. K1820-01) was used. In summary, for each cell line, $5x10^6$ cells were collected, resuspended in 200 μL PBS (Cat. No. 21-031 - Corning®, Life Sciences) after removal of all traces of growth medium and transferred to a sterile microcentrifuge tube containing 20 μL proteinase K. Then, 20 μL of RNase A was added to the sample and the mix was vortexed and incubated for 2 minutes at room temperature. Then 200 μL of PureLink™ Genomic Lysis/Binding Buffer provided by the kit was added to the sample. To allow proper protein digestion, the digestion mix was homogenized and incubated at 55°C for 10 minutes.

After a 10-minute incubation, 200 μL of 96-100% BioUltra ethanol, molecular biology grade ≥ 99,8% (Sigma Aldrich, Cat. No. 51976) was added to the lysate, and the whole digestion reaction was vortexed to obtain a homogeneous solution.

This lysate was loaded into the PureLink™ Spin Columns supplied with the kit. Subsequently, the columns were centrifuged at 10,000 g for 1 minute. The flow-through was then discarded and the genomic DNA blocked by the silica membrane was washed with two different wash buffers. Finally, genomic DNA was eluted by adding 50 µL of PureLink™ Genomic Elution Buffer to the column membrane and centrifuged at maximum speed for 1.5 minutes. The purified genomic DNA was stored at -20°C until further analysis.

To evaluate the expression levels of both RARB and ISL1, the total RNA of melanoma cell lines was extracted using TRIzol™ Reagent (Thermo Fisher Scientific, Cat. No. 15596018) according to the protocol provided by the manufacturer. Briefly, for each cell line, 400 µL of TRIzol™ reagent was added to $5x10^6$ cells. After a 5-minute incubation, 200 µL of chloroform molecular grade (SERVA Electrophoresis GmbH, Cat. No. 39553.01) was added and the reaction mix was incubated for additional 2 minutes was performed. The sample was then centrifuged at 12,000 x g at 4°C for 15 minutes to obtain a lower phenol-chloroform phase, an interphase and an upper aqueous phase containing RNA. In order to collect, the aqueous phase was transferred into a new tube and 500 µL of isopropanol was added to precipitate the RNA; the sample was incubated at room temperature for 10 minutes. After centrifugation, the supernatant was discarded, and the pellet was resuspended in 1 mL of 75% ethanol. A second centrifugation at 7,500 x g for 5 minutes at 4°C was performed to wash the RNA pellet and the supernatant was discarded to obtain a pure RNA pellet. Finally, the RNA was resuspended by adding 50 µL of molecular grade water (UltraPure™ Distilled Water DNase/RNase Free, Cat. No. 10977-035 - Invitrogen from Thermo Fisher Scientific). The RNA obtained was stored at -80°C until the next use.

As regards the extraction of DNA from FFPE samples, the QIAamp DNA FFPE Tissue Kit (Qiagen Cat. No./ID: 56404) was used following the manufacturer's protocol.

3.5 Reverse Transcription and Analysis of Gene Expression Levels by ddPCR

The RNA obtained from melanoma cell lines was reverse transcribed into cDNA using the SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, Cat. No. 18090010) according to the manufacturer's protocol in order to evaluate the expression levels of both RARB and ISL1. A reaction mix containing random hexamers, RNA template and dNTPs was incubated at 65°C for 5 minutes and then incubated on ice for 2 minutes. Then, the SuperScript IV reverse transcriptase was added and the reaction was incubated at 55°C for 10 minutes to reverse transcribe the RNA and at 80°C for 10 minutes to stop the reaction. The obtained cDNA was analyzed by using the droplet digital PCR high-sensitivity amplification system with EvaGreen chemistry.

Briefly, the amplification reaction was prepared using 11 µL of ddPCR QX200 ™ ddPCR ™ EvaGreen Supermix (Cat no. 186-4034 - Bio-Rad Laboratories, Inc.), 0.055 µL of the forward and reverse primers at a concentration of 100 µM, 0.6 µL of cDNA (10 ng/µL) and RNAse/DNAse-free H_2O to a final volume of 22 µL.

Subsequently, 20 µL of the reaction mix was loaded into a cartridge containing 70 µL QX200™ Droplet Generation Oil for EvaGreen (Cat. No. 1864006 - Bio-Rad Laboratories, Inc.), and droplets were generated using the QX200 Droplet Generator.

The obtained droplets were loaded into a PCR plate and amplified using C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc.) using the following thermal conditions: an initial phase of hot-start Taq polymerase activation at 95°C for 5 minutes; 40 cycles consisting of a denaturation step at 95°C for 30 seconds and an annealing and elongation step at 60°C for 1 minute; a signal stabilization phase consisting of a step at 4°C for 5 minutes and one at 90°C for 5 minutes. A ramp rate of 1.6°C/s was used to avoid thermal stress and the rupture of the droplets during the amplification steps.

After PCR amplification, the fluorescent signals of positive and negative droplets were detected using the QX200 Droplet Reader (Bio-Rad Laboratories, Inc). The results were then analyzed using QuantaSoft software, version 1.7.4

(QuantaSoft, Prague, Czech Republic), to obtain the absolute quantification of targets expressed as copies/μL.

For our analysis, beta-actin was used as the reference housekeeping gene. The primers used for each target are listed in Table 4 (Table 4).

Table 4. Lists of primers used for the MSRE-ddPCR assay.

Fwd RARB	5'-TTT CTC AGA CGG CCT TAC CC-3'
Rev RARB	5'-CAC GCT CTG CAC CTT TAG CA-3'
Fwd ISLI	5'-TTC CCA CTT AGC CAC AGC TC-3'
Rev ISLI	5'-ATT TGA TCC CGT ACA ACC TGA-3'
Fwd beta-actin	5'-CTG GGA CGA CAT GGA GAA AA-3'
Rev beta-actin	5'-AAG GAA GGC TGG AAG AGT-3'

3.6 Analysis of DNA Methylation Hotspots through MSRE-ddPCR

The analysis of the methylation levels of the DNA methylation hotspots identified through EpiMethEx was performed by using a novel assay developed during the research activities of the Ph.D. program and called Methylation-Sensitive Restriction Enzyme (MSRE) assay and ddPCR amplification (MSRE-ddPCR). This custom protocol is based on the use of methylation-sensitive restriction enzyme (MSRE) followed by ddPCR amplification in a one-step protocol. More in detail, a first restriction reaction using the methylation-sensitive enzyme HpaII is performed to discriminate between methylated and non-methylated DNA sequences. Unlike other existing methods, in the MSRE-ddPCR protocol the digestion and amplification reactions take place in the same tube. Therefore, this approach allows a one-step analysis to evaluate the percentage of methylation by reducing the potential errors of a multi-step method, such as the MSRE method, thus reducing the cost and volumes of the reaction.

In the MSRE-ddPCR protocol a synthetic DNA methylation control is used to evaluate the efficacy of the restriction enzyme reaction and custom TaqMan probes are used to selectively evaluate the methylation levels of the DNA methylation hotspots computationally selected. More in detail, the DNA methylation control is an artificially demethylated exogenous DNA fragment

obtained cloning a region of the coding sequence for the EGFP protein from the pcDNA3 clover plasmid (Addgene - Plasmid # 40259). This 210 bp unmethylated control is completely degraded by the HpaII enzyme, therefore, the identification of the copies/µL of the control after the ddPCR is a direct measurement of the restriction reaction efficacy. This 210 bp control was obtained by using the two primers reported in Table 5 (Table 5).

Table 5. Primers used for the generation of the DNA Methylation Control.

T7	5'-TAATACGACTCACTATAGGG-3'
EGFP-N bis	5'-CTTGCCGTTGGTGGCATCGC-3' (GCGATGCCACCAACGGCAAG)

The amplification signals for the DNA methylation control are revealed using a custom TaqMan probe binding the restriction site in the 210 bp sequence of the methylation. As already mentioned, this probe is useful to determine the copies/µL of the methylation control and to assess the presence of interference in the enzymatic cleavage reaction on the methylation sites.

The reaction mix of the MSRE-ddPCR assay is prepared as follows: for each sample, 20 ng of DNA was analyzed by MSRE-ddPCR using two different digestion-amplification mixes, one containing the restriction enzyme HpaII and the other containing no restriction enzyme (HpaII-free sample). Both reaction mixes contained 2X ddPCR Supermix for Probes (No dUTP), 900 nM (final concentration) of each primer and 450 nM (final concentration) of the probe specific for the target studied, a known amount of DNA Methylation Control (10 ng) and 20 IU of the restriction enzyme HpaII or DNAse/RNAse free H_2O for the HpaII-free sample mix.

The resulting reaction tubes were first incubated at 37°C for 30 minutes to allow enzymatic digestion mediated by HpaII. Subsequently, 20 µL of each digested sample was loaded into a cartridge for the generation of droplets as described in the previous chapter to generate about 20,000 droplets.

Subsequently, the generated droplets were transferred to a 96-well plate and amplified using a C 1000 Touch Thermal Cycler (Bio-Rad Laboratories Inc, Hercules, CA, United States Bio-Rad Laboratories, Inc., USA). The amplification protocol consisted of an initial denaturation for 10 minutes at

95°C, followed by 40 cycles consisting of 30 seconds of denaturation at 94°C, 1 minute of annealing and amplification at 55°C, 10 minutes of droplet and signal stabilization at 98°C; a ramp rate of 1.6°C/s between each step of the amplification was used.

Finally, the amplification signals were detected using the QX200 Droplet Reader (Bio-Rad Laboratories, Inc., USA) and the amplification results were analyzed through QuantaSoft software, version 1.7.4 (QuantaSoft, Prague, Czech Republic) to determine the exact copies/µL for the RARB and ISL1 genes and for the methylation control, used to establish the exact percentage of methylation of the analyzed hotspots.

To establish the percentage of methylation of each target, the following formula was used:

$$\%\ \text{of methylation} = \left(\frac{\text{HpaII}}{(-)}\right)^{\text{Target}} \times \left(1 - \left(\frac{\text{HpaII}}{(-)}\right)^{\text{Meth Ctrl}}\right) \times 100$$

In this formula, HpaII and (-) indicate the copies/µL obtained for the target and methylation control in each specimen treated or not treated with HpaII.

The use of MSRE-ddPCR ensures great advantages compared to standard protocols for the analysis of DNA methylation (e.g. MSRE or bisulfite conversion) as through MSRE-ddPCR protocol it is possible to evaluate the methylation levels of DNA of poor quality as that obtained from FFPE tissues or liquid biopsy samples which is often degraded or obtained in low concentration. The use of MSRE-ddPCR overcomes all these limitations ensuring a reliable evaluation of the methylation level of targets without any bias related to an inefficient restriction enzyme reaction that is monitored by using the synthetic DNA methylation control.

3.7 Statistical Analyses

The bioinformatics results obtained through EpiMethEx were already processed using different statistical tests as the R script developed already uses rigorous statistical calculations for the selection of hotspots whose methylation significantly correlates with the expression level of the gene. Indeed, EpiMethEx

only considers methylation hotspots with correlation levels higher than ± 0.6 (moderate correlation) with a p-value of $p < 0.05$.

As regards the experimental results obtained from melanoma cell lines and FFPE samples, the statistical differences between the methylation and expression levels observed for RARB and ISL1 in the five cell lines were established using the One-way ANOVA test and Tukey's multiple comparison test. Correlation analyses between DNA methylation and gene expression levels of RARB and ISL1 in the five cell lines were performed using Pearson's correlation test. The statistical difference between the DNA methylation levels observed in FFPE samples of cutaneous melanoma and FFPE samples of normal nevi was evaluated by using the Mann-Whitney test. The results were considered statistically significant when $p<0.05$. All statistical analyses were performed by using GraphPad Prism v.8.

4. RESULTS

4.1 Analysis of the Global Methylation Status in Cutaneous Melanoma and Computational Identification of DNA Methylation Hotspots

The EpiMethEx analysis performed on the methylation and expression data of melanoma patients obtained from the TCGA SKMC database and normal samples obtained from the GTEx database allowed the identification of 517 genes whose methylation status correlated negatively or positively with gene expression.

By analyzing the global methylation status observed in cutaneous melanoma, 66% of hyper-methylated CG probsets correlated negatively with the expression of genes ($p<0.05$) while 34% of hyper-methylated CG probsets correlated positively with gene expression ($p<0.05$) (Figure 10A). More in detail, the majority of the methylated CG probsets positively correlated with gene expression fell into intragenic portions (77%) confirming the hypothesis according to which intragenic hyper-methylation is associated with the over-expression of the methylated gene (Falzone L et al, 2016; Singer M et al, 2015) (Figure 10B). Conversely, the majority of methylated CG probsets negatively correlated with gene expression fell in the promoter regions (79% of all CG probstes of which 13% maps the TSS1500 region, 27% maps the TSS200 region, 22% maps the 5'UTR region and 17% maps the 1stExon). These latter data suggest that promoter methylation is associated with the down-regulation of gene expression (Figure 10C).

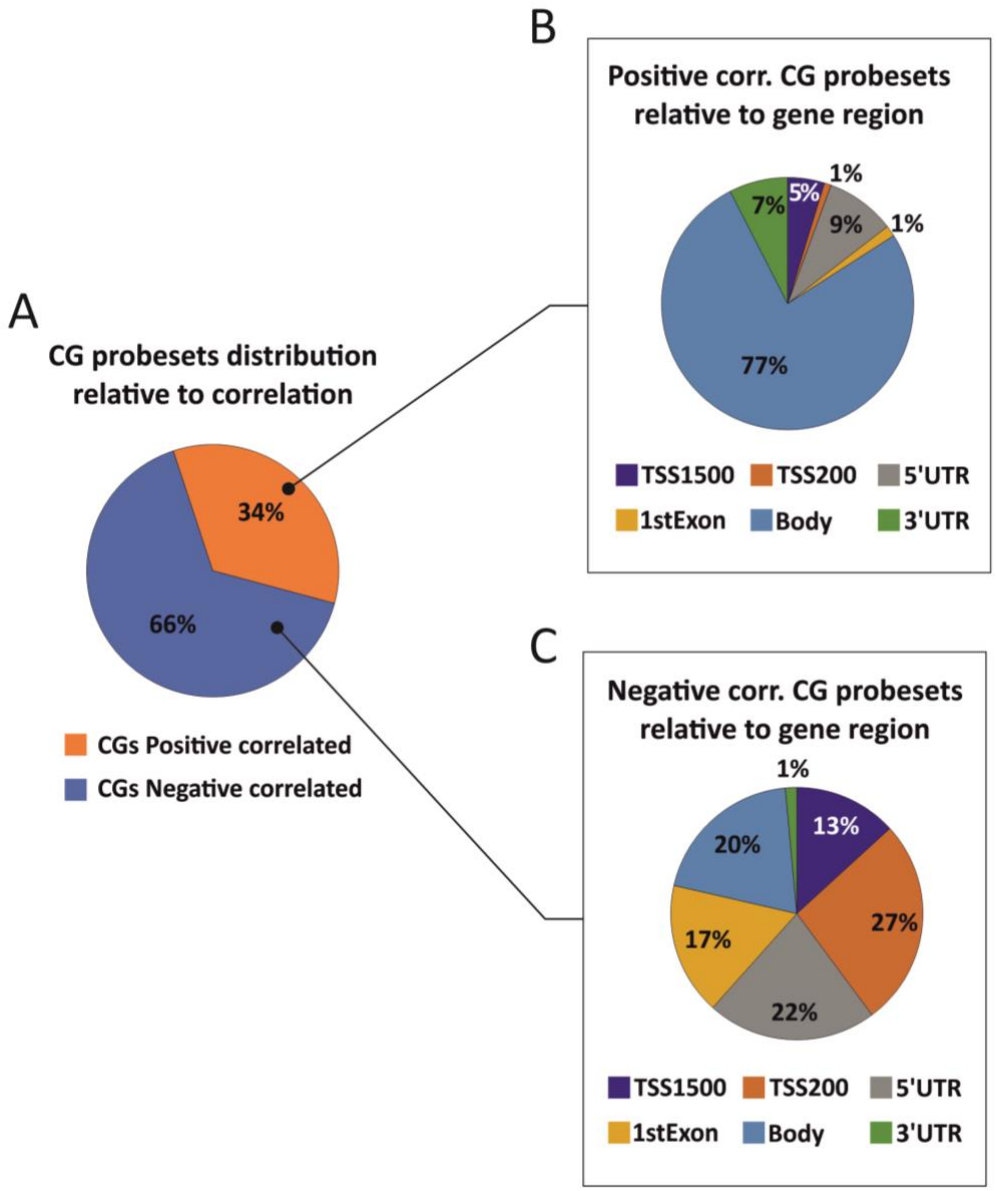

Figure 10. Global methylation status in cutaneous melanoma. A) Percentage of CG probsets positively or negatively correlated with gene expression; B) Gene region distribution of the methylated CG probsets positively correlated with gene expression; C) Gene region distribution of the methylated CG probsets negatively correlated with gene expression.

A more in-depth analysis of the functional role of the 517 genes identified through EpiMethEx and whose methylation significantly correlated with the expression levels performed by DAVID revealed an enrichment of 40 genes belonging to the ontological class of transcription factors (GO:0003700). In particular, 7% of all the genes identified through EpiMethEx were involved in the regulation of gene transcription (Figure 11).

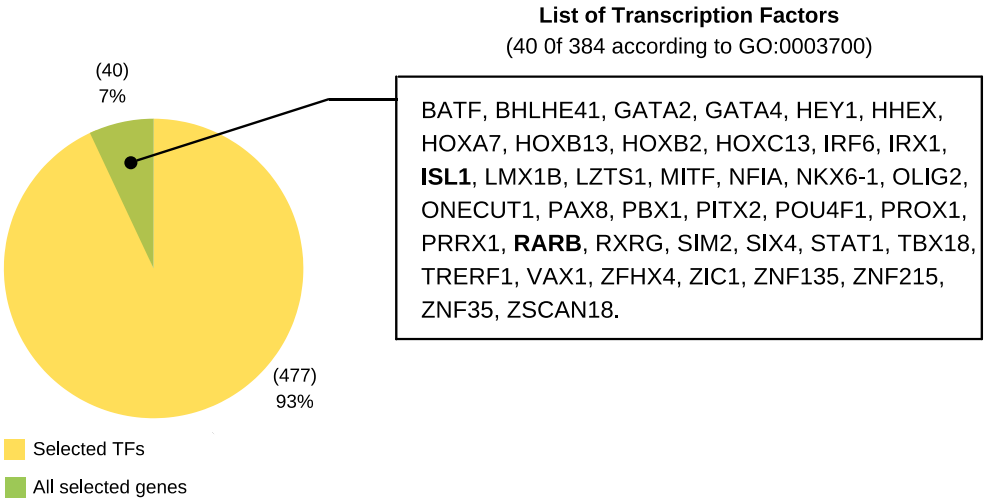

Figure 11. Percentage of transcription factors among the 517 genes identified through EpiMethEx.

This result is extremely interesting as it suggests how DNA methylation phenomena are able to alter not only the methylated genes but also a plethora of genes regulated by transcription factors in an indirect manner. Therefore, the epigenetic modification of some transcription factors occurring in melanoma could lead to the alteration of a high number of genes associated with these transcription factors and involved in various cellular processes, including cell proliferation, cell differentiation and apoptosis.

To better understand the role of these transcription factors in the potential development and progression of cutaneous melanoma, a further analysis of the molecular pathways and cellular processes in which the 40 transcription factors act was carried out. As shown in Figure 12, 28 of the 40 transcription factors identified by EpiMethEx (65% of transcription factors) were involved in the embryo morphogenesis and differentiation, 33% of these transcription factors (14 genes) were involved in the development of the neural tube and 1 transcription factor (2% of the identified transcription factors) MITF was directly involved in melanocyte differentiation (Figure 12).

By interpolating the lists of transcription factors involved in the three different pathways (embryo morphogenesis and differentiation, neural tube development and melanocyte differentiation) it was possible to identify 14 transcription factors involved both in embryonic morphogenesis and differentiation and in the development of the neural tube suggesting how these factors may play a

significant role in the development of melanomas but above all in the processes of tumor progression that lead to the migration of melanoma cells to distant sites of the organism and consequently to the formation of metastases (Figure 12).

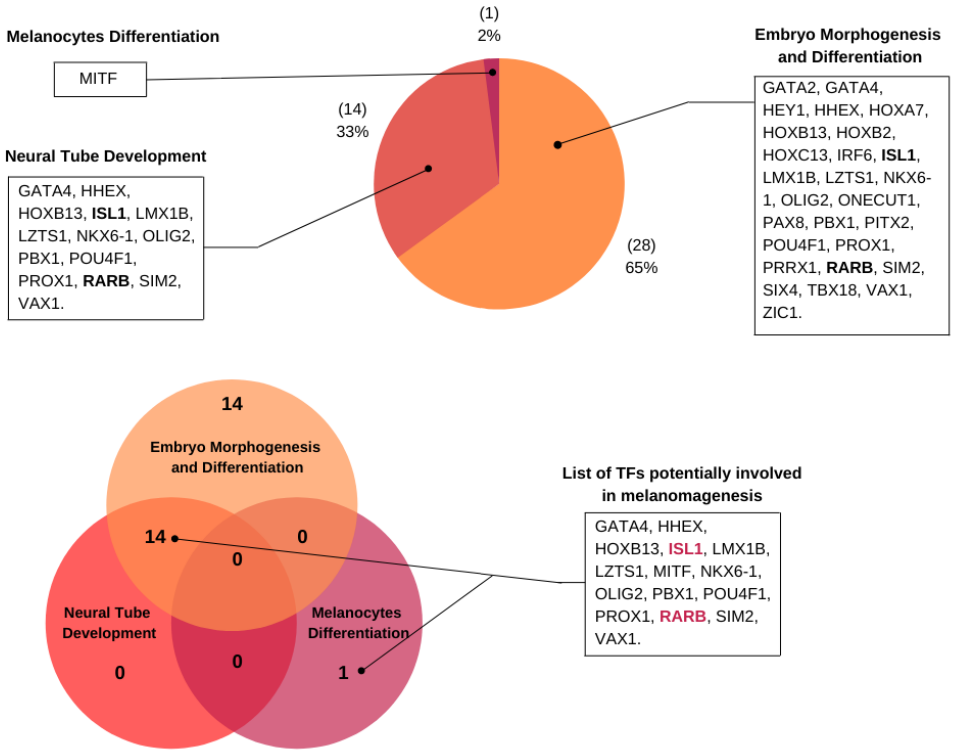

Figure 12. Functional role of the 40 transcription factors identified through EpiMethEx taking into account molecular pathways potentially involved and dysregulated in the development of cutaneous melanoma and in the formation of metastases.

Of note, also MIFT can be considered a key transcription factor involved in the development of melanoma as it is actively involved in the differentiation of melanocytes and its dysregulation could be associated with an increased risk of developing cutaneous melanoma. Therefore, these initial bioinformatic analyses allowed us to identify a set of 15 transcription factors whose expression levels are altered in melanoma as a result of hyper- or hypomethylation phenomena, which may be involved in various physio-pathological processes related to melanoma development and distant metastasis formation.

4.2 Dysregulation of the 15 Transcription Factors according to Different Clinical-Pathological Features of Cutaneous Melanoma

The dysregulation of the 15 transcription factors identified through EpiMethEx and DAVID analyses were analyzed taking into account different clinical-

pathological data of cutaneous melanoma contained in the TCGA SKCM database and normal samples contained in the GTEx database.

The differential analyses of the expression levels of these 15 transcription factors revealed how some genes in cutaneous melanoma compared to normal nevi. For example, HOXB13 and LZTS1 showed increased expression levels in the tumor compared to normal tissues with FC values of 10.81 and 20.75, respectively (Figure 13). By stratifying the melanoma samples contained in the TCGA SKCM database according to the Clark level, the presence of skin ulceration, the presence of subcutaneous lymph node, Breslow index and distant metastases, statistically significant changes in the expression levels of these 15 transcription factors were identified. In particular, by stratifying the samples according to the Clark level only one transcription factor was found to be down-regulated (HHEX) while stratifying by Breslow index nine different transcription factors were altered (Figure 13). The further stratifications performed clustering samples according to the presence and localization of metastases highlighted how many of the transcription factors identified have an altered expression (Figure 13). Among these transcription factors, RARB and ISL1 showed altered expression levels in almost all the stratifications performed, underlining how these two genes can be effectively involved in the progression and metastasis of cutaneous melanomas (Figure 13).

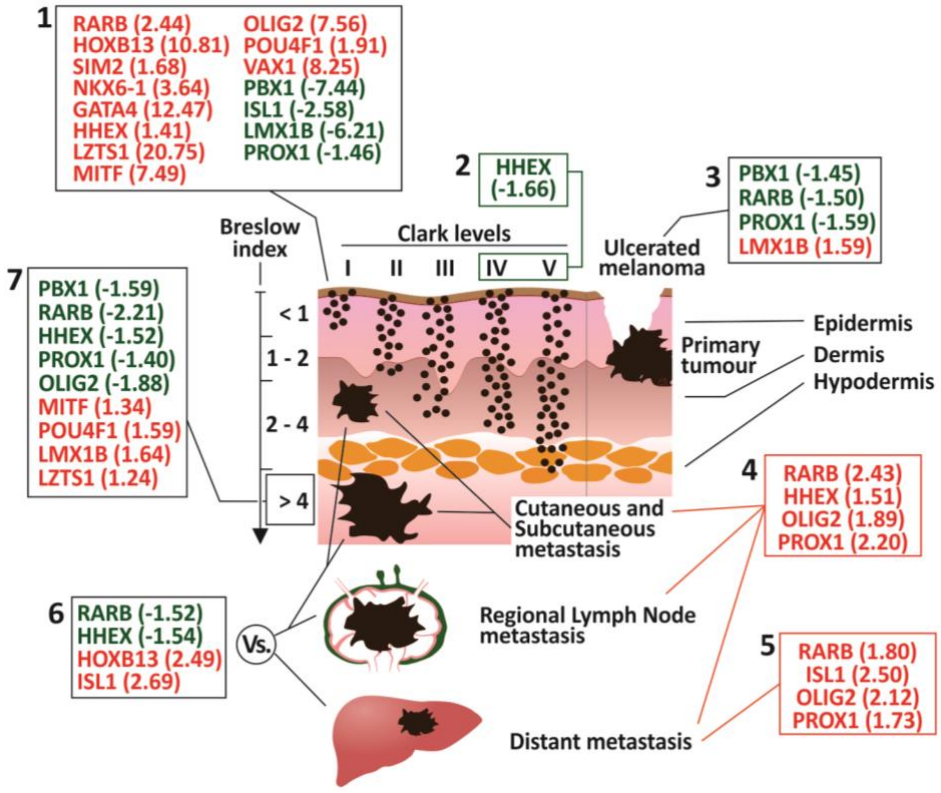

Figure 13. Alteration of the expression levels of the 15 transcription factors identified by EpiMethEx according to different melanoma features. 1) Tumor samples vs healthy tissues; 2) Stratification according to Clark levels; 3) Stratification according to the presence of skin ulcerations; 4, 5 and 6) Stratification according to the presence and localization of metastases; 7) Stratification according to Breslow index.

A more careful analysis of the correlation between DNA methylation and gene expression of these 15 transcription factors highlighted how some of them had a high presence of CG probset mapping the entire sequence of the gene and how most of these probsets correlated significantly with the gene expression in a concordant manner (Figure 14). The most interesting data concern the transcription factors RARB and ISL1. Indeed, the data reported in Figure 14 show that the CG probsets of RARB were located the promoter level and their methylation was negatively correlated with gene expression. Conversely, the CG probsets of ISL1 map the intragenic portion of the gene and the methylation of these probsets were positively correlated with the expression levels of ISL1 (Figure 14)

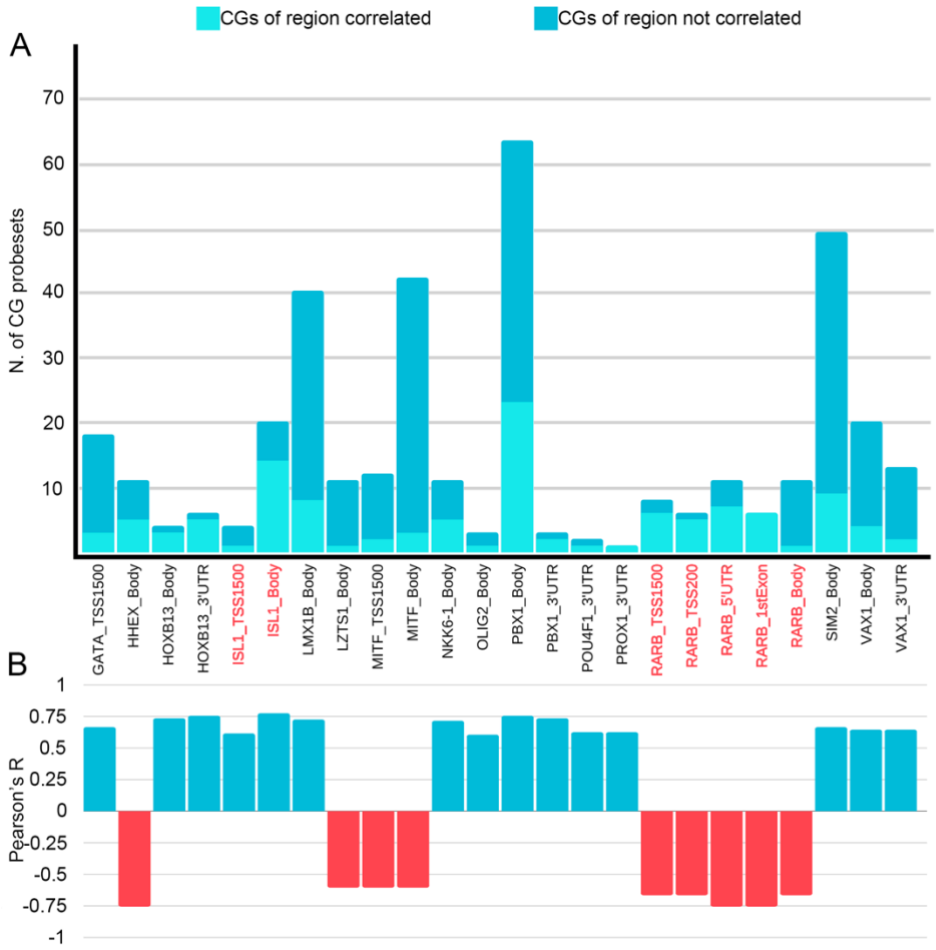

Figure 14. Correlation between DNA methylation and expression levels of the probsets mapping the 15 transcription factors investigated.

These additional data suggest that RARB and ISL1 represent excellent examples of negative correlation between promoter methylation and gene expression and of positive correlation between intragenic methylation and gene expression, respectively.

Overall, these data suggest that the methylation phenomena of these two genes and the consequent alteration of their expression levels could be related to the prognosis of patients with melanoma and therefore they can be used as biomarkers for this pathology. Therefore, the DNA methylation levels and expression levels of these two genes were validated in a panel of melanoma cell lines and in a pilot cohort of melanoma FFPE samples and healthy controls.

4.3 *In Vitro* Validation of the Correlation between DNA Methylation and Gene Expression of RARB and ISL1 in Melanoma Cell Lines

In order to validate the bioinformatic results obtained by EpiMethEx, the methylation and expression levels of RARB and ISL1 were analyzed using the MSRE-ddPCR and ddPCR method, respectively. Through the precise evaluation of the methylation levels of the DNA methylation hotspots identified and the respective expression levels of genes it was possible to establish the effective correlation between promoter methylation and down-regulation of RARB and intragenic methylation and over-expression of ISL1.

As regards the MSRE-ddPCR methylation analysis performed for the TSS200 and EX1 DNA methylation hotspots of RARB showed that these hotspots were strongly methylated in the A2058 and MeWo melanoma cell lines. On the contrary, the same methylation hotspots had methylation rates below 5 % in the A375, SK23MEL and M14 cell lines. The subsequent evaluation of the expression levels of RARB in all these five cell lines also revealed a greater expression of this gene in the hypo-methylated A375, SK23MEL and M14 cells; while the expression levels of RARB were down-regulated in the hyper-methylated A2058 and MeWo cell lines (Figure 15).

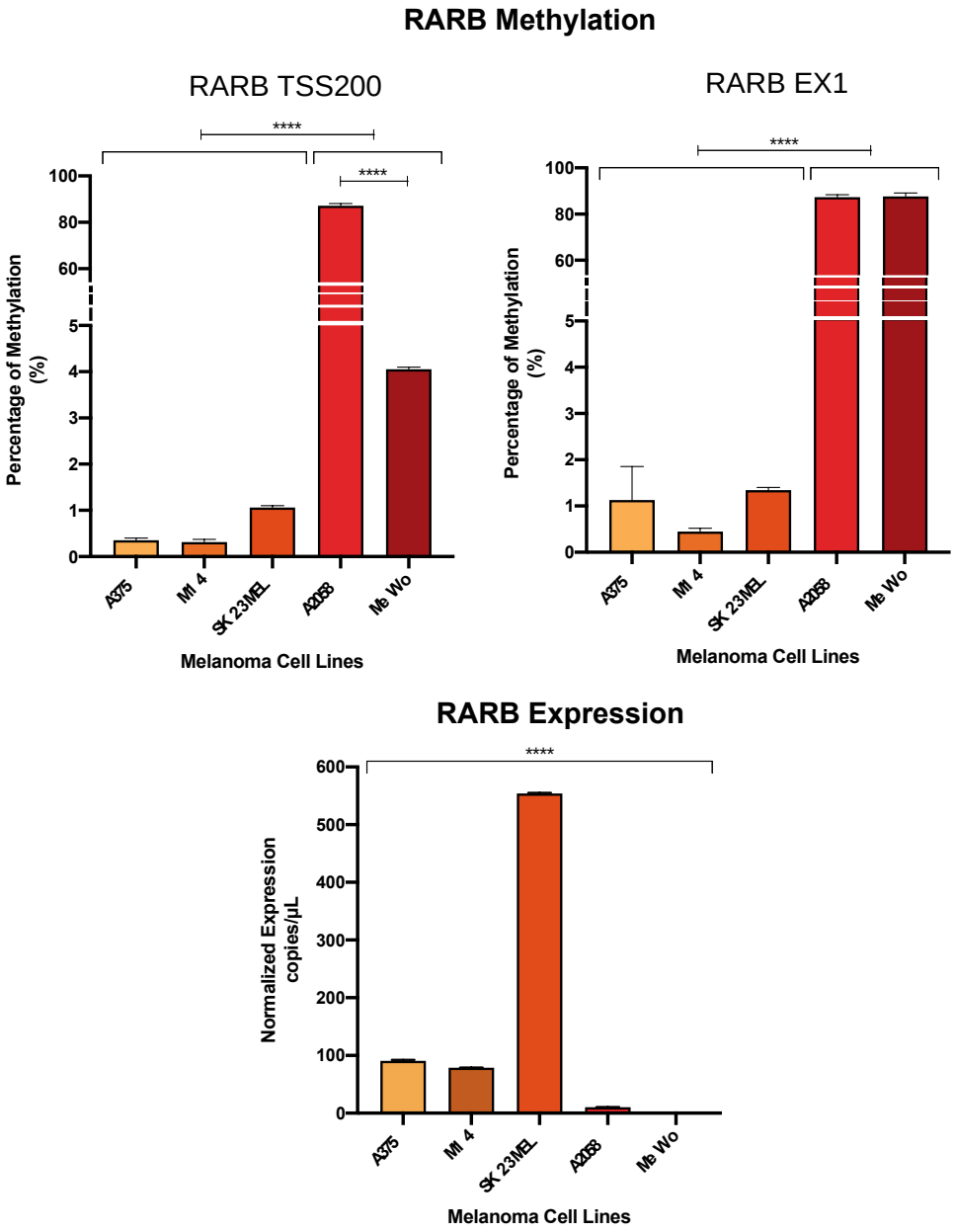

Figure 15. MSRE-ddPCR and ddPCR analysis of the methylation levels of the TSS200 and EX1 DNA methylation hotspots and of the expression levels of RARB in melanoma cell lines. **** $p<0.0001$.

As regards ISL1, the analysis of the methylation levels of the intragenic hotspots isl28 and isl53 highlighted higher methylation levels for the isl53 hotspot compared to the isl28 hotspot (78.25% vs 44%). In addition, high methylation levels were observed for A375, M14 and MeWo cells while slight levels of methylation (0.4% to 3.6%) were observed for SK23MEL and A2058 cell lines (Figure 16). By analyzing the expression levels of ISL1, normalized according to the expression levels of Beta-Actin used as a housekeeping gene, it was

possible to observe a direct association between methylation and gene expression. Indeed, higher levels of ISL1 were observed for the hyper-methylated A375 and MeWo cell lines, while the hypo-methylated cell lines M14, SK23MEL and A2058 showed a reduced expression of ISL1 (Figure 16).

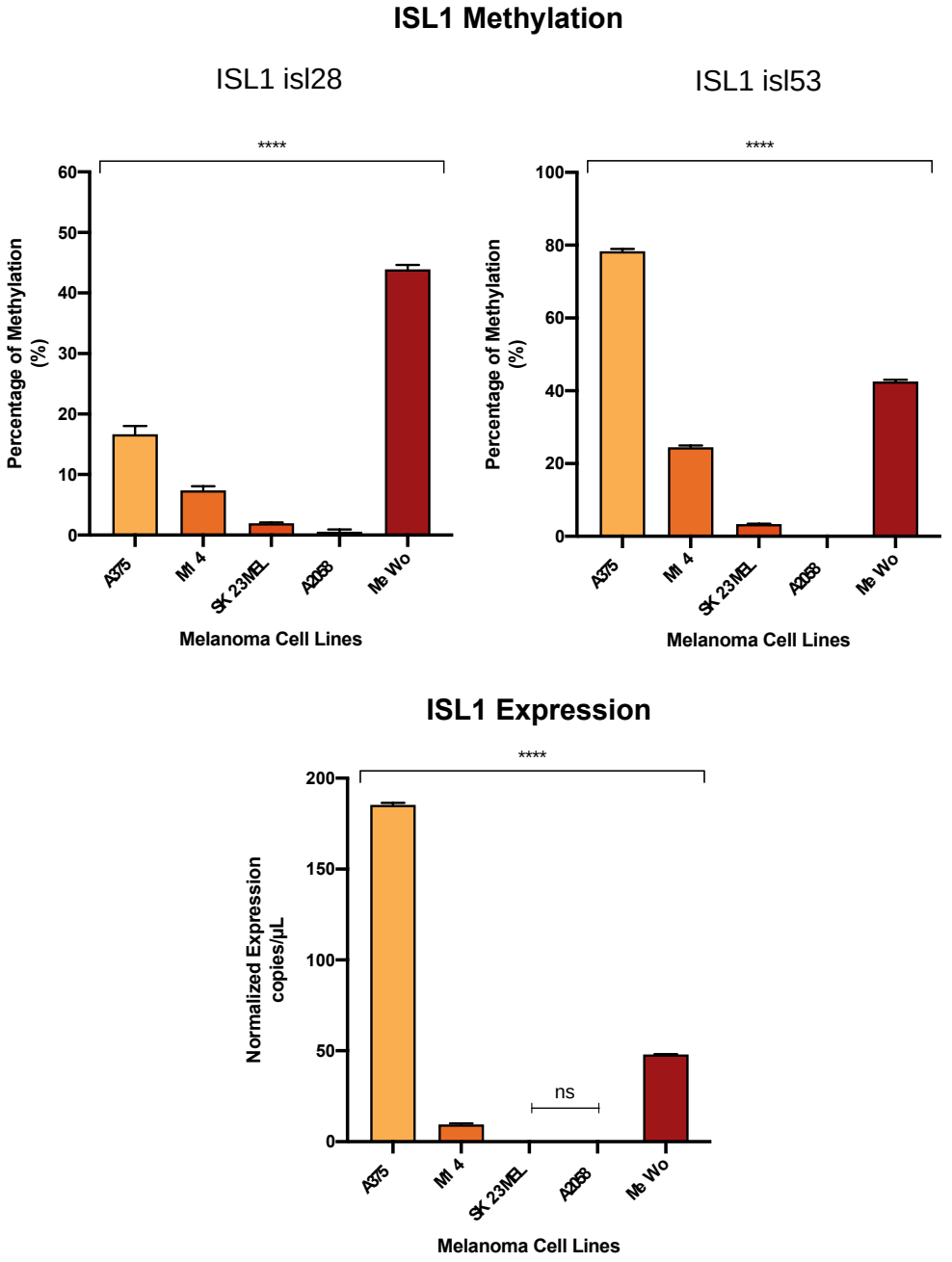

Figure 16. MSRE-ddPCR analysis of the methylation levels of the isl28 and isl53 intragenic DNA methylation hotspot of ISL1 and ddPCR analysis of the expression levels of the same gene in melanoma cell lines. **** $p<0.0001$.

To further evaluate the effective correlation patterns existing between promoter methylation and RARB expression and intragenic methylation and ISL1

expression, Pearson's correlation analyses were performed between the expression levels and methylation observed for the two genes in the five melanoma cell lines here analyzed.

The results of this further analysis further confirmed the effective negative correlation between promoter methylation and RARB expression and in particular a moderate negative correlation between the methylation levels of the TSS200 (r=-0.34) and EX1 (r=-0.55) hotspots with the expression of RARB (Figure 17A). Similarly, Pearson's correlation analyses performed between ISL1 expression and methylation levels highlighted a positive and moderate correlation between ISL1 expression and isl28 hotspot methylation (r=0.33) and a strong positive significant correlation between ISL1 expression and isl53 hotspot methylation (r=0.94; p=0.017) (Figure 17B).

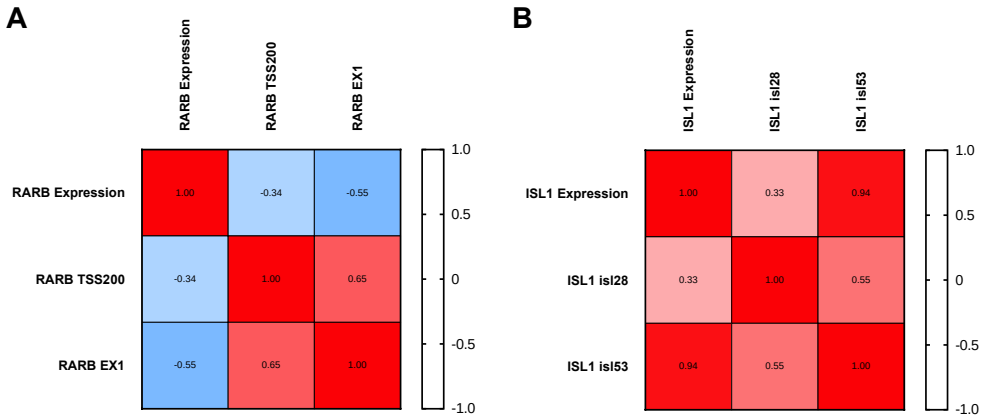

Figure 17. Pearson correlation analysis between expression and methylation levels. A) Correlation between expression and methylation levels of RARB; B) Correlation between expression and methylation levels of ISL1.

This further data confirmed once again the validity of the prediction bioinformatics analysis performed by using EpiMethEx, the validity of the MSRE-ddPCR protocol and the negative correlation between promoter methylation and gene expression as well as the positive correlation between intragenic methylation and expression of the methylated gene.

Once established the correlation existing between DNA methylation and gene expression, the potential diagnostic role of RARB and ISL1 DNA methylation hotspots was validated on FFPE samples obtained by both melanoma patients and healthy individuals with benign nevi.

4.4 Validation of the Diagnostic Value of RARB and ISL1 DNA Methylation Hotspots on FFPE Samples of Melanoma and Benign Nevi

To evaluate the translational impact of the *in silico* and *in vitro* findings obtained through EpiMethEx and the MSRE-ddPCR analyses performed on melanoma cell lines, the methylation levels of the DNA methylation hotspots of RARB and ISL1 were evaluated through MSRE-ddPCR in FFPE tissues obtained from 15 melanoma patients and 15 individuals with benign nevi.

The MSRE-ddPCR analyses performed on FFPE tissue samples demonstrated that both RARB TSS200 and RARB EX1 methylation hotspots were significantly methylated in melanoma samples compared to benign nevi (Figure 18). The mean percentage of methylation observed for the TSS200 methylation hotspot was 37.82% in melanoma samples compared to 16.66% of methylation observed in benign nevi (Figure 18A). Similarly, the percentage of methylation of RARB EX1 hotspot was significantly higher in melanoma samples compared to benign nevi (45.46% vs 20.68%) (Figure 18B). For both RARB promoter methylation hotspots, the increment of DNA methylation observed in melanoma samples compared to benign nevi was statistically significant ($p=0.0049$ and $p<0.0001$ for TSS200 and EX1, respectively) (Figure 18).

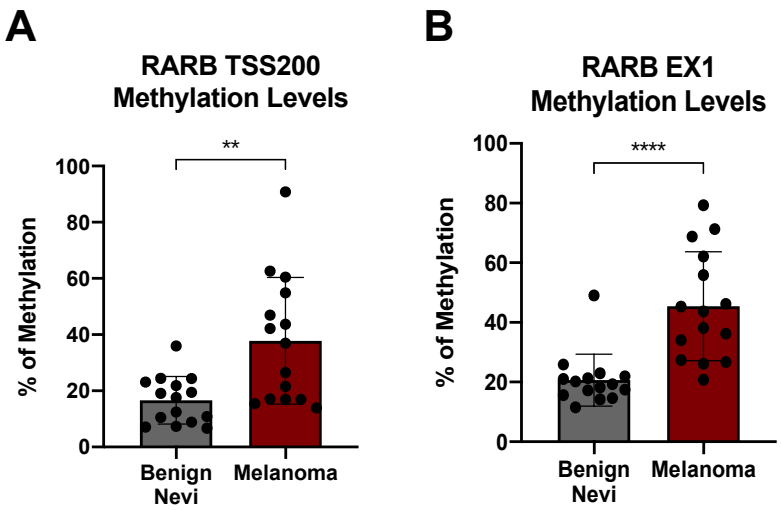

Figure 18. Methylation levels of RARB promoter methylation hotspots. A) Methylation levels of RARB TSS200 methylation hotspot in FFPE melanoma and benign nevi samples; B) Methylation levels of RARB EX1 methylation hotspot in FFPE melanoma and benign nevi samples. ** $p<0.01$; **** $p<0.0001$.

As regards ISL1 DNA methylation hotspots inconclusive results were obtained. Indeed, although a slight increment of the methylation levels of both isl28 and

isl53 methylation hotspots was observed, the results obtained were not statistically significant (Figure 19). In particular, the percentage of methylation observed for the isl28 DNA methylation hotspot was 34.95% in melanoma samples compared to 26.21% observed in benign nevi. However, such increment in the methylation levels observed in melanoma samples was not significant (Figure 19A). As regards the methylation levels observed for the DNA methylation hotspot isl53, the MSRE-ddPCR results revealed low methylation levels in both groups of samples. Also in this case, the slight increment of the methylation levels observed in melanoma samples (average methylation percentage of 5.36% vs 3.74% observed in benign nevi) was not significant (Figure 19B).

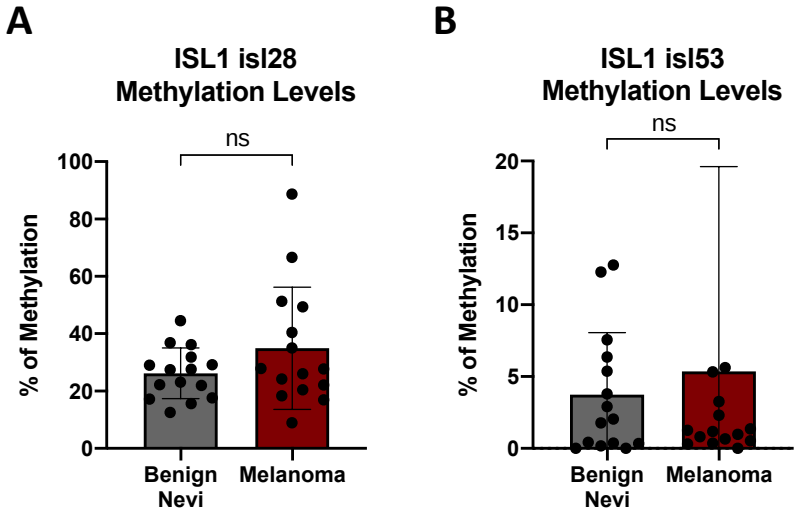

Figure 19. Methylation levels of ISL1 intragenic methylation hotspots. A) Methylation levels of ISL1 isl28 methylation hotspot in FFPE melanoma and benign nevi samples; B) Methylation levels of ISL1 isl53 methylation hotspot in FFPE melanoma and benign nevi samples. ns not statistically significant.

Overall, these further results obtained in clinical samples demonstrated the validity of the EpiMethEx bioinformatic prediction analysis for the methylation hotspots affecting RARB while the results obtained for ISL1 are controversial. Of note, the results obtained in both melanoma cell lines and FFPE samples demonstrated the great sensitivity and specificity of the MSRE-ddPCR protocol developed by the Experimental Oncology Laboratory of the University of Catania during the research activities of the Ph.D. program.

5. DISCUSSION

Cutaneous melanoma represents one of the most aggressive tumors due to its high invasive and metastatic potential. It has been widely demonstrated that the development and progression of melanomas are mainly due to the accumulation of various genetic and epigenetic alterations associated with several risk factors, of which chronic or intermittent sun exposure represent the most studied and recognized (Candido S et al, 2014). These molecular alterations lead to the neoplastic transformation of melanocytes which acquire a tumor-like phenotype characterized by abnormal cell proliferation and the loss of cell cycle control, senescence and apoptosis. All these processes lead to the accumulation of further oncogenic mutation responsible for the invasive properties of melanoma which invade surrounding tissues, also infiltrating the blood and lymphatic vessels.

Among the most frequent molecular alterations observed in cutaneous melanoma there are mutations affecting genes involved in various signal transduction pathways like the MAPK and PI3K/AKT pathways, which are involved in cell proliferation and survival. The dysregulation of these pathways also determines the increased expression of proteins that are involved in the degradation of the extracellular matrix, favoring the infiltration of the tumor into the subcutaneous layers and surrounding tissues (Napoli S et al, 2020).

The accumulation of both driver and passenger mutations in melanoma cells lead also to the stimulation of other cellular and molecular processes involved in the formation of melanoma metastases. In particular, the epithelial-mesenchymal transition (EMT), i.e. the process of de-differentiation of melanocytes into mesenchymal cells, is commonly observed in melanoma cells which have the ability to migrate away from the primitive tumor bulk and form distant metastases surviving in the bloodstream (Yan S et al, 2016).

Recently, it was demonstrated that the formation of distant metastases in melanoma patients is sustained by the reactivation of transcription factors that were active during the embryogenic differentiation of melanocytes (Tang Y et al, 2020). Of note, melanocytes originate from cells of the neural crest which, following EMT processes, migrate to different locations in the body during embryogenesis. Therefore, adult melanocytes that acquire a neoplastic possess

embryological traces of the mesenchymal and migratory abilities of neural crest cells mediated by transcription factors that if reactivated are responsible for the high rate of metastases observed in cutaneous melanoma (Bailey CM et al, 2012; Leong HS et al, 2012). The reactivation of these embryological transcription factors in melanoma can be related to both genetic and epigenetic alterations. In this context, different studies have demonstrated a strong impact of epigenetic alterations on the development of cutaneous melanoma (Giunta EF et al, 2021; Strub T et al, 2020). Among the epigenetic alterations involved in melanoma development, the methylation of DNA is one of the most studied. In particular, the methylation of cytosines is responsible for the alteration of gene expression levels resulting in genome-wide DNA hypomethylation and more localized hypermethylation at CpG-rich genomic loci known as CpG islands (Jones PA and Baylin SB, 2002) Recent studies have also shown that DNA methylation is actively involved in the development of several cancers, including cutaneous melanoma (Akhavan-Niaki H et al, 2013).

As regards the epigenetic role of DNA methylation, it was demonstrated that intragenic DNA methylation is positively correlated with gene expression while methylation phenomena affecting gene promoters are negatively correlated with the expression of genes in the majority of cases. More in detail, it was speculated that intragenic methylation may play a role in the mechanisms of transcription elongation, intragenic activation (enhancer), and alternative splicing. (Singer M et al, 2015).

Starting from these observations, our research group has demonstrated that intragenic DNA methylation phenomena affecting MMP9 gene are correlated with its over-expression suggesting how DNA methylation and epigenetic modifications can be involved in the development of melanoma and in the aggressive behavior of this tumor (Falzone L et al, 2016). These data obtained on MMP9 prompted us to investigate the role of DNA methylation in the development and progression of cutaneous melanoma. Therefore, through the adoption of both *in silico* and *in vitro* evaluations the pathogenetic role of DNA methylation was investigated in cutaneous melanoma.

Recently, different bioinformatics and computational tools have been developed to fasten the analysis of omics data collected on public databases. Through the use of these tools, it is possible to predict the global methylation status in specific pathologies and to identify potential biomarkers or altered genes responsible for the development and progression of tumors.

Starting from these observations and our preliminary studies on DNA methylation and cutaneous melanoma, the aim of the present study was to evaluate the global DNA methylation status in cutaneous melanoma in order to identify DNA methylation hotspots potentially involved in the development and progression of this tumor. For this purpose, a bioinformatics analysis was first performed using the bioinformatics tool EpiMethEx, an R-package developed by the Experimental Oncology laboratory as part of the research project of the Ph.D. program (Candido S et al, 2019). In particular, EpiMethEx was used to evaluate the DNA methylation and gene expression data contained in the TCGA SKCM and GTEx databases in order to identify genes whose methylation status correlated positively or negatively with gene expression. The EpiMethEx analysis revealed 517 genes whose methylation status significantly alter gene expression. Subsequently, a gene ontology analysis performed by using the prediction tool DAVID revealed that among these 517 methylated genes 40 belong to the class of transcription factors. Of these 40 transcription factors, particular attention was paid to the study of DNA methylation phenomena affecting genes involved in melanocyte differentiation and epithelial to mesenchymal transition. Overall, the bioinformatics analyses allowed the selection of 15 different transcription factors strongly involved in embryonic morphogenesis and differentiation and in the development of the neural tube suggesting how these factors may play a significant role in the differentiation of melanocytes and in the development of melanomas as well as in the tumor progression and the underlying mechanisms responsible for the migration of melanoma cells and the formation of metastases. The in-depth analysis of the methylation and expression levels of these 15 transcriptions factors revealed as RARB and ISL1 represent excellent examples of genes whose, respectively, promoter methylation is negatively correlated with gene expression and intragenic methylation is positively correlated with gene

expression. Therefore, these two genes were selected for the further *in vitro* and clinical validation analyses performed on melanoma cell lines and FFPE samples, respectively.

As regards RARB and ISL1, these genes appeared dysregulated in melanoma taking into account different clinical-pathological features of patients. Of note, RARB is involved in the regulation of retinoic acid (RA) function. Several studies have demonstrated that (RA) is a key factor involved in embryogenesis and organogenesis through the regulation the expression levels of different tissue-specific genes. RARB is one of the nuclear receptors able to bind RA. In particular, RA can bind different nuclear receptors, RAR receptors α, β and γ, which are involved in the regulation of the expression levels of different genes (Chambon P, 1996; Mangelsdorf DJ et al, 1991). It has been demonstrated that RA-signaling is actively involved in the regulatory mechanisms of neural crest cells migration during primary neural development. In addition, retinoic acid has negative effects on the secretion and expression of MMPs (Nguyen J et al, 2006). These data suggest how RA and RARB have a strong anti-tumor effect, therefore methylation phenomena affecting RARB promoter could lead to gene silencing and consequently promote tumor development or aggression (Xu XC, 2007). In this context, some studies have demonstrated that RARB is highly expressed in melanocytes of the skin and methylation phenomena at the promoter level could alter its expression levels (Boehm N et al, 2004; Fan J et al, 2010).

As regards ISL1, recently some studies have evaluated its involvement in the migration process of cancer cells (Li M et al, 2021). The insulin gene enhancer protein (ISL1) is a transcription factor particularly expressed during the formation of the neural tube. In particular, ISL1 acts as a signal molecule for primary sensory neurons in the dorsal region, the D2 class of dorsal interneurons and motor neurons in the ventral region.

In addition, ISL1 has been shown to be highly expressed in a number of malignant tumors promoting cell proliferation and EMT transformation through the PI3K/AKT pathway and the upregulating Aurora kinase A protein (Li M et al, 2021). Another study demonstrated that ISL1 positively regulates the expression of MMP-2 and MMP-9 in A375 cells, suggesting how the over-

expression of these transcription factors in cutaneous melanoma could be considered a negative prognostic factor (Zhu X et al, 2018).

All these data suggest that methylation phenomena affecting these two transcription factors may be involved in the de-differentiation of melanoma cells and in the migratory processes responsible for the high metastatic rates observed in melanoma patients.

Based on the functional role of RARB and ISL1 in different tumors, including cutaneous melanoma, the methylation and gene expression levels of these two factors were validated on melanoma cell lines and clinical samples obtained from both melanoma patients and healthy individuals. In particular, in order to validate the bioinformatic results obtained by EpiMethEx and establish the effective correlation between promoter methylation and down-regulation of RARB and intragenic methylation and over-expression of ISL1, the methylation levels of two RARB promoter methylation hotspots (TSS200 and EX1) and two ISL1 intragenic methylation hotspots (isl28 and isl53) were evaluated in a panel of five melanoma cell lines using the innovative method MSRE-ddPCR developed by our laboratory. In parallel, the expression levels of both RARB and ISL1 were observed by ddPCR. The results of the analysis performed on melanoma cell lines confirmed the data obtained through EpiMethEx demonstrating the negative correlation existing between RARB promoter methylation and its expression and the positive correlation existing between ISL1 intragenic methylation and its expression. These *in vitro* results ìdemonstrated the high predictive value of the analyses performed with EpiMethEx as well as the reproducibility of the results obtained by the highly sensitive MSRE-ddPCR methylation analysis protocol here developed.

Finally, the *in silico* and *in vitro* results were further confirmed on FFPE melanoma samples and normal nevi. In particular, the MSRE-ddPCR analyses performed on clinical samples demonstrated that the TSS200 and EX1 DNA methylation hotspots of RARB are effectively hyper-methylated in melanoma samples compared to benign nevi further validating the results obtained on melanoma cell lines. However, inconclusive results were obtained as regards ISL1 methylation levels as no statistical differences between the methylation

levels of isl28 and isl53 methylation hotspots were observed in melanoma samples and normal controls. These latter results suggest that the number of FFPE samples should be increased to obtain statistically significant data.

Overall, the results obtained in FFPE samples also demonstrated that the MSRE-ddPCR protocol is effective in correctly establishing DNA methylation levels also in DNA samples of poor quality as those obtained from FFPE tissues. Indeed, the DNA obtained from fixed samples is highly fragmented and often the methylation levels of this type of sample cannot be analyzed through classic MSRE protocols or bisulfite conversion of methylated cytosines.

On these bases, the results here obtained encourage the adoption of the MSRE-ddPCR protocol for the effective identification and analysis of DNA methylation hotspots in low-quality samples. In this manner, it is possible to evaluate the diagnostic potential of all the DNA methylation hotspots identified through EpiMethEx this identifying novel potential biomarkers for the management of cutaneous melanoma. However, further validation experiments performed on a wide cohort of melanoma patients and normal individuals are mandatory to confirm these preliminary results.

6. CONCLUSIONS

The computational and molecular results obtained in the present study confirm the high predictive value of the analysis performed by EpiMethEx and the reproducibility of the results obtained with the innovative MSRE-ddPCR methylation assay here developed.

In particular, here it was demonstrated that methylation phenomena occurring in promoter or intragenic regions are significantly correlated with gene silencing and over-expression, respectively. Therefore, methylation phenomena occurring in melanoma cells could be responsible for the reactivation of transcription factors involved in the de-differentiation of melanocytes and in the promotion of EMT and metastasis formation processes.

On these bases, the analysis of DNA methylation levels in individuals at risk for the development of melanoma or with a diagnosis of cutaneous melanoma could be useful to predict the risk of development of this tumor and the prognosis of patients.

Of note, the preliminary results here obtained need to be further validated in a wider cohort of melanoma patients and healthy controls. In particular, the analysis of the DNA methylation status of RARB and ISL1, as well as of other transcription factors here identified, should be evaluated in a higher number of FFPE samples and in liquid biopsy samples of individuals at risk for this tumor as well by using the MSRE-ddPCR here developed. In this way, the diagnostic and prognostic role of DNA methylation hotspots will be further clarified. In addition, besides the evaluation of the methylation levels of the hotspots here identified it is important to also evaluate the expression levels of the transcription factors in order to establish their diagnostic and prognostic significance in cutaneous melanoma patients.

In conclusion, the results here obtained pave the way for the development of new strategies for the identification of diagnostic and prognostic epigenetic biomarkers for cutaneous melanoma.

7. REFERENCES

Abbas Q, Emre Celebi M, Garcia IF, Ahmad W. Melanoma recognition framework based on expert definition of ABCD for dermoscopic images. Skin Res Technol. 2013 Feb;19(1):e93-102. doi: 10.1111/j.1600-0846.2012.00614.x.

Aitken JF, Youlden DR, Baade PD, Soyer HP, Green AC, Smithers BM. Generational shift in melanoma incidence and mortality in Queensland, Australia, 1995-2014. Int J Cancer. 2018 Apr 15;142(8):1528-1535. doi: 10.1002/ijc.31141.

Akhavan-Niaki H, Samadani AA. DNA methylation and cancer development: molecular mechanism. Cell Biochem Biophys. 2013 Nov;67(2):501-13. doi: 10.1007/s12013-013-9555-2.

Alendar T, Kittler H. Morphologic characteristics of nevi associated with melanoma: a clinical, dermatoscopic and histopathologic analysis. Dermatol Pract Concept. 2018 Apr 30;8(2):104-108. doi: 10.5826/dpc.0802a07.

Antequera F, Bird A. CpG Islands: A Historical Perspective. Methods Mol Biol. 2018;1766:3-13. doi: 10.1007/978-1-4939-7768-0_1.

Ascha M, Ascha MS, Tanenbaum J, Bordeaux JS. Risk Factors for Melanoma in Renal Transplant Recipients. JAMA Dermatol. 2017 Nov 1;153(11):1130-1136. doi: 10.1001/jamadermatol.2017.2291.

Ascierto PA, Kirkwood JM, Grob JJ, Simeone E, Grimaldi AM, Maio M, Palmieri G, Testori A, Marincola FM, Mozzillo N. The role of BRAF V600 mutation in melanoma. J Transl Med. 2012 Jul 9;10:85. doi: 10.1186/1479-5876-10-85.

Audia JE, Campbell RM. Histone Modifications and Cancer. Cold Spring Harb Perspect Biol. 2016 Apr 1;8(4):a019521. doi: 10.1101/cshperspect.a019521.

Azevedo H, Pessoa GC, de Luna Vitorino FN, Nsengimana J, Newton-Bishop J, Reis EM, da Cunha JPC, Jasiulionis MG. Gene co-expression and histone modification signatures are associated with melanoma progression, epithelial-to-mesenchymal transition, and metastasis. Clin Epigenetics. 2020 Aug 24;12(1):127. doi: 10.1186/s13148-020-00910-9.

Azoury SC, Lange JR. Epidemiology, risk factors, prevention, and early detection of melanoma. Surg Clin North Am. 2014 Oct;94(5):945-62, vii. doi: 10.1016/j.suc.2014.07.013.

Bailey CM, Morrison JA, Kulesa PM. Melanoma revives an embryonic migration program to promote plasticity and invasion. Pigment Cell Melanoma Res. 2012 Sep;25(5):573-83. doi: 10.1111/j.1755-148X.2012.01025.x.

Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009 Jan 23;136(2):215-33. doi: 10.1016/j.cell.2009.01.002.

Behrens CL, Thorgaard C, Philip A, Bentzen J. Sunburn in children and adolescents: associations with parents' behaviour and attitudes. Scand J Public Health. 2013 May;41(3):302-10. doi: 10.1177/1403494813476158.

Bertolotto C, Lesueur F, Giuliano S, Strub T, de Lichy M, Bille K, Dessen P, d'Hayer B, Mohamdi H, Remenieras A, Maubec E, de la Fouchardière A, Molinié V, Vabres P, Dalle S, Poulalhon N, Martin-Denavit T, Thomas L, Andry-Benzaquen P, et al. A SUMOylation-defective MITF germline mutation predisposes to melanoma and renal carcinoma. Nature. 2011 Oct 19;480(7375):94-8. doi: 10.1038/nature10539.

Bertolotto C. Melanoma: from melanocyte to genetic alterations and clinical options. Scientifica (Cairo). 2013;2013:635203. doi: 10.1155/2013/635203.

Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002 Jan 1;16(1):6-21. doi: 10.1101/gad.947102.

Boehm N, Samama B, Cribier B, Rochette-Egly C. Retinoic-acid receptor beta expression in melanocytes. Eur J Dermatol. 2004 Jan-Feb;14(1):19-23.

Boniol M, Autier P, Boyle P, Gandini S. Cutaneous melanoma attributable to sunbed use: systematic review and meta-analysis. BMJ. 2012 Jul 24;345:e4757. doi: 10.1136/bmj.e4757.

Bourneuf E. The MeLiM Minipig: An Original Spontaneous Model to Explore Cutaneous Melanoma Genetic Basis. Front Genet. 2017 Oct 13;8:146. doi: 10.3389/fgene.2017.00146.

Braeuer RR, Watson IR, Wu CJ, Mobley AK, Kamiya T, Shoshan E, Bar-Eli M. Why is melanoma so metastatic? Pigment Cell Melanoma Res. 2014 Jan;27(1):19-36. doi: 10.1111/pcmr.12172.

Breslow A. Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. Ann Surg. 1970 Nov;172(5):902-8. doi: 10.1097/00000658-197011000-00017.

Bronner ME, LeDouarin NM. Development and evolution of the neural crest: an overview. Dev Biol. 2012 Jun 1;366(1):2-9. doi: 10.1016/j.ydbio.2011.12.042.

Bruno W, Pastorino L, Ghiorzo P, Andreotti V, Martinuzzi C, Menin C, Elefanti L, Stagni C, Vecchiato A, Rodolfo M, Maurichi A, Manoukian S, De Giorgi V, Savarese I, Gensini F, Borgognoni L, et al. Multiple primary melanomas (MPMs) and criteria for genetic assessment: MultiMEL, a multicenter study of the Italian Melanoma Intergroup. J Am Acad Dermatol. 2016 Feb;74(2):325-32. doi: 10.1016/j.jaad.2015.09.053.

Cabrita R, Mitra S, Sanna A, Ekedahl H, Lövgren K, Olsson H, Ingvar C, Isaksson K, Lauss M, Carneiro A, Jönsson G. The Role of PTEN Loss in Immune Escape, Melanoma Prognosis and Therapy Response. Cancers (Basel). 2020 Mar 21;12(3):742. doi: 10.3390/cancers12030742.

Cadet J, Douki T, Ravanat JL. Oxidatively generated damage to cellular DNA by UVB and UVA radiation. Photochem Photobiol. 2015 Jan-Feb;91(1):140-55. doi: 10.1111/php.12368.

Caini S, Gandini S, Sera F, Raimondi S, Fargnoli MC, Boniol M, Armstrong BK. Meta-analysis of risk factors for cutaneous melanoma according to anatomical site and

clinico-pathological variant. Eur J Cancer. 2009 Nov;45(17):3054-63. doi: 10.1016/j.ejca.2009.05.009.

Cameron F, Whiteside G, Perry C. Ipilimumab: first global approval. Drugs. 2011 May 28;71(8):1093-104. doi: 10.2165/11594010-000000000-00000.

Campagnari M, Jafelicci AS, Carneiro HA, Brechtbühl ER, Bertolli E, Duprat Neto JP. Dermal Substitutes Use in Reconstructive Surgery for Skin Tumors: A Single-Center Experience. Int J Surg Oncol. 2017;2017:9805980. doi: 10.1155/2017/9805980.

Candido S, Rapisarda V, Marconi A, Malaponte G, Bevelacqua V, Gangemi P, Scalisi A, McCubrey JA, Maestro R, Spandidos DA, Fenga C, Libra M. Analysis of the B-RafV600E mutation in cutaneous melanoma patients with occupational sun exposure. Oncol Rep. 2014 Mar;31(3):1079-82. doi: 10.3892/or.2014.2977.

Carrera C, Gual A, Díaz A, Puig-Butillé JA, Noguès S, Vilalta A, Conill C, Rull R, Vilana R, Arguis P, Vidal-Sicart S, Alós L, Palou J, Castel T, Malvehy J, Puig S. Prognostic role of the histological subtype of melanoma on the hands and feet in Caucasians. Melanoma Res. 2017 Aug;27(4):315-320. doi: 10.1097/CMR.0000000000000340.

Chambon P. A decade of molecular biology of retinoic acid receptors. FASEB J. 1996 Jul;10(9):940-54.

Chandler VL. Paramutation: from maize to mice. Cell. 2007 Feb 23;128(4):641-5. doi: 10.1016/j.cell.2007.02.007.

Chicas-Sett R, Morales-Orue I, Rodriguez-Abreu D, Lara-Jimenez P. Combining radiotherapy and ipilimumab induces clinically relevant radiation-induced abscopal effects in metastatic melanoma patients: A systematic review. Clin Transl Radiat Oncol. 2017 Dec 23;9:5-11. doi: 10.1016/j.ctro.2017.12.004.

Choi YS, Fisher DE. UV and melanoma: the TP53 link. Cell Res. 2014 Oct;24(10):1157-8. doi: 10.1038/cr.2014.95.

Christofi T, Baritaki S, Falzone L, Libra M, Zaravinos A. Current Perspectives in Cancer Immunotherapy. Cancers (Basel). 2019 Sep 30;11(10):1472. doi: 10.3390/cancers11101472.

Ciarletta P, Foret L, Ben Amar M. The radial growth phase of malignant melanoma: multi-phase modelling, numerical simulations and linear stability analysis. J R Soc Interface. 2011 Mar 6;8(56):345-68. doi: 10.1098/rsif.2010.0285.

Ciarrocchi A, Pietroletti R, Carlei F, Amicucci G. Extensive surgery and lymphadenectomy do not improve survival in primary melanoma of the anorectum: results from analysis of a large database (SEER). Colorectal Dis. 2017 Feb;19(2):158-164. doi: 10.1111/codi.13412.

Costa FF. Non-coding RNAs: lost in translation? Gene. 2007 Jan 15;386(1-2):1-10. doi: 10.1016/j.gene.2006.09.028.

Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. FASEB J. 2007 Apr;21(4):976-94. doi: 10.1096/fj.06-6649rev.

Cui W, Cai Y, Zhou X. Advances in subunits of PI3K class I in cancer. Pathology. 2014 Apr;46(3):169-76. doi: 10.1097/PAT.0000000000000066.

Curchin DJ, Harris VR, McCormack CJ, Smith SD. Changing trends in the incidence of invasive melanoma in Victoria, 1985-2015. Med J Aust. 2018 Apr 2;208(6):265-269. doi: 10.5694/mja17.00725.

Curiel-Lewandrowski C, Speetzen LS, Cranmer L, Warneke JA, Loescher LJ. Multiple primary cutaneous melanomas in Li-Fraumeni syndrome. Arch Dermatol. 2011 Feb;147(2):248-50. doi: 10.1001/archdermatol.2010.428.

Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KH, Aiba S, Bröcker EB, LeBoit PE, Pinkel D, Bastian BC. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005 Nov 17;353(20):2135-47. doi: 10.1056/NEJMoa050092.

Davies JR, Randerson-Moor J, Kukalizch K, Harland M, Kumar R, Madhusudan S, Nagore E, Hansson J, Höiom V, Ghiorzo P, Gruis NA, Kanetsky PA, Wendt J, Pjanova D, Puig S, Saiag P, et al. Inherited variants in the MC1R gene and survival from cutaneous melanoma: a BioGenoMEL study. Pigment Cell Melanoma Res. 2012 May;25(3):384-94. doi: 10.1111/j.1755-148X.2012.00982.x.

Davis NM, Sokolosky M, Stadelman K, Abrams SL, Libra M, Candido S, Nicoletti F, Polesel J, Maestro R, D'Assoro A, Drobot L, Rakus D, et al. Deregulation of the EGFR/PI3K/PTEN/Akt/mTORC1 pathway in breast cancer: possibilities for therapeutic intervention. Oncotarget. 2014 Jul 15;5(13):4603-50. doi: 10.18632/oncotarget.2209.

de Polo A, Luo Z, Gerarduzzi C, Chen X, Little JB, Yuan ZM. AXL receptor signalling suppresses p53 in melanoma through stabilization of the MDMX-MDM2 complex. J Mol Cell Biol. 2017 Apr 1;9(2):154-165. doi: 10.1093/jmcb/mjw045.

De Simone P, Valiante M, Silipo V. Familial melanoma and multiple primary melanoma. G Ital Dermatol Venereol. 2017 Jun;152(3):262-265. doi: 10.23736/S0392-0488.17.05554-7.

Deken MA, Gadiot J, Jordanova ES, Lacroix R, van Gool M, Kroon P, Pineda C, Geukes Foppen MH, Scolyer R, Song JY, Verbrugge I, Hoeller C, Dummer R, Haanen JB, Long GV, Blank CU. Targeting the MAPK and PI3K pathways in combination with PD1 blockade in melanoma. Oncoimmunology. 2016 Oct 14;5(12):e1238557. doi: 10.1080/2162402X.2016.1238557.

Demenais F, Mohamdi H, Chaudru V, Goldstein AM, Newton Bishop JA, Bishop DT, Kanetsky PA, Hayward NK, Gillanders E, Elder DE, Avril MF, Azizi E, van Belle P, Bergman W, Bianchi-Scarrà G, et al. Association of MC1R variants and host phenotypes with melanoma risk in CDKN2A mutation carriers: a GenoMEL study. J Natl Cancer Inst. 2010 Oct 20;102(20):1568-83. doi: 10.1093/jnci/djq363.

Dhomen N, Marais R. New insight into BRAF mutations in cancer. Curr Opin Genet Dev. 2007 Feb;17(1):31-9. doi: 10.1016/j.gde.2006.12.005.

Edwards RH, Ward MR, Wu H, Medina CA, Brose MS, Volpe P, Nussen-Lee S, Haupt HM, Martin AM, Herlyn M, Lessin SR, Weber BL. Absence of BRAF mutations in UV-protected mucosal melanomas. J Med Genet. 2004 Apr;41(4):270-2. doi: 10.1136/jmg.2003.016667.

Elwood JM, Lee JA. Trends in mortality from primary tumours of skin in Canada. Can Med Assoc J. 1974 Apr 20;110(8):913-5.

Emri G, Paragh G, Tósaki Á, Janka E, Kollár S, Hegedűs C, Gellén E, Horkay I, Koncz G, Remenyik É. Ultraviolet radiation-mediated development of cutaneous melanoma: An update. J Photochem Photobiol B. 2018 Aug;185:169-175. doi: 10.1016/j.jphotobiol.2018.06.005.

Erkurt MA, Aydogdu I, Kuku I, Kaya E, Basaran Y. Nodular melanoma presenting with rapid progression and widespread metastases: a case report. J Med Case Rep. 2009 Feb 6;3:50. doi: 10.1186/1752-1947-3-50.

Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet. 2007 Apr;8(4):286-98. doi: 10.1038/nrg2005.

Fabbri M, Garzon R, Cimmino A, Liu Z, Zanesi N, Callegari E, Liu S, Alder H, Costinean S, Fernandez-Cymering C, Volinia S, Guler G, Morrison CD, Chan KK, Marcucci G, Calin GA, Huebner K, Croce CM. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. Proc Natl Acad Sci U S A. 2007 Oct 2;104(40):15805-10. doi: 10.1073/pnas.0707628104.

Fajuyigbe D, Young AR. The impact of skin colour on human photobiological responses. Pigment Cell Melanoma Res. 2016 Nov;29(6):607-618. doi: 10.1111/pcmr.12511.

Falzone L, Grimaldi M, Celentano E, Augustin LSA, Libra M. Identification of Modulated MicroRNAs Associated with Breast Cancer, Diet, and Physical Activity. Cancers (Basel). 2020 Sep 8;12(9):2555. doi: 10.3390/cancers12092555.

Falzone L, Lupo G, La Rosa GRM, Crimi S, Anfuso CD, Salemi R, Rapisarda E, Libra M, Candido S. Identification of Novel MicroRNAs and Their Diagnostic and Prognostic Significance in Oral Cancer. Cancers (Basel). 2019 Apr 30;11(5):610. doi: 10.3390/cancers11050610.

Falzone L, Salemi R, Travali S, Scalisi A, McCubrey JA, Candido S, Libra M. MMP-9 overexpression is associated with intragenic hypermethylation of MMP9 gene in melanoma. Aging (Albany NY). 2016 May;8(5):933-44. doi: 10.18632/aging.100951.

Falzone L, Salomone S, Libra M. Evolution of Cancer Pharmacological Treatments at the Turn of the Third Millennium. Front Pharmacol. 2018 Nov 13;9:1300. doi: 10.3389/fphar.2018.01300.

Fan J, Eastham L, Varney ME, Hall A, Adkins NL, Chetel L, Sollars VE, Georgel P, Niles RM. Silencing and re-expression of retinoic acid receptor beta2 in human melanoma. Pigment Cell Melanoma Res. 2010 Jun;23(3):419-29. doi:

10.1111/j.1755-148X.2010.00702.x. Epub 2010 Mar 29. Erratum in: Pigment Cell Melanoma Res. 2010 Oct;23(5):722. Chetel, Lora [added].

Fang S, Wang Y, Dang Y, Gagel A, Ross MI, Gershenwald JE, Cormier JN, Wargo J, Haydu LE, Davies MA, McQuade JL, Sui D, Bassett RL, Reveille JD, Wei Q, Amos CI, Lee JE. Association between Body Mass Index, C-Reactive Protein Levels, and Melanoma Patient Outcomes. J Invest Dermatol. 2017 Aug;137(8):1792-1795. doi: 10.1016/j.jid.2017.04.007. Epub 2017 Apr 23. PMID: 28442307.

Fang WL, Huang KH, Lan YT, Lin CH, Chang SC, Chen MH, Chao Y, Lin WC, Lo SS, Li AF, Wu CW, Chiou SH, Shyr YM. Mutations in PI3K/AKT pathway genes and amplifications of PIK3CA are associated with patterns of recurrence in gastric cancers. Oncotarget. 2016 Feb 2;7(5):6201-20. doi: 10.18632/oncotarget.6641.

Fattouh K, Ducroux E, Decullier E, Kanitakis J, Morelon E, Boissonnat P, Sebbag L, Jullien D, Euvrard S. Increasing incidence of melanoma after solid organ transplantation: a retrospective epidemiological study. Transpl Int. 2017 Nov;30(11):1172-1180. doi: 10.1111/tri.13011.

Fechete O, Ungureanu L, Șenilă S, Vornicescu D, Dănescu S, Vasilovici A, Candrea E, Vesa ȘC, Cosgarea R. Risk factors for melanoma and skin health behaviour: An analysis on Romanian melanoma patients. Oncol Lett. 2019 May;17(5):4139-4144. doi: 10.3892/ol.2018.9737.

Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015 Mar 1;136(5):E359-86. doi: 10.1002/ijc.29210.

Flynn M, Pickering L, Larkin J, Turajlic S. Immune-checkpoint inhibitors in melanoma and kidney cancer: from sequencing to rational selection. Ther Adv Med Oncol. 2018 Jun 12;10:1758835918777427. doi: 10.1177/1758835918777427.

Fowles JS, Denton CL, Gustafson DL. Comparative analysis of MAPK and PI3K/AKT pathway activation and inhibition in human and canine melanoma. Vet Comp Oncol. 2015 Sep;13(3):288-304. doi: 10.1111/vco.12044.

Friedman JM, Liang G, Liu CC, Wolff EM, Tsai YC, Ye W, Zhou X, Jones PA. The putative tumor suppressor microRNA-101 modulates the cancer epigenome by repressing the polycomb group protein EZH2. Cancer Res. 2009 Mar 15;69(6):2623-9. doi: 10.1158/0008-5472.CAN-08-3114.

Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafrè SA, Farace MG. miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. J Biol Chem. 2007 Aug 10;282(32):23716-24. doi: 10.1074/jbc.M701805200.

Garg M. Epithelial-mesenchymal transition - activating transcription factors - multifunctional regulators in cancer. World J Stem Cells. 2013 Oct 26;5(4):188-95. doi: 10.4252/wjsc.v5.i4.188.

Gevaert O. MethylMix: an R package for identifying DNA methylation-driven genes. Bioinformatics. 2015 Jun 1;31(11):1839-41. doi: 10.1093/bioinformatics/btv020.

Giambò F, Leone GM, Gattuso G, Rizzo R, Cosentino A, Cinà D, Teodoro M, Costa C, Tsatsakis A, Fenga C, Falzone L. Genetic and Epigenetic Alterations Induced by Pesticide Exposure: Integrated Analysis of Gene Expression, microRNA Expression, and DNA Methylation Datasets. Int J Environ Res Public Health. 2021 Aug 17;18(16):8697. doi: 10.3390/ijerph18168697.

Giunta EF, Arrichiello G, Curvietto M, Pappalardo A, Bosso D, Rosanova M, Diana A, Giordano P, Petrillo A, Federico P, Fabozzi T, Parola S, Riccio V, Mucci B, Vanella V, Festino L, Daniele B, Ascierto PA, Ottaviano M, On Behalf Of Scito Youth. Epigenetic Regulation in Melanoma: Facts and Hopes. Cells. 2021 Aug 11;10(8):2048. doi: 10.3390/cells10082048.

Glazer AM, Rigel DS, Winkelmann RR, Farberg AS. Clinical Diagnosis of Skin Cancer: Enhancing Inspection and Early Recognition. Dermatol Clin. 2017 Oct;35(4):409-416. doi: 10.1016/j.det.2017.06.001.

Goldstein AM, Tucker MA. Dysplastic nevi and melanoma. Cancer Epidemiol Biomarkers Prev. 2013 Apr;22(4):528-32. doi: 10.1158/1055-9965.EPI-12-1346.

Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. Annu Rev Biochem. 2005;74:481-514. doi: 10.1146/annurev.biochem.74.010904.153721.

Gomez-Lira M, Ferronato S, Orlandi E, Dal Molin A, Malerba G, Frigerio S, Rodolfo M, Romanelli MG. Association of microRNA 146a polymorphism rs2910164 and the risk of melanoma in an Italian population. Exp Dermatol. 2015 Oct;24(10):794-5. doi: 10.1111/exd.12778.

Gong F, Guo Y, Niu Y, Jin J, Zhang X, Shi X, Zhang L, Li R, Chen L, Ma RZ. Epigenetic silencing of TET2 and TET3 induces an EMT-like process in melanoma. Oncotarget. 2017 Jan 3;8(1):315-328. doi: 10.18632/oncotarget.13324.

Green AC, Viros A, Hughes MCB, Gaudy-Marqueste C, Akhras V, Cook MG, Marais R. Nodular Melanoma: A Histopathologic Entity? Acta Derm Venereol. 2018 Apr 16;98(4):460-462. doi: 10.2340/00015555-2855.

Greenwald HS, Friedman EB, Osman I. Superficial spreading and nodular melanoma are distinct biological entities: a challenge to the linear progression model. Melanoma Res. 2012 Feb;22(1):1-8. doi: 10.1097/CMR.0b013e32834e6aa0.

Grigore M, Furtunescu F, Minca D, Costache M, Garbe C, Simionescu O. The iris signal: blue periphery, tan collaret and freckles pattern - strong indicators for epidermal skin cancer in South-Eastern Europe. J Eur Acad Dermatol Venereol. 2018 Oct;32(10):1662-1667. doi: 10.1111/jdv.14929.

Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet. 2009 Jan;10(1):32-42. doi: 10.1038/nrg2485.

Hafsi S, Pezzino FM, Candido S, Ligresti G, Spandidos DA, Soua Z, McCubrey JA, Travali S, Libra M. Gene alterations in the PI3K/PTEN/AKT pathway as a

mechanism of drug-resistance (review). Int J Oncol. 2012 Mar;40(3):639-44. doi: 10.3892/ijo.2011.1312.

Hayes AJ, Larkin J. BMI and outcomes in melanoma: more evidence for the obesity paradox. Lancet Oncol. 2018 Mar;19(3):269-270. doi: 10.1016/S1470-2045(18)30077-9.

He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004 Jul;5(7):522-31. doi: 10.1038/nrg1379. Erratum in: Nat Rev Genet. 2004 Aug;5(8):631. PMID: 15211354.

Hodis E, Watson IR, Kryukov GV, Arold ST, Imielinski M, Theurillat JP, Nickerson E, Auclair D, Li L, Place C, Dicara D, Ramos AH, Lawrence MS, Cibulskis K, Sivachenko A, Voet D, Saksena G, Stransky N, et al. A landscape of driver mutations in melanoma. Cell. 2012 Jul 20;150(2):251-63. doi: 10.1016/j.cell.2012.06.024.

İyidal AY, Gül Ü, Kılıç A. Number and size of acquired melanocytic nevi and affecting risk factors in cases admitted to the dermatology clinic. Postepy Dermatol Alergol. 2016 Oct;33(5):375-380. doi: 10.5114/ada.2016.62845.

Jackett LA, Scolyer RA. A Review of Key Biological and Molecular Events Underpinning Transformation of Melanocytes to Primary and Metastatic Melanoma. Cancers (Basel). 2019 Dec 17;11(12):2041. doi: 10.3390/cancers11122041.

Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet. 2003 Mar;33 Suppl:245-54. doi: 10.1038/ng1089.

Jensen DE, Proctor M, Marquis ST, Gardner HP, Ha SI, Chodosh LA, Ishov AM, Tommerup N, Vissing H, Sekido Y, Minna J, Borodovsky A, Schultz DC, Wilkinson KD, Maul GG, Barlev N, Berger SL, Prendergast GC, Rauscher FJ 3rd. BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. Oncogene. 1998 Mar 5;16(9):1097-112.

Jiang C, Pugh BF. Nucleosome positioning and gene regulation: advances through genomics. Nat Rev Genet. 2009 Mar;10(3):161-72. doi: 10.1038/nrg2522.

Jiao Y, Widschwendter M, Teschendorff AE. A systems-level integrative framework for genome-wide DNA methylation and gene expression data identifies differential gene expression modules under epigenetic control. Bioinformatics. 2014 Aug 15;30(16):2360-6. doi: 10.1093/bioinformatics/btu316.

Jin L, Hu WL, Jiang CC, Wang JX, Han CC, Chu P, Zhang LJ, Thorne RF, Wilmott J, Scolyer RA, Hersey P, Zhang XD, Wu M. MicroRNA-149*, a p53-responsive microRNA, functions as an oncogenic regulator in human melanoma. Proc Natl Acad Sci U S A. 2011 Sep 20;108(38):15840-5. doi: 10.1073/pnas.1019312108.

Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nat Rev Genet. 2002 Jun;3(6):415-28. doi: 10.1038/nrg816.

Kagohara LT, Stein-O'Brien GL, Kelley D, Flam E, Wick HC, Danilova LV, Easwaran H, Favorov AV, Qian J, Gaykalova DA, Fertig EJ. Epigenetic regulation of gene

expression in cancer: techniques, resources and analysis. Brief Funct Genomics. 2018 Jan 1;17(1):49-63. doi: 10.1093/bfgp/elx018.

Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, Sasaki H. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. Nature. 2004 Jun 24;429(6994):900-3. doi: 10.1038/nature02633.

Karim RZ, Li W, Sanki A, Colman MH, Yang YH, Thompson JF, Scolyer RA. Reduced p16 and increased cyclin D1 and pRb expression are correlated with progression in cutaneous melanocytic tumors. Int J Surg Pathol. 2009 Oct;17(5):361-7. doi: 10.1177/1066896909336177.

Kato S, Weng QY, Insco ML, Chen KY, Muralidhar S, Pozniak J, Diaz JMS, Drier Y, Nguyen N, Lo JA, van Rooijen E, Kemeny LV, Zhan Y, Feng Y, Silkworth W, Powell CT, Liau BB, Xiong Y, Jin J, Newton-Bishop J, Zon LI, Bernstein BE, Fisher DE. Gain-of-Function Genetic Alterations of G9a Drive Oncogenesis. Cancer Discov. 2020 Jul;10(7):980-997. doi: 10.1158/2159-8290.CD-19-0532.

Kawczyk-Krupka A, Bugaj AM, Latos W, Zaremba K, Sieroń A. Photodynamic therapy in treatment of cutaneous and choroidal melanoma. Photodiagnosis Photodyn Ther. 2013 Dec;10(4):503-9. doi: 10.1016/j.pdpdt.2013.05.006.

Keijzer W, Mulder MP, Langeveld JC, Smit EM, Bos JL, Bootsma D, Hoeijmakers JH. Establishment and characterization of a melanoma cell line from a xeroderma pigmentosum patient: activation of N-ras at a potential pyrimidine dimer site. Cancer Res. 1989 Mar 1;49(5):1229-35.

Keung EZ, Gershenwald JE. The eighth edition American Joint Committee on Cancer (AJCC) melanoma staging system: implications for melanoma treatment and care. Expert Rev Anticancer Ther. 2018 Aug;18(8):775-784. doi: 10.1080/14737140.2018.1489246.

Khan F, Strohl A, Allen PD, Doerr TD. Desmoplastic Melanoma of the Head and Neck: Incidence and Survival, 1992-2013. Otolaryngol Head Neck Surg. 2017 Oct;157(4):648-656. doi: 10.1177/0194599817725696.

Koga Y, Pelizzola M, Cheng E, Krauthammer M, Sznol M, Ariyan S, Narayan D, Molinaro AM, Halaban R, Weissman SM. Genome-wide screen of promoter methylation identifies novel markers in melanoma. Genome Res. 2009 Aug;19(8):1462-70. doi: 10.1101/gr.091447.109.

Kosary CL, Altekruse SF, Ruhl J, Lee R, Dickie L. Clinical and prognostic factors for melanoma of the skin using SEER registries: collaborative stage data collection system, version 1 and version 2. Cancer. 2014 Dec 1;120 Suppl 23:3807-14. doi: 10.1002/cncr.29050.

Kulesa PM, Gammill LS. Neural crest migration: patterns, phases and signals. Dev Biol. 2010 Aug 15;344(2):566-8. doi: 10.1016/j.ydbio.2010.05.005.

Kulichová D, Dáňová J, Kunte C, Ruzicka T, Celko AM. Risk factors for malignant melanoma and preventive methods. Cutis. 2014 Nov;94(5):241-8.

Lambert AW, Pattabiraman DR, Weinberg RA. Emerging Biological Principles of Metastasis. Cell. 2017 Feb 9;168(4):670-691. doi: 10.1016/j.cell.2016.11.037.

Lancaster HO. Some geographical aspects of the mortality from melanoma in Europeans. Med J Aust. 1956 Jun 30;43(26):1082-7.

Larkin J, Hodi FS, Wolchok JD. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. N Engl J Med. 2015 Sep 24;373(13):1270-1. doi: 10.1056/NEJMc1509660. Erratum in: N Engl J Med. 2018 Nov 29;379(22):2185. PMID: 26398076.

Laughery MF, Brown AJ, Bohm KA, Sivapragasam S, Morris HS, Tchmola M, Washington AD, Mitchell D, Mather S, Malc EP, Mieczkowski PA, Roberts SA, Wyrick JJ. Atypical UV Photoproducts Induce Non-canonical Mutation Classes Associated with Driver Mutations in Melanoma. Cell Rep. 2020 Nov 17;33(7):108401. doi: 10.1016/j.celrep.2020.108401.

Leonardi GC, Candido S, Falzone L, Spandidos DA, Libra M. Cutaneous melanoma and the immunotherapy revolution (Review). Int J Oncol. 2020 Sep;57(3):609-618. doi: 10.3892/ijo.2020.5088.

Leonardi GC, Falzone L, Salemi R, Zanghì A, Spandidos DA, Mccubrey JA, Candido S, Libra M. Cutaneous melanoma: From pathogenesis to therapy (Review). Int J Oncol. 2018 Apr;52(4):1071-1080. doi: 10.3892/ijo.2018.4287.

Leong HS, Chambers AF, Lewis JD. Assessing cancer cell migration and metastatic growth in vivo in the chick embryo using fluorescence intravital imaging. Methods Mol Biol. 2012;872:1-14. doi: 10.1007/978-1-61779-797-2_1.

Levenson VV. DNA methylation as a universal biomarker. Expert Rev Mol Diagn. 2010 May;10(4):481-8. doi: 10.1586/erm.10.17.

Lewis JM, Truong TN, Schwartz MA. Integrins regulate the apoptotic response to DNA damage through modulation of p53. Proc Natl Acad Sci U S A. 2002 Mar 19;99(6):3627-32. doi: 10.1073/pnas.062698499.

Li FZ, Dhillon AS, Anderson RL, McArthur G, Ferrao PT. Phenotype switching in melanoma: implications for progression and therapy. Front Oncol. 2015 Feb 13;5:31. doi: 10.3389/fonc.2015.00031.

Li L, Sun F, Chen X, Zhang M. ISL1 is upregulated in breast cancer and promotes cell proliferation, invasion, and angiogenesis. Onco Targets Ther. 2018 Feb 14;11:781-789. doi: 10.2147/OTT.S144241.

Li M, Sun C, Bu X, Que Y, Zhang L, Zhang Y, Zhang L, Lu S, Huang J, Zhu J, Wang J, Sun F, Zhang Y. ISL1 promoted tumorigenesis and EMT via Aurora kinase A-induced activation of PI3K/AKT signaling pathway in neuroblastoma. Cell Death Dis. 2021 Jun 15;12(6):620. doi: 10.1038/s41419-021-03894-3.

Li S, Zhang J, Huang S, He X. Genome-wide analysis reveals that exon methylation facilitates its selective usage in the human transcriptome. Brief Bioinform. 2018 Sep 28;19(5):754-764. doi: 10.1093/bib/bbx019.

Lin JC, Jeong S, Liang G, Takai D, Fatemi M, Tsai YC, Egger G, Gal-Yam EN, Jones PA. Role of nucleosomal occupancy in the epigenetic silencing of the MLH1 CpG island. Cancer Cell. 2007 Nov;12(5):432-44. doi: 10.1016/j.ccr.2007.10.014.

Lin YT, Wu KJ. Epigenetic regulation of epithelial-mesenchymal transition: focusing on hypoxia and TGF-β signaling. J Biomed Sci. 2020 Mar 2;27(1):39. doi: 10.1186/s12929-020-00632-3.

Liu S, Tetzlaff MT, Cui R, Xu X. miR-200c inhibits melanoma progression and drug resistance through down-regulation of BMI-1. Am J Pathol. 2012 Nov;181(5):1823-35. doi: 10.1016/j.ajpath.2012.07.009.

Liu-Smith F, Farhat AM, Arce A, Ziogas A, Taylor T, Wang Z, Yourk V, Liu J, Wu J, McEligot AJ, Anton-Culver H, Meyskens FL. Sex differences in the association of cutaneous melanoma incidence rates and geographic ultraviolet light exposure. J Am Acad Dermatol. 2017 Mar;76(3):499-505.e3. doi: 10.1016/j.jaad.2016.08.027.

Long GV, Menzies AM, Nagrial AM, Haydu LE, Hamilton AL, Mann GJ, Hughes TM, Thompson JF, Scolyer RA, Kefford RF. Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. J Clin Oncol. 2011 Apr 1;29(10):1239-46. doi: 10.1200/JCO.2010.32.4327.

Lu M, Miller P, Lu X. Restoring the tumour suppressive function of p53 as a parallel strategy in melanoma therapy. FEBS Lett. 2014 Aug 19;588(16):2616-21. doi: 10.1016/j.febslet.2014.05.008.

Lujambio A, Calin GA, Villanueva A, Ropero S, Sánchez-Céspedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, Gallagher WM, Eccles SA, Croce CM, Esteller M. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A. 2008 Sep 9;105(36):13556-61. doi: 10.1073/pnas.0803055105.

Mangelsdorf DJ, Umesono K, Kliewer SA, Borgmeyer U, Ong ES, Evans RM. A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. Cell. 1991 Aug 9;66(3):555-61. doi: 10.1016/0092-8674(81)90018-0.

Markovic SN, Erickson LA, Rao RD, Weenig RH, Pockaj BA, Bardia A, Vachon CM, Schild SE, McWilliams RR, Hand JL, Laman SD, Kottschade LA, Maples WJ, Pittelkow MR, Pulido JS, Cameron JD, Creagan ET; Melanoma Study Group of the Mayo Clinic Cancer Center. Malignant melanoma in the 21st century, part 1: epidemiology, risk factors, screening, prevention, and diagnosis. Mayo Clin Proc. 2007 Mar;82(3):364-80. doi: 10.4065/82.3.364.

Martens MC, Seebode C, Lehmann J, Emmert S. Photocarcinogenesis and Skin Cancer Prevention Strategies: An Update. Anticancer Res. 2018 Feb;38(2):1153-1158. doi: 10.21873/anticanres.12334.

Mataca E, Migaldi M, Cesinaro AM. Impact of Dermoscopy and Reflectance Confocal Microscopy on the Histopathologic Diagnosis of Lentigo Maligna/Lentigo Maligna Melanoma. Am J Dermatopathol. 2018 Dec;40(12):884-889. doi: 10.1097/DAD.0000000000001212.

Maunakea AK, Chepelev I, Zhao K. Epigenome mapping in normal and disease States. Circ Res. 2010 Aug 6;107(3):327-39. doi: 10.1161/CIRCRESAHA.110.222463.

McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Franklin RA, Montalto G, Cervello M, Libra M, Candido S, Malaponte G, Mazzarino MC, Fagone P, Nicoletti F, Bäsecke J, Mijatovic S, Maksimovic-Ivanic D, Milella M, Tafuri A, Chiarini F, Evangelisti C, Cocco L, Martelli AM. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR cascade inhibitors: how mutations can result in therapy resistance and how to overcome resistance. Oncotarget. 2012 Oct;3(10):1068-111. doi: 10.18632/oncotarget.659.

McCubrey JA, Steelman LS, Kempf CR, Chappell WH, Abrams SL, Stivala F, Malaponte G, Nicoletti F, Libra M, Bäsecke J, Maksimovic-Ivanic D, Mijatovic S, Montalto G, Cervello M, Cocco L, Martelli AM. Therapeutic resistance resulting from mutations in Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR signaling pathways. J Cell Physiol. 2011 Nov;226(11):2762-81. doi: 10.1002/jcp.22647.

McEvoy AC, Pereira MR, Reid A, Pearce R, Cowell L, Al-Ogaili Z, Khattak MA, Millward M, Meniawy TM, Gray ES, Ziman M. Monitoring melanoma recurrence with circulating tumor DNA: a proof of concept from three case studies. Oncotarget. 2019 Jan 4;10(2):113-122. doi: 10.18632/oncotarget.26451.

Mendizabal I, Zeng J, Keller TE, Yi SV. Body-hypomethylated human genes harbor extensive intragenic transcriptional activity and are prone to cancer-associated dysregulation. Nucleic Acids Res. 2017 May 5;45(8):4390-4400. doi: 10.1093/nar/gkx020.

Mercatelli N, Coppola V, Bonci D, Miele F, Costantini A, Guadagnoli M, Bonanno E, Muto G, Frajese GV, De Maria R, Spagnoli LG, Farace MG, Ciafrè SA. The inhibition of the highly expressed miR-221 and miR-222 impairs the growth of prostate carcinoma xenografts in mice. PLoS One. 2008;3(12):e4029. doi: 10.1371/journal.pone.0004029.

Meyle KD, Guldberg P. Genetic risk factors for melanoma. Hum Genet. 2009 Oct;126(4):499-510. doi: 10.1007/s00439-009-0715-9.

Miller AJ, Mihm MC Jr. Melanoma. N Engl J Med. 2006 Jul 6;355(1):51-65. doi: 10.1056/NEJMra052166.

Molina-Vila MA, de-Las-Casas CM, Bertran-Alamillo J, et al. cfDNA analysis from blood in melanoma. Annals of Translational Medicine. 2015 Nov;3(20):309. DOI: 10.3978/j.issn.2305-5839.2015.11.23.

Moore LD, Le T, Fan G. DNA methylation and its basic function. Neuropsychopharmacology. 2013 Jan;38(1):23-38. doi: 10.1038/npp.2012.112.

Muthusamy V, Duraisamy S, Bradbury CM, Hobbs C, Curley DP, Nelson B, Bosenberg M. Epigenetic silencing of novel tumor suppressors in malignant melanoma. Cancer Res. 2006 Dec 1;66(23):11187-93. doi: 10.1158/0008-5472.CAN-06-1274.

Myles ZM, Buchanan N, King JB, Singh S, White A, Wu M, Ajani U. Anatomic distribution of malignant melanoma on the non-Hispanic black patient, 1998-2007. Arch Dermatol. 2012 Jul;148(7):797-801. doi: 10.1001/archdermatol.2011.3227.

Nakamura Y, Fujisawa Y. Diagnosis and Management of Acral Lentiginous Melanoma. Curr Treat Options Oncol. 2018 Jun 27;19(8):42. doi: 10.1007/s11864-018-0560-y.

Nakano T, Xu X, Salem AMH, Shoulkamy MI, Ide H. Radiation-induced DNA-protein cross-links: Mechanisms and biological significance. Free Radic Biol Med. 2017 Jun;107:136-145. doi: 10.1016/j.freeradbiomed.2016.11.041.

Napoli S, Scuderi C, Gattuso G, Bella VD, Candido S, Basile MS, Libra M, Falzone L. Functional Roles of Matrix Metalloproteinases and Their Inhibitors in Melanoma. Cells. 2020 May 7;9(5):1151. doi: 10.3390/cells9051151.

Nguyen J, Dumont J, Bauvois B. Comparative effects of interferon-gamma and all- trans retinoic acid on secreted and surface-associated matrix metalloproteinase-9 expression of human monocytes. Cell Mol Biol (Noisy-le-grand). 2006 May 15;52(1):51-8.

Nieto MA, Huang RY, Jackson RA, Thiery JP. EMT: 2016. Cell. 2016 Jun 30;166(1):21-45. doi: 10.1016/j.cell.2016.06.028.

Njauw CN, Kim I, Piris A, Gabree M, Taylor M, Lane AM, DeAngelis MM, Gragoudas E, Duncan LM, Tsao H. Germline BAP1 inactivation is preferentially associated with metastatic ocular melanoma and cutaneous-ocular melanoma families. PLoS One. 2012;7(4):e35295. doi: 10.1371/journal.pone.0035295.

Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999 Oct 29;99(3):247-57. doi: 10.1016/s0092-8674(00)81656-6.

Palmieri G, Ombra M, Colombino M, Casula M, Sini M, Manca A, Paliogiannis P, Ascierto PA, Cossu A. Multiple Molecular Pathways in Melanomagenesis: Characterization of Therapeutic Targets. Front Oncol. 2015 Aug 10;5:183. doi: 10.3389/fonc.2015.00183.

Pastushenko I, Brisebarre A, Sifrim A, Fioramonti M, Revenco T, Boumahdi S, Van Keymeulen A, Brown D, Moers V, Lemaire S, De Clercq S, Minguijón E, Balsat C, Sokolow Y, Dubois C, De Cock F, Scozzaro S, Sopena F, Lanas A, D'Haene N, Salmon I, Marine JC, Voet T, Sotiropoulou PA, Blanpain C. Identification of the tumour transition states occurring during EMT. Nature. 2018 Apr;556(7702):463-468. doi: 10.1038/s41586-018-0040-3.

Pejkova S, Dzokic G, Tudzarova-Gjorgova S, Panov S. Molecular Biology and Genetic Mechanisms in the Progression of the Malignant Skin Melanoma. Pril (Makedon Akad Nauk Umet Odd Med Nauki). 2016 Nov 1;37(2-3):89-97. doi: 10.1515/prilozi-2016-0021.

Pergoli L, Favero C, Pfeiffer RM, Tarantini L, Calista D, Cavalleri T, Angelici L, Consonni D, Bertazzi PA, Pesatori AC, Landi MT, Bollati V. Blood DNA

methylation, nevi number, and the risk of melanoma. Melanoma Res. 2014 Oct;24(5):480-7. doi: 10.1097/CMR.0000000000000112.

Petersen B, Wulf HC, Triguero-Mas M, Philipsen PA, Thieden E, Olsen P, Heydenreich J, Dadvand P, Basagaña X, Liljendahl TS, Harrison GI, Segerbäck D, Schmalwieser AW, Young AR, Nieuwenhuijsen MJ. Sun and ski holidays improve vitamin D status, but are associated with high levels of DNA damage. J Invest Dermatol. 2014 Nov;134(11):2806-2813. doi: 10.1038/jid.2014.223.

Pham DDM, Guhan S, Tsao H. KIT and Melanoma: Biological Insights and Clinical Implications. Yonsei Med J. 2020 Jul;61(7):562-571. doi: 10.3349/ymj.2020.61.7.562.

Pinault L, Fioletov V. Sun exposure, sun protection and sunburn among Canadian adults. Health Rep. 2017 May 17;28(5):12-19.

Prendergast GC, Ziff EB. Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. Science. 1991 Jan 11;251(4990):186-9. doi: 10.1126/science.1987636. PMID: 1987636.

Rastrelli M, Tropea S, Rossi CR, Alaibac M. Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. In Vivo. 2014 Nov-Dec;28(6):1005-11.

Ravanat JL, Breton J, Douki T, Gasparutto D, Grand A, Rachidi W, Sauvaigo S. Radiation-mediated formation of complex damage to DNA: a chemical aspect overview. Br J Radiol. 2014 Mar;87(1035):20130715. doi: 10.1259/bjr.20130715.

Reichrath J, Rass K. Ultraviolet damage, DNA repair and vitamin D in nonmelanoma skin cancer and in malignant melanoma: an update. Adv Exp Med Biol. 2014;810:208-33. doi: 10.1007/978-1-4939-0437-2_12.

Retseck J, Nasr A, Lin Y, Lin H, Mendiratta P, Butterfield LH, Tarhini AA. Long term impact of CTLA4 blockade immunotherapy on regulatory and effector immune responses in patients with melanoma. J Transl Med. 2018 Jul 4;16(1):184. doi: 10.1186/s12967-018-1563-y.

Rigel DS. Epidemiology of melanoma. Semin Cutan Med Surg. 2010 Dec;29(4):204-9. doi: 10.1016/j.sder.2010.10.005.

Roche J. The Epithelial-to-Mesenchymal Transition in Cancer. Cancers (Basel). 2018 Feb 16;10(2):52. doi: 10.3390/cancers10020052. Erratum in: Cancers (Basel). 2018 Mar 19;10(3).

Rodriguez J, Frigola J, Vendrell E, Risques RA, Fraga MF, Morales C, Moreno V, Esteller M, Capellà G, Ribas M, Peinado MA. Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. Cancer Res. 2006 Sep 1;66(17):8462-9468. doi: 10.1158/0008-5472.CAN-06-0293.

Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, Vultur A, Basu D, Gimotty P, Vogt T, Herlyn M. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell. 2010 May 14;141(4):583-94. doi: 10.1016/j.cell.2010.04.020.

Rongioletti F, Smoller BR. Unusual histological variants of cutaneous malignant melanoma with some clinical and possible prognostic correlations. J Cutan Pathol. 2005 Oct;32(9):589-603. doi: 10.1111/j.0303-6987.2005.00418.x.

Ruiz-Garcia E, Matus-Santos JA, Guadarrama-Orozco JA, Alvarez-Avitia MA, Aguilar-Ponce JL, Fernandez-Figueroa E, Maldonado-Mendoza J, Lopez-Camarillo C, Marchat LA, Lino-Silva S, Cuellar-Hubbe M, de la Garza-Salazar J, Meneses-García A, Astudillo-de la Vega H, Martinez-Said H. Frequency of BRAF V600E Mutation in the Mexican Population of Patients With Metastatic Melanoma. J Glob Oncol. 2018 Sep;4:1-5. doi: 10.1200/JGO.2016.008912.

Russo A, Ficili B, Candido S, Pezzino FM, Guarneri C, Biondi A, Travali S, McCubrey JA, Spandidos DA, Libra M. Emerging targeted therapies for melanoma treatment (review). Int J Oncol. 2014 Aug;45(2):516-24. doi: 10.3892/ijo.2014.2481.

Russo AE, Torrisi E, Bevelacqua Y, Perrotta R, Libra M, McCubrey JA, Spandidos DA, Stivala F, Malaponte G. Melanoma: molecular pathogenesis and emerging target therapies (Review). Int J Oncol. 2009 Jun;34(6):1481-9. doi: 10.3892/ijo_00000277.

Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell. 2006 Jun;9(6):435-43. doi: 10.1016/j.ccr.2006.04.020.

Salemi R, Falzone L, Madonna G, Polesel J, Cinà D, Mallardo D, Ascierto PA, Libra M, Candido S. MMP-9 as a Candidate Marker of Response to BRAF Inhibitors in Melanoma Patients With BRAFV600E Mutation Detected in Circulating-Free DNA. Front Pharmacol. 2018 Aug 14;9:856. doi: 10.3389/fphar.2018.00856.

Santenard A, Torres-Padilla ME. Epigenetic reprogramming in mammalian reproduction: contribution from histone variants. Epigenetics. 2009 Feb 16;4(2):80-4. doi: 10.4161/epi.4.2.7838.

Sarma K, Reinberg D. Histone variants meet their match. Nat Rev Mol Cell Biol. 2005 Feb;6(2):139-49. doi: 10.1038/nrm1567.

Satzger I, Mattern A, Kuettler U, Weinspach D, Niebuhr M, Kapp A, Gutzmer R. microRNA-21 is upregulated in malignant melanoma and influences apoptosis of melanocytic cells. Exp Dermatol. 2012 Jul;21(7):509-14. doi: 10.1111/j.1600-0625.2012.01510.x.

Schones DE, Cui K, Cuddapah S, Roh TY, Barski A, Wang Z, Wei G, Zhao K. Dynamic regulation of nucleosome positioning in the human genome. Cell. 2008 Mar 7;132(5):887-98. doi: 10.1016/j.cell.2008.02.022. PMID: 18329373.

Schuch AP, Garcia CC, Makita K, Menck CF. DNA damage as a biological sensor for environmental sunlight. Photochem Photobiol Sci. 2013 Aug;12(8):1259-72. doi: 10.1039/c3pp00004d.

Sergentanis TN, Antoniadis AG, Gogas HJ, Antonopoulos CN, Adami HO, Ekbom A, Petridou ET. Obesity and risk of malignant melanoma: a meta-analysis of cohort and

case-control studies. Eur J Cancer. 2013 Feb;49(3):642-57. doi: 10.1016/j.ejca.2012.08.028.

Shain AH, Bastian BC. From melanocytes to melanomas. Nat Rev Cancer. 2016 Jun;16(6):345-58. doi: 10.1038/nrc.2016.37. Erratum in: Nat Rev Cancer. 2020 Jun;20(6):355.

Shanmugam MK, Arfuso F, Arumugam S, Chinnathambi A, Jinsong B, Warrier S, Wang LZ, Kumar AP, Ahn KS, Sethi G, Lakshmanan M. Role of novel histone modifications in cancer. Oncotarget. 2017 Dec 17;9(13):11414-11426. doi: 10.18632/oncotarget.23356. Erratum in: Oncotarget. 2018 Apr 10;9(27):19460.

Shen J, Song R, Wan J, Huff C, Fang S, Lee JE, Zhao H. Global methylation of blood leukocyte DNA and risk of melanoma. Int J Cancer. 2017 Apr 1;140(7):1503-1509. doi: 10.1002/ijc.30577.

Shi W. Radiation Therapy for Melanoma. In: Ward WH, Farma JM, editors. Cutaneous Melanoma: Etiology and Therapy [Internet]. Brisbane (AU): Codon Publications; 2017 Dec 21. Chapter 8.

Shi Y. Histone lysine demethylases: emerging roles in development, physiology and disease. Nat Rev Genet. 2007 Nov;8(11):829-33. doi: 10.1038/nrg2218.

Shivaswamy S, Bhinge A, Zhao Y, Jones S, Hirst M, Iyer VR. Dynamic remodeling of individual nucleosomes across a eukaryotic genome in response to transcriptional perturbation. PLoS Biol. 2008 Mar 18;6(3):e65. doi: 10.1371/journal.pbio.0060065.

Sigalotti L, Covre A, Fratta E, Parisi G, Colizzi F, Rizzo A, Danielli R, Nicolay HJ, Coral S, Maio M. Epigenetics of human cutaneous melanoma: setting the stage for new therapeutic strategies. J Transl Med. 2010 Jun 11;8:56. doi: 10.1186/1479-5876-8-56.

Singer M, Kosti I, Pachter L, Mandel-Gutfreund Y. A diverse epigenetic landscape at human exons with implication for expression. Nucleic Acids Res. 2015 Apr 20;43(7):3498-508. doi: 10.1093/nar/gkv153.

Smith CL, Peterson CL. ATP-dependent chromatin remodeling. Curr Top Dev Biol. 2005;65:115-48. doi: 10.1016/S0070-2153(04)65004-6.

Soto JL, Cabrera CM, Serrano S, López-Nevot MA. Mutation analysis of genes that control the G1/S cell cycle in melanoma: TP53, CDKN1A, CDKN2A, and CDKN2B. BMC Cancer. 2005 Apr 8;5:36. doi: 10.1186/1471-2407-5-36.

Soura E, Eliades PJ, Shannon K, Stratigos AJ, Tsao H. Hereditary melanoma: Update on syndromes and management: Genetics of familial atypical multiple mole melanoma syndrome. J Am Acad Dermatol. 2016 Mar;74(3):395-407; quiz 408-10. doi: 10.1016/j.jaad.2015.08.038.

Spain L, Schmid T, Gore M, Larkin J. Efficacy of the combination of ipilimumab and nivolumab following progression on pembrolizumab in advanced melanoma with poor risk features. Eur J Cancer. 2017 Apr;75:243-244. doi: 10.1016/j.ejca.2016.12.040.

Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Milella M, Tafuri A, Bonati A, Bäsecke J, Cocco L, Evangelisti C, Martelli AM, Montalto G, Cervello M, McCubrey JA. Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. Aging (Albany NY). 2011 Mar;3(3):192-222. doi: 10.18632/aging.100296.

Stiegel E, Xiong D, Ya J, Funchain P, Isakov R, Gastman B, Vij A. Prognostic value of sentinel lymph node biopsy according to Breslow thickness for cutaneous melanoma. J Am Acad Dermatol. 2018 May;78(5):942-948. doi: 10.1016/j.jaad.2018.01.030.

Stone TW, McPherson M, Gail Darlington L. Obesity and Cancer: Existing and New Hypotheses for a Causal Connection. EBioMedicine. 2018 Apr;30:14-28. doi: 10.1016/j.ebiom.2018.02.022.

Strub T, Ballotti R, Bertolotto C. The "ART" of Epigenetics in Melanoma: From histone "Alterations, to Resistance and Therapies". Theranostics. 2020 Jan 1;10(4):1777-1797. doi: 10.7150/thno.36218.

Sturm RA, Fox C, McClenahan P, Jagirdar K, Ibarrola-Villava M, Banan P, Abbott NC, Ribas G, Gabrielli B, Duffy DL, Peter Soyer H. Phenotypic characterization of nevus and tumor patterns in MITF E318K mutation carrier melanoma patients. J Invest Dermatol. 2014 Jan;134(1):141-149. doi: 10.1038/jid.2013.272.

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin. 2021 May;71(3):209-249. doi: 10.3322/caac.21660.

Sweetlove M, Wrightson E, Kolekar S, Rewcastle GW, Baguley BC, Shepherd PR, Jamieson SM. Inhibitors of pan-PI3K Signaling Synergize with BRAF or MEK Inhibitors to Prevent BRAF-Mutant Melanoma Cell Growth. Front Oncol. 2015 Jun 16;5:135. doi: 10.3389/fonc.2015.00135.

Takai D, Jones PA. Comprehensive analysis of CpG islands in human chromosomes 21 and 22. Proc Natl Acad Sci U S A. 2002 Mar 19;99(6):3740-5. doi: 10.1073/pnas.052410099.

Tang Y, Durand S, Dalle S, Caramel J. EMT-Inducing Transcription Factors, Drivers of Melanoma Phenotype Switching, and Resistance to Treatment. Cancers (Basel). 2020 Aug 4;12(8):2154. doi: 10.3390/cancers12082154.

Testa U, Castelli G, Pelosi E. Melanoma: Genetic Abnormalities, Tumor Progression, Clonal Evolution and Tumor Initiating Cells. Med Sci (Basel). 2017 Nov 20;5(4):28. doi: 10.3390/medsci5040028.

Thomas NE, Edmiston SN, Alexander A, Millikan RC, Groben PA, Hao H, Tolbert D, Berwick M, Busam K, Begg CB, Mattingly D, Ollila DW, Tse CK, Hummer A, Lee-Taylor J, Conway K. Number of nevi and early-life ambient UV exposure are

associated with BRAF-mutant melanoma. Cancer Epidemiol Biomarkers Prev. 2007 May;16(5):991-7. doi: 10.1158/1055-9965.EPI-06-1038.

Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, Tokino T. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res. 2008 Jun 1;68(11):4123-32. doi: 10.1158/0008-5472.CAN-08-0325.

Tsao H, Olazagasti JM, Cordoro KM, Brewer JD, Taylor SC, Bordeaux JS, Chren MM, Sober AJ, Tegeler C, Bhushan R, Begolka WS, American Academy of Dermatology Ad Hoc Task Force for the ABCDEs of Melanoma. Early detection of melanoma: reviewing the ABCDEs. J Am Acad Dermatol. 2015 Apr;72(4):717-23. doi: 10.1016/j.jaad.2015.01.025.

Udayakumar D, Mahato B, Gabree M, Tsao H. Genetic determinants of cutaneous melanoma predisposition. Semin Cutan Med Surg. 2010 Sep;29(3):190-5. doi: 10.1016/j.sder.2010.06.002.

Van Allen EM, Wagle N, Sucker A, Treacy DJ, Johannessen CM, Goetz EM, Place CS, Taylor-Weiner A, Whittaker S, Kryukov GV, Hodis E, Rosenberg M, McKenna A, Cibulskis K, et al. The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. Cancer Discov. 2014 Jan;4(1):94-109. doi: 10.1158/2159-8290.CD-13-0617.

Varamo C, Occelli M, Vivenza D, Merlano M, Lo Nigro C. MicroRNAs role as potential biomarkers and key regulators in melanoma. Genes Chromosomes Cancer. 2017 Jan;56(1):3-10. doi: 10.1002/gcc.22402.

Ventura A, Jacks T. MicroRNAs and cancer: short RNAs go a long way. Cell. 2009 Feb 20;136(4):586-91. doi: 10.1016/j.cell.2009.02.005.

Wagner NB, Forschner A, Leiter U, Garbe C, Eigentler TK. S100B and LDH as early prognostic markers for response and overall survival in melanoma patients treated with anti-PD-1 or combined anti-PD-1 plus anti-CTLA-4 antibodies. Br J Cancer. 2018 Aug;119(3):339-346. doi: 10.1038/s41416-018-0167-x.

Wang Y, Leung FC. An evaluation of new criteria for CpG islands in the human genome as gene markers. Bioinformatics. 2004 May 1;20(7):1170-7. doi: 10.1093/bioinformatics/bth059.

Wapinski O, Chang HY. Long noncoding RNAs and human disease. Trends Cell Biol. 2011 Jun;21(6):354-61. doi: 10.1016/j.tcb.2011.04.001. Epub 2011 May 6. Erratum in: Trends Cell Biol. 2011 Oct;21(10):561.

Ward-Peterson M, Acuña JM, Alkhalifah MK, Nasiri AM, Al-Akeel ES, Alkhaldi TM, Dawari SA, Aldaham SA. Association Between Race/Ethnicity and Survival of Melanoma Patients in the United States Over 3 Decades: A Secondary Analysis of SEER Data. Medicine (Baltimore). 2016 Apr;95(17):e3315. doi: 10.1097/MD.0000000000003315.

Watt F, Molloy PL. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes Dev. 1988 Sep;2(9):1136-43. doi: 10.1101/gad.2.9.1136.

Wellbrock C. MAPK pathway inhibition in melanoma: resistance three ways. Biochem Soc Trans. 2014 Aug;42(4):727-32. doi: 10.1042/BST20140020.

Xu XC. Tumor-suppressive activity of retinoic acid receptor-beta in cancer. Cancer Lett. 2007 Aug 8;253(1):14-24. doi: 10.1016/j.canlet.2006.11.019.

Yan S, Holderness BM, Li Z, Seidel GD, Gui J, Fisher JL, Ernstoff MS. Epithelial-Mesenchymal Expression Phenotype of Primary Melanoma and Matched Metastases and Relationship with Overall Survival. Anticancer Res. 2016 Dec;36(12):6449-6456. doi: 10.21873/anticanres.11243.

Yokoyama S, Woods SL, Boyle GM, Aoude LG, MacGregor S, Zismann V, Gartside M, Cust AE, Haq R, Harland M, Taylor JC, Duffy DL, Holohan K, Dutton-Regester K, Palmer JM, Bonazzi V, et al. A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. Nature. 2011 Nov 13;480(7375):99-103. doi: 10.1038/nature10630.

Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol. 2007 Feb 1;302(1):1-12. doi: 10.1016/j.ydbio.2006.08.028.

Zhang M, Qureshi AA, Geller AC, Frazier L, Hunter DJ, Han J. Use of tanning beds and incidence of skin cancer. J Clin Oncol. 2012 May 10;30(14):1588-93. doi: 10.1200/JCO.2011.39.3652.

Zhang T, Dutton-Regester K, Brown KM, Hayward NK. The genomic landscape of cutaneous melanoma. Pigment Cell Melanoma Res. 2016 May;29(3):266-83. doi: 10.1111/pcmr.12459.

Zhu X, Li Y, Meng Q. Islet-1 promotes the proliferation and invasion, and inhibits the apoptosis of A375 human melanoma cells. Int J Mol Med. 2018 Jun;41(6):3680-3690. doi: 10.3892/ijmm.2018.3569.

Zlotorynski E. Epigenetics: DNA methylation prevents intragenic transcription. Nat Rev Mol Cell Biol. 2017 Mar 21;18(4):212-213. doi: 10.1038/nrm.2017.25.