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Genomics of Human Malignant Melanoma

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1. Introduction

Malignant melanoma is considered the most aggressive form of skin cancer. The incidence rate of the disease has steadily risen over the past few decades throughout the world. If melanoma is diagnosed early, it can be cured by surgical resection, but as soon as the first distant metastasis appears, the disease becomes one of the most aggressive types of metastatic, chemoresistant lesions. Cutaneous melanocytes originate from highly motile neural crest progenitors that migrate to the skin during embryonic development. They are pigment-producing skin cells that reside between keratinocytes in the basal layer of the epidermis, producing melanin in response to a variety of external stimuli, such as ultraviolet (UV) radiation. Although UV radiation is the main exogenous etiological risk factor for the development of the disease, other presently unknown factors are also involved. As estimated by the World Health Organization worldwide number of newly diagnosed skin cancer cases is between 2 and 3 million each year, of which 132,000 are melanoma. Additionally, in most western countries, the incidence of melanoma doubles roughly every decade.

Malignant melanoma progresses through a series of well-defined clinical and histopathological stages, advancing in a stepwise manner from either a common acquired or a dysplastic nevus through the primary radial growth phase (RGP) and the vertical growth phase (VGP) to distant metastasis [Welch et al., 1997]. Different subtypes of the disease represent diverse entities, as there are marked differences in their biological behaviours. While the most common superficial spreading subtype (SSM) is characterised by a prolonged RGP, nodular melanoma (NM) begins to grow vertically from its onset. Clinical staging of primary cutaneous melanoma is based on measurements of tumour thickness (in millimetres), the presence or absence of ulceration, penetration through cutaneous layers, mitotic rate and evidence of lymph node, cutaneous or distant metastasis [Chin et al., 2006]. The vertical progression of lesions is representative of the degree of tumour progression and is measured by the Breslow thickness, which was first used in the early 1970s and measures the thickness of the tumour from the top of the epidermal granular layer (or from the ulcer base if the tumour is ulcerated) to the innermost depth of invasion. Ulceration of the tumour surface of melanoma covering the epidermis is one of the most sensitive parameters of metastatic potential. The currently used diagnostic and prognostic approaches to recognise

the disease at an early stage are based on morphological observations supplemented by sentinel lymph node biopsy, which can define the prognosis of melanoma and assist in choosing the optimal surgical treatment. However, despite the extensive research approaches that have been employed to study this disease, current prognostic biological markers, either alone or in combination, are not adequate for accurately performing individualised assessment of the predicted risk of melanoma progression and are often not helpful in defining the most effective therapy [Chin et al., 2006]. Similar to other solid tumours, it is assumed that the morphologic heterogeneity of melanoma originates from distinct genetic alterations that lead to diverse pathways of melanoma development and progression. To design effective therapeutic approaches, it is of critical importance to identify the genetic determinants of disease initiation and progression. Additionally, it is crucial to define the functionally important genetic/genomic alterations that result in melanoma initiation and progression [Ghosh & Chin, 2009]. Advances in genetic and genomic methodologies during the past decade have exponentially increased our understanding of the molecular genetic alterations associated with this disease.

2. Molecular pathways involved in melanoma initiation and progression

The genomic heterogeneity of melanoma and the complexity of the molecular pathways involved in disease development and progression suggest that no individual genetic or molecular alteration is crucial in these processes per se. The accumulation of and interactions between such alterations in combination with the interactions between the tumour cells and the microenvironment are involved in the generation of a specific set of biological outcomes [Palmieri et al., 2009].

Current knowledge about the main molecular pathways at the DNA level and their interactions during melanomagenesis are summarised below. However, many other drivers of melanomagenesis remain to be discovered.

2.1 Main genes involved in melanomagenesis and related signalling pathways

2.1.1 CDKN2A and CDK4 in familial melanoma

In addition to recognised heritable traits, such as skin, hair and eye colour, and the presence of a large number of nevi (benign, atypical or giant congenital), a familial history of melanoma (at least 3 affected relatives) is a significant risk factor for disease development [Chin et al., 2006; Sekulic et al., 2008]. Approximately 10% of individuals diagnosed with melanoma have a familial predisposition associated with a 2.24-fold increase in the risk of tumour development [Igbokwe & Lopez-Terrada, 2011]. Single-base mutations and deletions of the *CDKN2A* (cyclin-dependent kinase inhibitor 2A) gene at the 9p21 locus have been found to be the major germline alterations involved in these tumours, contributing to 10% to 40% of familial melanoma cases. The penetrance of *CDKN2A* mutations appears to be influenced by geographical location [Meyle & Guldberg, 2009]. Other melanoma-prone families harbour mutations in different genes, including germline mutations in *CDK4* (cyclin-dependent kinase 4) or unidentified genes on chromosomes 1p22 and 20q11.22 [Meyle & Guldberg, 2009; Sekulic et al., 2008].

CDKN2A encodes 2 overlapping, but distinct tumour-suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$, utilising alternative promoters and first exons (1α for INK4a and 1β for ARF) [Chin et al., 2006]. As most pathogenic mutations occur in exon 2 of these genes, they are often simultaneously altered in multiple tumours [Palmieri et al., 2009].

p16^{INK4a}-CDK4/6-RB pathway

RB1 (retinoblastoma 1) is an essential gatekeeper at the G1/S transition point of the cell cycle. In its underphosphorylated form, RB1 binds to the E2F transcription factor, preventing induction of the expression of genes crucial for the G1/S transition. However, when RB1 is phosphorylated by the activation of the CCND1 (cyclin D1) - CDK4/6 complex, E2F is released, leading the cell cycle to progress through the G1/S transition [Sekulic et al., 2008]. The key regulatory molecule involved in this mechanism is p16^{INK4a}, which inhibits the activity of CDK4/6 kinases in a dose-dependent manner and consequently causes cell cycle arrest [Chin et al., 2006]. Recently, it has been shown that p16^{INK4a} functions as an alternate RB-independent regulator and suppressor of UV-induced DNA damage by reactive oxygen species and reduces the effect of oxidative stress in melanocytes via the p38 stress-activated protein kinase [Jenkins et al., 2011].

Frequent point mutations in the coding region of the *CDKN2A* locus typically target and inactivate p16^{INK4a}, while preserve p14^{ARF} in 25% to 40% of melanoma-prone families and 0.2% to 2% of sporadic melanomas [Chin et al., 2006; Meyle & Guldberg, 2009]. Additionally, *CDKN2A* appears to be homozygously deleted in approximately 50% of melanomas or silenced by promoter hypermethylation in 20% to 75% of tumours [Moore et al., 2008; Sekulic et al., 2008].

Other members of the pathway rarely harbour mutations. Germline mutations in **CDK4**, an RB kinase inhibited by INK4a, have been only found in 15 melanoma-prone families [Meyle & Guldberg, 2009]. Most of these rare mutations target a conserved arginine residue at position 24 and substitute it with cysteine (R24C). Therefore, the mutant protein lacks the ability to bind to INK4a, but the CDK4-CCND1 interaction is preserved, resulting in the constitutive activation of this complex [Chin et al., 2006; Sekulic et al., 2008]. It is important to note that inactivating *p16^{INK4a}* mutations and activating *CDK4^{R24C}* mutations are not observed simultaneously, which suggests that they are mutually exclusive [Chin et al., 2006; Sekulic et al., 2008]. Alterations of *CDK4* are also observed in sporadic melanomas (more commonly in the acral and mucosal types) in the form of focal gene amplification without *p16^{INK4a}* deletion [Sekulic et al., 2008]. Other molecular alterations that are sometimes observed are **CDK6** overexpression, rare mutations in **CCND1** (approximately 4% of tumours) and inactivating mutations in **RB1** in approximately 6% of sporadic melanomas, but germline mutation in **RB1** is also observed, with an increased risk of tumour development [Sekulic et al., 2008].

p14^{ARF}-MDM2-p53 pathway

Inactivation of the p53 pathway is a common trait of cancers. The p53 protein is often referred to as the guardian of the genome because as a transcription factor, it regulates several genes involved in cell cycle arrest, senescence, DNA repair and apoptosis [Sekulic et al., 2008]. This broad set of functions entails precise control of the intracellular protein level by a complex network of positive and negative regulators. The main negative regulator is the MDM2 protein, which by binding to p53, promotes its ubiquitination and consequent proteosomal degradation. MDM2 is inhibited by p14^{ARF}, which leads to the stabilisation of p53 [Sekulic et al., 2008]. Following DNA damage, p53 activates p21$^{WAF1/CIP1}$, which blocks the CCNDE-CDK2 complex, causing cell cycle arrest at the G1/S transition point through the decreased phosphorylation of RB1, thus allowing DNA repair or apoptosis to occur [Sekulic et al., 2008].

p53 is one of the most commonly mutated genes in human cancers. However, in contrast to most solid tumours, in which the pathway is inactivated at the level of p53 itself, point mutations in this gene are very rare in melanoma (occurring in approximately 9% of cases) and appear to rely on the inactivation of *CDKN2A* [Chin et al., 2006]. Although most mutations affect $p16^{INK4a}$ with or without $p14^{ARF}$ alterations, some melanoma-prone families exhibit mutations in $p14^{ARF}$ alone, which suggests that the loss of this gene itself could be sufficient for melanoma formation, and both $p16^{INK4a}$ and $p14^{ARF}$ are essential for melanoma suppression [Meyle & Guldberg, 2009; Sekulic et al., 2008]. Some data suggest that the tumour suppressor function of $p14^{ARF}$ might be fulfilled by other, unidentified p53-independent mechanisms, which could also have important clinical implications [Sekulic et al., 2008].

2.1.2 Receptor tyrosine kinases (RTKs)

RTKs are important targets of molecular alterations in various cancers. Although most of these changes occur at the expression level, several RTKs localise to regions affected by DNA copy number gains or amplifications [Chin et al., 2006]. In malignant melanoma, the most frequently altered RTKs are EGFR (epidermal growth factor receptor), c-MET (the oncogenic form of the hepatocyte growth factor receptor) and c-KIT (stem cell factor receptor) [Ghosh & Chin, 2009].

EGFR (7p12) can be activated by EGF family ligands, such as EGF, TGF-α, amphiregulin or heparin-binding EGF [Ghosh & Chin, 2009]. Amplification, usually via copy number gains of the entire chromosome 7, and overexpression of EGFR have been frequently observed in late-stage melanomas and in association with the nodular subtype, which suggests that EGFR may support metastatic potential [Chin et al., 2006; Ghosh & Chin, 2009; Rother & Jones, 2009; Timar et al., 2010]. However, no focal *EGFR* amplification or mutation has been reported in melanoma [Chin et al., 2006; Ghosh & Chin, 2009]. Additionally, the EGFR signalling loop plays an important role in the RAS-driven tumourigenecity of melanoma cells. Animal models have suggested that autocrine EGFR signalling is essential for RAS-mediated transformation by promoting survival through PI3K activation of AKT [Chin et al., 2006; Ghosh & Chin, 2009].

The **c-MET** receptor (7q31) is normally expressed on epithelial cells and melanocytes and is activated by its ligand, HGF (hepatocyte growth factor). Similar to EGFR, c-MET overexpression and copy number gains of the locus are late-stage events in tumour progression that contribute to the metastatic character of skin melanomas, but no focal gene amplification and/or activating mutations have been demonstrated [Chin et al., 2006; Ghosh & Chin, 2009; Timar et al., 2010]. Oncogenic c-MET activation mainly occurs in an autocrine manner with the establishment of a HGF-MET autocrine loop, which drives the development of metastatic disease rather than the initial steps of melanomagenesis [Chin et al., 2006; Ghosh & Chin, 2009]. Finally, it was recently shown that c-MET can be a direct target of microphthalmia-associated transcription factor (MITF), which can be itself amplified in a subset of melanomas [Chin et al., 2006; Ghosh & Chin, 2009].

The **c-KIT** gene (4q12) encodes an RTK that binds stem cell factor (SCF). Interestingly, c-KIT does not represent a typical RTK in malignant melanoma, as a progressive loss of its expression can frequently be observed during tumour progression. Furthermore, c-KIT-expressing metastatic melanomas exhibit increased sensitivity to SCF-mediated apoptosis [Chin et al., 2006; Ghosh & Chin, 2009]. It has recently been reported that in some melanomas, *c-KIT* harbours a known GIST-associated point mutation, L576P, which maps to

the 5′ juxtamembrane domain, where most activating *c-KIT* mutations cluster [Chin et al., 2006; Ghosh & Chin, 2009]. A recent analysis showed that approximately 21% of mucosal, 11% of acral and 28% of melanoma developing on chronic sun-damaged skin exhibited an alteration in *c-KIT*. This suggests that the mutational and amplification status of *c-KIT*, which is usually mutually exclusive with *BRAF* and *NRAS* status, might have a significant clinical impact and may identify a patient subpopulation that would benefit from imatinib (c-KIT inhibitor) therapy [Ghosh & Chin, 2009; Igbokwe & Lopez-Terrada, 2011; Rother & Jones, 2009; Ko & Fisher, 2011].

2.1.3 Main pathways involved in melanoma: RAS/MAPK and PI3K signalling

Two major signalling cascades exist in melanoma: the RAS/MAP kinase pathway, which is mainly responsible for cellular proliferation, and the PI3 kinase pathway, which plays an important role in tumour cell survival. However, these regulatory functions are not exclusive, as interactions between the members of the two cascades enhance tumourigenesis, cell growth, chemoresistance, invasion, migration and cell cycle dysregulation [Sekulic et al., 2008].

2.1.3.1 RAS/MAPK pathway

The RAS/MAPK signalling cascade is carried out through various RTKs, including FGFR, c-KIT, c-MET or EGFR, and is strongly affected during tumourigenesis and progression. A receptor-ligand interaction initiates the phosphorylation cascade through RAS-RAF-MEK-MAPK kinases and finally activates enzymes (involved in metabolic regulation), cytoskeletal components (affecting cell shape and migration) and specific transcription factors, such as ETS1/2, that are essential for the initiation of the expression of target genes related to cell proliferation and survival [Chin et al., 2006; Sekulic et al., 2008]. Hyperphosphorylated ERKs are common features of human cancers, including melanoma (approximately 90% of cases). This enforced activation is usually achieved through activating mutations related to upstream mediators, such as *RAF* and *RAS* [Chin et al., 2006; Igbokwe & Lopez-Terrada, 2011; Palmieri et al., 2009].

Activating mutations in the **RAS** proto-oncogene family (*HRAS*, *NRAS* and *KRAS*) are detected in melanoma with an incidence of between 10% and 15%. Point mutations in **NRAS** (Q61L) are the most common type of these mutations and are found in 56% of congenital nevi (but are rare in dysplastic and benign acquired nevi), as well as 33% of primary and 26% of metastatic melanomas correlated with a nodular subtype and intermittent sun exposure [Chin et al., 2006; Ghosh & Chin, 2009; Rother & Jones, 2009; Sekulic et al., 2008]. **HRAS** point mutations, together with genomic 11p amplifications, are rarely associated with melanomas. However, they are more frequent in Spitz nevi and indicate benign behaviour, whereas *KRAS* activations have not been observed in melanocytic lesions [Blokx et al., 2010; Chin et al., 2006; Ghosh & Chin, 2009]. Mouse models have revealed that RAS family members have distinct roles in melanocyte biology: *HRAS* mutations in combination with the inactivation of $p16^{INK4a}$, $p14^{ARF}$ and/or *p53* promote the development of nonmetastatic melanoma, whereas *NRAS* activations together with $p16^{INK4a}$ and $p14^{ARF}$ deficiencies cause cutaneous melanoma with a high penetrance and short latency [Chin et al., 2006; Ghosh & Chin, 2009].

The **RAF** proto-oncogene family consists of *ARAF*, *BRAF* and *CRAF*. Activating mutations in **BRAF** are the most widespread genetic alterations observed in human melanoma, with up to a 70% incidence, and they are associated with intermittent sun exposure and influence the

pattern of metastasis. In contrast, mutations in *ARAF* and *CRAF* have not been observed in human melanomas [Chin et al., 2006; Ghosh & Chin, 2009; Ko & Fisher, 2011; Meyle & Guldberg, 2009; Palmieri et al., 2009; Sekulic et al., 2008; Viros et al., 2008]. Among these point mutations, the most common is V600E (a valine to glutamic acid substitution), which accounts for >90% of all *BRAF* mutations [Meyle & Guldberg, 2009]. The $BRAF^{V600E}$ mutation is induced by UV damage, but its specific relationship with UV exposure has not yet been identified. It has recently been suggested that because *MC1R* variants are strongly associated with *BRAF* activation, they may modify the connection between *BRAF* mutation, nevus burden and melanoma risk, indicating that activating mutations for *BRAF* are somehow indirectly induced by UV radiation [Ko & Fisher, 2011; Palmieri et al., 2009]. *BRAF* mutations are common in nodular and superficial spreading melanomas arising in skin in association with intermittent sun exposure (59%), in contrast to the lentigo maligna melanomas that occur in chronically sun-exposed areas (11%) and acral (23%) and mucosal (11%) melanomas, and they are absent in uveal melanomas [Chin et al., 2006; Ghosh & Chin, 2009; Rother & Jones, 2009]. Activating mutations are also seen in benign and dysplastic nevi, which often remain growth-arrested throughout an individual's life time and rarely progress into melanoma, suggesting a role in the early neoplastic stages of melanoma and that there may be a $BRAF^{V600E}$–induced checkpoint for malignant transformation [Chin et al., 2006; Ghosh & Chin, 2009]. Congenital nevi are often positive for the senescence marker SA-β-Gal and for $p16^{INK4a}$. In vitro studies have shown that in normal cells, $BRAF^{V600E}$ can induce $p16^{INK4a}$, SA-β-Gal and cell cycle arrest, resulting in oncogene-induced senescence [Chin et al., 2006; Ghosh & Chin, 2009; Palmieri et al., 2009]. However, $p16^{INK4a}$ positivity is not 100% correlated with SA-β-Gal positivity, suggesting the presence of other factors involved in cell senescence, such as those regulated by IGFBP7 (IGF-binding protein 7). Benign nevi contain both $BRAF^{V600E}$ and high levels of IGFBP7, while $BRAF^{V600E}$ melanomas do not [Ghosh & Chin, 2009]. Additionally, BRAF can also cooperate with the $p14^{ARF}$-MDM2-p53 pathway because in p53-deficient zebrafish, mutant BRAF can induce invasive melanoma [Ko & Fisher, 2011; Palmieri et al., 2009].

BRAF and *NRAS* mutations are mutually exclusive at the single-cell level in melanoma, but they can be found in the same tumour with different segregation into neoplastic cells, potentially resulting in distinct biological properties and heterogeneous responses to therapy [Palmieri et al., 2007]. These alterations are also found in benign and dysplastic nevi, indicating that their activation is an early event in melanoma and is sufficient for initiation but is not involved in progression; therefore, additional genetic alterations are necessary for malignant transformation [Conway et al., 2010; Igbokwe & Lopez-Terrada, 2011]. This exclusion may be based on the ability of melanoma cells carrying an *NRAS* mutation to bypass BRAF and signal via CRAF [Rother et al., 2009]. *BRAF* or *NRAS* mutations and concurrent genetic aberrations in *BRAF* and *PTEN* or *MC1R* are highly associated with intermittent sun exposure, whereas in acral melanomas or melanomas arising from chronic sun-exposed skin, wild-type *BRAF* and *N-RAS* can usually be found and are associated with copy number gains of *CCND1* and *CDK4*, indicating the greater importance of the $p16^{INK4a}$-RB cascade in these melanocytic lesions [Palmieri et al., 2007]. Finally, *RAF-RAS* mutations, possibly in association with mutant *c-KIT* and $p16^{INK4a}$ alterations, might be useful in distinguishing secondary new and metastatic melanomas, which could have considerable prognostic and clinical significance, as these melanomas are prone to systemic spread and are often incurable [Blokx et al., 2010].

2.1.3.2 PI3K/AKT pathway

The PI3 kinase signalling cascade is activated by a number of extracellular signals in both paracrine and autocrine manners, such as signals from integrins, extracellular matrix proteins, HGF and insulin-like growth factors. This pathway is often hyperactive in melanoma and antagonises the intrinsic apoptotic pathway [Chin et al., 2006; Ghosh & Chin, 2009; Sekulic et al., 2008]. Genetic alterations targeting members of the PI3K pathway are not as frequent as in the case of the RAS/MAPK pathway. PI3 kinase mutation occurs at a low frequency in melanoma (5%) [Palmieri et al., 2009]. The major genetic alteration observed is the deletion of **PTEN** (phosphatase and tensin homologue) at the 10q23 locus, which encodes a lipid and protein phosphatase. Loss of a *PTEN* allele or a change in its expression have been observed in approximately 20% and 40% of lesions, respectively; however, homozygous deletion and point mutations are rare in melanomas [Chin et al., 2006; Ghosh & Chin, 2009; Igbokwe & Lopez-Terrada, 2011; Timar et al., 2010]. As a negative regulator of the PI3 kinase pathway, PTEN reduces the intracellular level of PIP_3 induced by PI3K and consequently blocks AKT activation and phosphorylation of the mTOR transcription factor, which promotes the expression of target genes involved in cellular division, cell migration and survival. Loss of *PTEN* may represent an essential aberration in the formation of invasive melanomas [Ko & Fisher, 2011].

Another important member of the PI3 kinase pathway is **AKT**, which exists in three isoforms: AKT1, AKT2 and AKT3. Although the complexity of this signalling pathway is not fully understood, it appears that the activation of different AKT isoforms may play distinct roles in tumour cell proliferation and survival and is associated with *in situ* melanomas and lesions arising from sun-exposed skin [Chin et al., 2006; Ghosh & Chin, 2009; Rother & Jones, 2009]. Following PIP_3 formation, AKT becomes phosphorylated by PDK1 (pyruvate dehydrogenase kinase isozyme 1, mitochondrial) and consequently inactivates proapoptotic proteins, such as BAD, and activates the NFκB and FOXO1 transcription factors, which promote the expression of genes involved in survival [Sekulic et al., 2008]. Constitutive activation of **AKT3** through DNA copy number gains may be present in 40% to 60% of sporadic melanomas and stimulates a vertical growth phenotype, survival, migration, angiogenesis, glycolytic metabolism and cell cycle progression through the upregulation of CCND1 [Ghosh & Chin, 2009; Ko & Fisher, 2011; Palmieri et al., 2009; Sekulic et al., 2008]. However, it has been shown that **AKT1** activation inhibits tumour cell migration and the invasion of particular cell lines, including melanomas [Chin et al., 2006; Ghosh & Chin, 2009].

PTEN can also regulate RAS/MAPK signalling and promotes cell cycle arrest at the G1/S transition point through the upregulation of $p27^{KIP1}$. Moreover, activating mutant RAS can phosphorylate PI3K, resulting in increased AKT activity. Therefore, *PTEN* loss and the presence of oncogenic *RAS* are redundant [Rother & Jones, 2009; Sekulic et al., 2008].

2.1.4 Other important pathways in melanoma: WNT and MSH signalling

2.1.4.1 WNT pathway

WNT (wingless-type MMTV integration site family) signalling has significant developmental functions, especially in neural crest cells, such as melanocytes [Chin et al., 2006; Sekulic et al., 2008]. Activation of the canonical WNT pathway inactivates GSK3β (glycogen synthase kinase 3 beta), which normally promotes the proteosomal degradation of β-catenin via phosphorylation and results in an increased level of β-catenin that enhances the expression

of target genes, such as *MITF* and *CCND1*, through activation of the TCF-LEF transcriptional complex [Chin et al., 2006; Sekulic et al., 2008]. Despite the frequent stabilising β-catenin mutations that occur in melanoma cell lines, the incidence of these genetic alterations is much lower in primary melanoma samples. However, immunohistochemical studies have provided evidence of nuclear accumulation of β-catenin, which reflects the activation of the canonical WNT pathway and the contribution of MITF as an important upstream regulator of melanoma survival/proliferation [Chin et al., 2006]. Overexpression of **WNT5a** is often observed in melanomas; nevertheless, its function is associated with protein kinase C activation rather than β-catenin [Chin et al., 2006; Timar et al., 2010]. Furthermore, upregulation of **WNT2** has also been found in melanomas, contributing to the inhibition of normal apoptotic progress, and determination of its protein level can be useful in distinguishing between melanoma and nevi [Palmieri et al., 2009].

2.1.4.2 MSH pathway

MSH signalling is a key regulator of pigmentation through binding to its specific receptor, MC1R (melanocortin 1 receptor). **MC1R**, which is highly polymorphic, is expressed on epidermal melanocytes and determines the UV-induced skin response through modulating the induction efficiency of intracellular cAMP production [Chin et al., 2006; Meyle & Guldberg, 2009; Sekulic et al., 2008]. People with a red-hair-colour phenotype are unable to tan and exhibit more freckles; therefore, they display a 2.7 to 16.0-fold increase in risk of developing melanoma [Sekulic et al., 2008]. Some investigators have suggested that certain *MC1R* alleles have a pigment-independent effect on melanomagenesis [Chin et al., 2006; Meyle & Guldberg, 2009]. MSH-MC1R binding activates the canonical PKA (protein kinase A) response by inducing adenylate cyclase to produce cAMP, phosphorylates Rsk and/or PKA, which finally stimulate the CREB/ATF1 transcription factors. This complex both induces the expression of specific genes involved in pigmentation through direct regulation and activates the *MITF* promoter, which defines the tissue-specific expression of genes involved in pigmentation. The expression level of *MITF* affects the resulting phenotype; a low level defines pheomelanin, red-blond pigments, whereas a high level defines eumelanin, brown-black pigments [Chin et al., 2006]. In addition to PKA phosphorylation, activation of the RAS/MAPK pathway and inactivation of the PI3K pathway also appear to be consequences of cAMP stimulation. The general nature of cAMP and CREB/ATF1 activation, compared to the highly tissue restricted nature of MITF activation, suggests the presence of a cooperating site that maps to the Sox10 consensus element in the *MITF* promoter [Chin et al., 2006].

2.1.5 Novel pathways in melanoma: Notch1 and iNOS signalling

2.1.5.1 Notch1 pathway

The Notch signalling pathway plays key roles in tissue homeostasis and the regulation of cell fate [Bedogni & Powell, 2009]. Depending on cell-cell interactions and the extracellular environment, Notch can act as either a tumour suppressor or an oncogene [Palmieri et al., 2009]. Upon activation, the Notch receptor undergoes two consecutive enzymatic cleavages, which result in the formation of N^{ICD}, a truncated variant of the receptor. N^{ICD} translocates to the nucleus and forms a complex with the CBF1 transcription factor and other co-activators, which influences the intensity and duration of Notch signals and consequently induces transcription from promoters containing CBF1-responsive elements [Bedogni &

Powell, 2009; Palmieri et al., 2009]. Normally, Notch1 signalling induces growth arrest and differentiation in keratinocytes via $p21^{WAF1/CIP1}$ induction and blocking WNT signalling. In melanomas and atypical nevi, Notch1 and 2 and their ligands are significantly upregulated. Notch1 exerts its effects through interactions with several pathways [Bedogni & Powell, 2009; Palmieri et al., 2009]. Binding to β-catenin mediates oncogenic activity either via the WNT pathway or by regulating N-cadherin. Notch1 also exhibits cross-talk with and inhibits the MAPK and PI3K pathways, contributing to the vertical growth phase. Furthermore, direct interactions between N^{ICD} and NFκB cause the nuclear retention of NFκB transcription factor and enhance melanoma cell survival (late Notch-dependent activation). N^{ICD} can also directly regulate IFNγ expression by creating a complex with both NFκB and IFNγ promoters. Finally, RAS-mediated malignant transformation also requires intact Notch signalling [Palmieri et al., 2009]. Taken together, these data suggest that *Notch* may represent a novel target involved in melanomagenesis.

2.1.5.2 iNOS pathway

NO (nitric oxide) free radical production by NOS (nitric oxide synthase) plays an important role in immune responses. Three isoforms can be distinguished: nNOS/NOS I (neuronal) and eNOS/NOS III (endothelial) are constitutively expressed, and iNOS/NOS II (inducible) is transcriptionally regulated by several mediators [Palmieri et al., 2009]. In normal melanocytes, free radicals are completely inactivated via the redox function of eumelanin. Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been identified in melanoma [Jenkins et al., 2011]. The effect of NO in tumour progression is dose dependent; a high NO concentration can lead to apoptosis via its effect on multiple apoptosis-related proteins, such as p53 and Bcl-2, and growth inhibition, whereas a low NO concentration may contribute to tumour growth and angiogenesis [Palmieri et al., 2009]. However, the role of **iNOS** in melanoma progression is still controversial. A higher level of iNOS has been found in subcutaneous and lymph node metastases of nonprogressive melanomas compared to that in progressive tumours, but the degree of iNOS expression is lower in metastases than in nevi or primary melanomas [Palmieri et al., 2009]. Finally, **nNOS** may also have an impact on melanomagenesis because approximately 82% of primary tumours, 72% of atypical nevi and 49% of benign nevi have been reported to express this protein, whereas normal melanocytes do not exhibit nNOS expression. Therefore, *de novo* expression of nNOS may be a marker for early stage tumours [Palmieri et al., 2009].

Currently, the most effective therapy against melanoma is surgical resection of primary tumours, followed by observation of a sentinel lymph node [Gerami et al., 2009]. The cytogenetic heterogeneity of human melanomas results in significant problems in the classification of histologically ambiguous primary tumours and in differentiation between melanomas and other lesions, such as Spitz nevi, blue nevi or proliferating congenital nevi [Braun-Falco et al., 2009; Scolyer et al., 2010]. Understanding these alterations (Figure 1) and evaluating all of the known molecular targets in melanoma through the application of new molecular biological techniques, such as FISH, PCR, sequencing and microarrays, in addition to the gold standard of histopathologic examination, will be crucial in predicting subsets of patients with particular biological and clinical characteristics who may respond favourably to a therapy that specifically targets a characteristic molecular signature [Gerami et al., 2009; Ghosh & Chin, 2009; Palmieri et al., 2007].

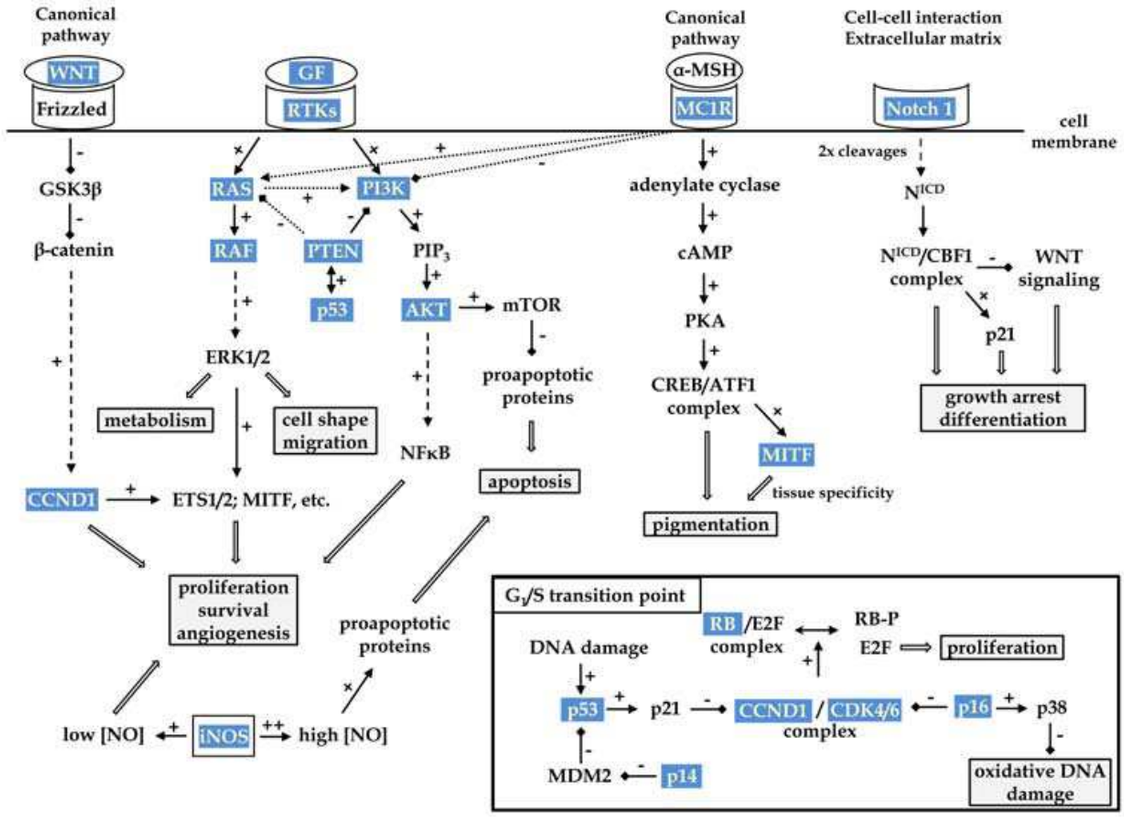

Fig. 1. Schematic representation of the signalling pathways affected in melanoma progression. Members showed (epi)genetic and/or expressional alterations are highlighted with blue

3. Genetic alterations in malignant melanoma

Molecular biological techniques developed during the last two decades have greatly improved our understanding of the genetic background of malignant melanoma. A number of studies using classical chromosome banding, fluorescence *in situ* hybridisation (FISH) and chromosomal and array comparative genomic hybridisation (CGH) have been performed to define the genetic alterations that underlie the development and progression of melanoma [Trent et al., 1991; Bastian et al., 1998; Chin et al., 2006]. These studies have identified a large number of non-random alterations on chromosomes 1p, 6, 7, 8q, 9, 10q, 11q and 17, the frequencies of which are reflective of the malignant potential of the sporadic form of the disease. As a result of detailed analysis, a number of tumour suppressors and oncogenes have been implicated as key factors in melanoma pathogenesis, including *CDKN2A*, *PTEN, BRAF*, *NRAS*, *MITF*, *AIM1*, *CCND1*, and *MYC* [Poetch et al., 2003; Casarso et al., 2005; Rákosy et al., 2008; Lázár et al., 2009; Dalton et al. 2010].

3.1 Non-random copy number alterations

Comparative genomic hybridisation represents the best approach for searching for DNA sequence copy number alterations in cancer genomes. Improvement of the resolution and sensitivity of CGH during the last decade has allowed the discovery of recurrent copy number alterations and new genetic targets in different cancer types, including malignant

melanoma. Although chromosomal and array CGH have revealed a large number of common non-random alterations (DNA sequence amplifications and deletions, Figure 2), no obvious or validated melanoma-relevant molecular targets have yet been identified [Gosh & Chin, 2009]. The non-random nature of melanoma-specific copy number alterations may allow the segregation of melanomas into subtypes based on distinct clinical and biological behaviours. The array CGH investigation of primary melanoma producing the greatest number of findings was reported by Curtin et al. [Curtin et al., 2005]. At the beginning of this study, it was hypothesised that the clinical heterogeneity of the disease could be explained by genetically distinct types of melanomas with different susceptibilities to ultraviolet light.

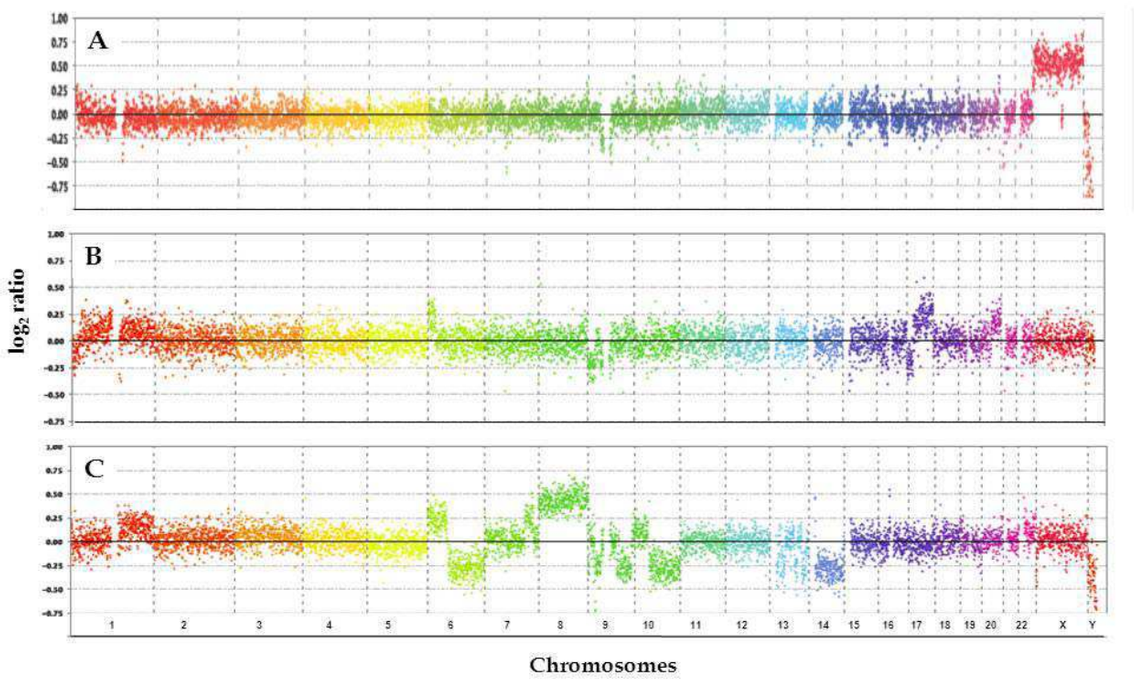

Fig. 2. Array CGH profiles of malignant melanoma. A.) Control sample without copy number alteration. B.) Array CGH profile of a primary malignant melanoma sample. C.) melanoma metastasis

Chromosome copy number alterations and the mutational status of the *BRAF* and *NRAS* genes were compared in 126 primary melanomas, and melanoma tissue samples were grouped based on their degree of exposure to ultraviolet light. The four types of melanoma included in these groupings were as follows: acral melanoma (melanoma occurring on the non-hair-bearing skin of the palms or soles), mucosal melanoma (tumours arising on mucosal membranes), tumours arising from skin with chronic sun-induced damage and lesions arising from skin without chronic sun-induced damage. Melanomas without chronic sun-induced damage frequently showed mutations in the *BRAF* oncogene, together with losses of chromosome 10q (site of *PTEN*), or mutations in the *NRAS* gene alone. In contrast, melanomas arising from skin with chronic sun-induced damage (mucosal and acral melanomas) did not exhibit *BRAF* or *NRAS* mutations but instead displayed an increased number of copies of the *CCND1* or *CDK4* genes. The different genetic alterations were

identified in different anatomical sites of the skin and with varying levels of ultraviolet exposure indicate that distinct molecular pathways are involved in the development and progression of the disease [Curtin et al., 2005].

3.1.1 Genetic differences between benign and malignant melanocytic lesions

The cytogenetic heterogeneity of the different subtypes of benign and malignant skin lesions were recently summarised by Blokx et al. [2010]. Some of the important DNA copy number alterations that distinguish melanomas from nevi can be successfully detected by interphase FISH [Bauer & Bastian, 2006; Gerami et al., 2010]. Based on previous array CGH observations, four DNA regions were selected, targeting 3 loci on chromosome 6 (*MYB1*: 6q23, *RREB1*: 6p25 and centromere 6) and *CCND1* on chromosome 11q13 as being the most powerful discriminators between melanomas and nevi that can assist in the diagnostic classification of melanocytic tumours that cannot be consistently classified by other currently available methods. The sensitivity of the four DNA probes was further tested by FISH in 110 nevi and 123 melanomas, and it was found that the overall sensitivity of the tested probes was 83%, and the specificity was 94% [Gerami et al., 2010]. In another study, the same group reported that copy number changes at 11q13 (*CCND1*) and 8q34 (harbouring the *MYC* oncogene) were highly associated with prognosis and could discriminate between metastasising and no metastasising melanomas [Gerami et al., 2011].

The histopathologic subtypes of cutaneous melanomas (superficial spreading, nodular, acral lentiginous and lentigo maligna melanomas) are characterised based on the growth pattern of the radial growth phase of melanoma. Using interphase cytogenetics, several groups have showed that the different subtypes exhibit differences in chromosomal aberrations [Poetsch et al., 1998; Bastian et al., 2000; Treszl et al., 2004; Bastian et al., 2006] that may also contribute to distinct outcomes. Comparing the CGH profiles of AMs (acral melanomas) and SSMs, a significant difference was seen between the amplified loci/samples and between the two subtypes of melanomas. While all AMs exhibited at least one gene amplification, less than 15% of SSM samples showed amplification [Bastian, 2000]. The most frequently amplified region was 11q13, harbouring the *CCND1* oncogene, which probably arises early in the progression of AM. The biological relevance of the *CCND1* amplification was further investigated, and the results showed that *CCND1* amplification was strongly correlated with protein expression and indicated that *CCND1* is an oncogene involved in malignant melanoma [Bastian, 2002]. Recently, we reported that coamplification of *CCND1* with other genes (especially *TAOS1*) within the 11q13 amplicon could contribute to a more aggressive phenotype than that of *CCND1* alone [Lazar et al., 2009].

Targeting a combination of these loci using FISH may become a useful standardised prognostic test for melanoma skin cancer in the future. However, it is important to note that FISH analysis should be performed in combination with standard clinical and histopathological evaluations [Gerami et al., 2010].

3.1.2 New melanoma genes discovered by high-resolution array CGH

A systematic analysis of the melanoma genome led to the discovery of a new lineage survival oncogene, *MITF* (3p14), which is amplified or shows copy number gains in 10% of primary and 15% to 20% of metastatic melanomas [Garraway et al., 2005]. Based on FISH analysis, the *MITF* copy number fluctuates between 4 and 13 copies per cell. However, no amplification can be detected in nevi samples. Comparing *MITF* copy number alterations and clinical parameters, it was found that patients presenting tissue carrying the amplified gene exhibited

survival of less than five years. A similar correlation was seen for MITF protein expression, which implicates *MITF* gene amplification in the progression and lethality of a subset of melanomas. It was clearly demonstrated that MITF plays a crucial role in melanocyte biology and melanoma progression [Hoek et al., 2008] and potentially acts as a dominant oncogene. *MITF* regulates the expression of a large variety of genes, including genes involved in pigmentation, cell cycle regulation, differentiation, survival and migration. In addition to amplification, altered *MITF* function during melanomagenesis can be achieved by single-base substitutions or by mutation of its regulator, SOX10 [Cronin et al., 2009]. Targeting MITF in combination with BRAF or cyclin-dependent kinase inhibitors may offer a rational therapeutic opportunity to successfully treat this aggressive, chemoresistant disease.

The discovery of new targets by array CGH was also reported by Gast et al. [2010]. In this study, SNP arrays with 250,000 targets were applied to 60 cell lines derived from metastasised melanomas. Amplifications were found to be more common than deletions in these cell lines. Similar to the findings of other studies, homozygous and heterozygous deletions of the *CDKN2A* gene were the most frequent type of deletion found at the 9p21 locus, and these alterations were associated with a lack of gene expression. In addition to the common alterations described above, it was observed that melanoma cell lines without *BRAF* and *NRAS* oncogenic mutations exhibited losses of the entire 13q and 16q chromosome regions. These data further confirm that distinct molecular pathways are involved in malignant melanomas, driving melanoma initiation and progression in association with either oncogenic *BRAF* or *NRAS* mutations complemented mainly by the loss of tumour suppressor genes, including *CDKN2A* and *PTEN.* Alternatively, they may implicate the amplification of *CCDN1* (11q13) and *CDK4*, together with deletions of the 13q and 16q chromosome arms, which contain two major players, the *RB1* and *MC1R* genes respectively [Gast et al., 2010]. Losses of the entire chromosome 13q region have been predominantly observed in melanoma cell lines without *BRAF* and *NRAS* oncogenic mutations. Uncovering the potential role of the deletion of the q arms of both chromosomes 13 and 16 requires further investigations in primary melanomas.

A chromosome 6p gain is one of the most frequent alterations found in melanomas, as determined by CGH and classical cytogenetic studies. A novel melanoma metastasis gene on 6p25-p24 (*NEDD9*: neural precursor cell expressed, developmentally downregulated 9) showing recurrent focal amplification in 35% of human melanomas and melanoma cell lines was discovered by a cross-species comparison [Kim et al., 2006]. The expression of *NEDD9* is significantly upregulated relative to nontransformed melanocytes and benign melanocytic neoplasia, and this gene is overexpressed in 52% of melanomas, but only 14% of nevi. It was found that *NEDD9* amplification and overexpression were strongly associated with enhanced invasion and metastasis formation related to malignant melanomas. The large regional gain on chromosome 6p and the high recurrence of the alteration likely indicate the presence and synergistic activities of multiple oncogenes on 6p, in addition to *NEDD9.* The observation that more than 50% of human primary melanomas exhibit higher NEDD9 expression relative to benign nevi suggests the possibility that the amplification or overexpression of NEDD9 in a regional/focal manner may identify a subset of primary melanoma tumours with increased risk of metastasis [Kim et al., 2006].

4. Gene expression profiling of melanoma

Molecular classification of cutaneous malignant melanoma by gene expression profiling was first described in 2000 [Bittner et al., 2000]. Based on the obtained gene expression data, two

major clusters were identified, but no correlation was found between the cluster groups and any clinical variable associated with their tumour sets. Another group investigated the relationship between the gene expression profiles and clinical outcomes of 58 primary melanomas; 254 genes were found whose expression might have a role in predicting the clinical outcome of melanoma patients [Winnepenninckx et al., 2006]. A study was recently conducted with the major aim of identifying new prognostic markers and therapeutic targets that might aid clinical cancer diagnosis and management [Jeffs et al., 2009]. Global transcript profiling identified a signature characterised by decreased expression of developmental and lineage specification genes, including MITF, EDNRB, DCT, and TYR, and increased expression of genes involved in interactions with the extracellular environment, such as PLAUR, VCAN, and HIF1a. Migration assays showed that the gene signature was correlated with the invasive potential of the cell lines, and external validation using publicly available data indicated that tumours with the invasive gene signature were less melanocytic and might be more aggressive. It is significant that the invasion signature could be detected in both primary and metastatic tumours, suggesting that gene expression conferring increased invasive potential in melanoma may occur independently of tumour stage [Jeffs et al., 2009].

The impact of genomics on understanding human melanoma progression and metastasis was summarised by Ren et al. [2008]. Several groups have found distinct differences in gene expression patterns along the spectrum of melanoma tumour progression, with many showing distinct sets of over- and underexpressed genes that have been validated as having distinct, key roles in melanoma progression. Several research groups have attempted to develop large gene classifier sets composed of several hundreds or often thousands of genes. A critical analysis of gene expression studies relating to malignant melanoma progression was performed recently by Timár et al. [2010]. Despite the stunning success of genomics in defining genomic markers or gene signatures for breast cancer prognosis and for predicting therapies, there has been virtually no similar progress related to malignant melanoma. In summarising the microarray studies that have been performed on skin melanomas, it is noticeable that different groups used different microarray platforms in highly heterogeneous patient cohorts and pathological sample collections. Additionally, the investigated tumours may be heterogeneous even within a single study, containing sometimes limited numbers of primary melanomas or cutaneous, lymphatic or visceral metastases. Furthermore, the biological behaviour and histological appearance of the tumours were not taken into account in some studies, although some authors concluded that the gene expression signatures of superficial spreading and nodular melanomas are considerably different from each other [Jaeger et al., 2007]. Underlying the discrepancies in these data the problem is that the defined prognostic gene sets have not been validated in independent cohorts or datasets, with the exception of one study performed in primary tumours [Winnepenninckx et al., 2009].

The most recent systematic approach to characterise the spectrum of cancer-associated mRNA alterations through the integration of transcriptomic and structural genomic data has revealed new insights into melanoma biology and will likely lead to a new era of discovery in melanoma genomics that promises to reveal molecular mechanisms associated with the disease [Berger et al., 2010]. More than 700 non-synonymous coding variants have been identified. However, only a subset of these was validated to clarify whether they were bona fide somatic mutations. Based on the results described above, it is expected that most of these variants are inherited SNPs and that approximately 30% are somatic mutations. The

most interesting variants include a mutation observed in the melanoma cell line 501 Mel (*CTNNB1, chr3:41241117, C/T*), which was noted 135 times in the COSMIC database of somatic mutations in cancer. A new approach, paired-end massively parallel sequencing of cDNA, together with analyses of high-resolution chromosomal copy number data was used, as a result 11 novel melanoma gene fusions were identified, that produced by underlying genomic rearrangements and 12 novel read-through transcripts. These chimeric transcripts were mapped at base-pair resolution and traced to their genomic origins using matched chromosomal copy number information. Furthermore, these data were used to discover and validate base-pair mutations that accumulated in these melanomas, revealing a surprisingly high rate of somatic mutations and lending support to the notion that point mutations constitute the major driver of melanoma progression. Taken together, these results may indicate new avenues for target gene discovery related to melanoma, while also providing a template for large-scale transcriptome studies across diseases associated with many tumour types [Berger et al., 2010].

5. Epigenetic events: an explanation for altered gene expression in melanoma

Over the past two decades, gene expression studies have revealed a relatively large number of genes that show altered expression at both the mRNA and protein levels. Despite some contradictory results that are probably due to the genetic variability and heterogeneity of primary melanomas, the altered expression of a group of transcripts can be affirmed to play a crucial role in the carcinogenesis and progression of melanomas. In addition to the success of mutation and copy number variation studies related to certain genes, the demonstration of gene deregulation has prompted scientists to explore other alterations that could be the cause of altered expression and that could further act as biomarkers for early diagnosis and represent a characteristic of less favourable clinical outcomes. The commonly used term "epigenetics" has been rapidly spreading over the last decade. It emerged to define heritable changes in genome function that cannot be explained by direct influence over DNA sequences. As epigenetic mechanisms affect gene expression, resulting in different phenotypes without directly altering the underlying DNA sequence, it is reasonable that they should have an impact on cancer development. Because the importance of epigenetic processes in gene regulation is currently a topic of doubt and discussion, standard nomenclature for different types of epigenetic modifications is not available in the literature, although it is reasonable to distinguish between them based on the phase during which a specific event influences gene expression (Figure 3). There are two types of such alterations that are known to occur at the transcriptional level: DNA methylation and chromatin modification (post-transcriptional covalent change and chromatin rearrangement without chemical alteration). The third type of epigenetic alteration that has been discovered to date is RNA interference, which only affects a phenotype later, at the post-transcriptional level, as it directly cleaves mRNA, resulting in disrupted or no translation.

5.1 DNA methylation

5.1.1 Methyltransferases: potential epigenetic drug candidates

The best described factor involved in epigenetic inheritance is DNA methylation, a covalent modification of cytosines (mainly at position 5, but also at position 4 or 6) that results in 5-methyl-cytosine occurs in CpG dinucleotides that are part of CpG islands that can most

commonly be found at or near promoter regions in mammals. This process is accomplished through specific molecules: DNA methyltransferases (DNMTs), which are responsible for establishing and maintaining the unique methylation pattern on DNA. Three different types of DNMT have been reported to date. DNMT2 has rather low activity and functions only in tRNA methylation. DNMT1 and DNMT3 play essential roles in both mammalian development and in cancer biology, as they catalyse the addition of a methyl group to cytosines from the donor S-adenosyl-methionine. However, they require different substrates. Because DNMT1 is methylation dependent, it predominantly methylates hemimethylated CpGs. Therefore, DNMT1 is responsible for maintaining the methylation pattern, which is extremely important in cell division. DNMT3a and DNMT3b are referred to as *de novo* methylases because they act independently of previous methylation of the complementary strand. Both DNMT3a and DNMT3b could be important factors in establishing a new CpG methylation pattern, though DNMT3 exhibits a preference for centromeric regions [Cheng & Blumental, 2008]. Additionally, if a methyl group has already been

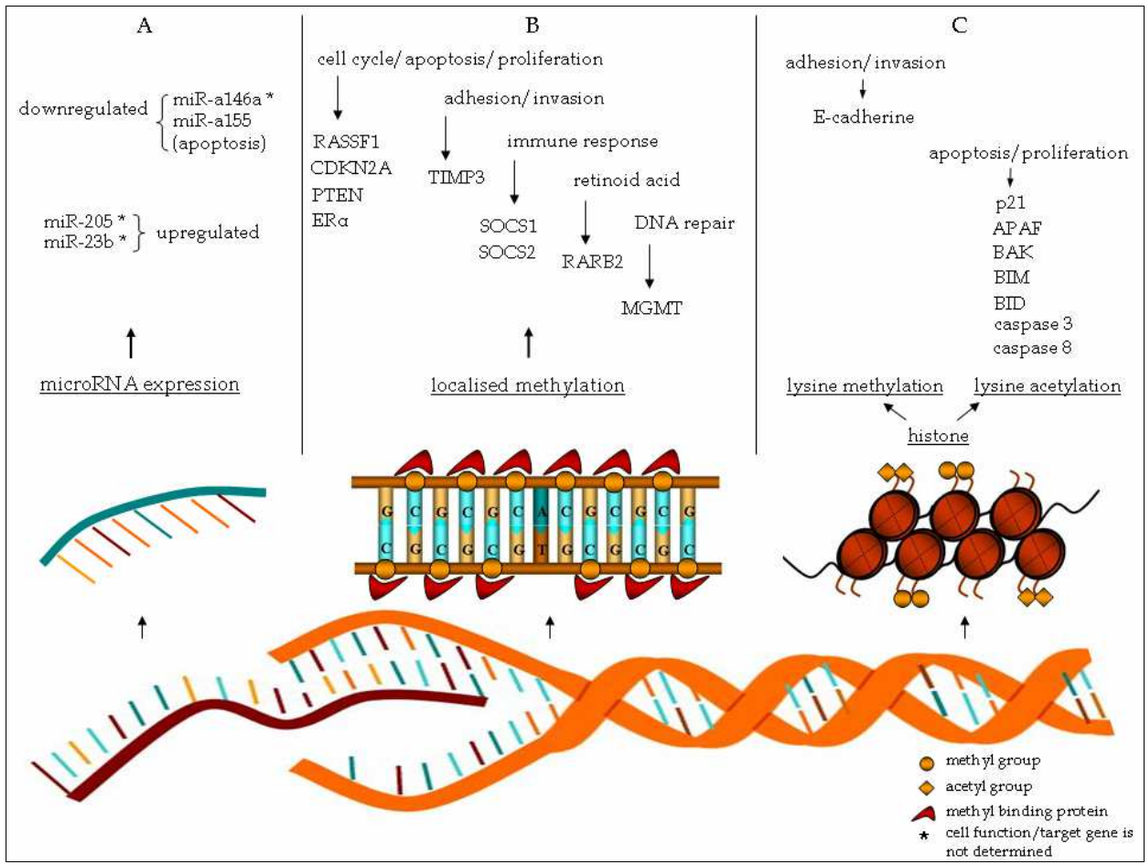

Fig. 3. Main regulatory elements and pathways involved in the three main epigenetic mechanisms in melanoma. A: microRNA regulation at post-transcriptional level results in decreasing in gene expression. B: localised methylation occuring at the promoter regions attracts methyl-CpG-binding proteins to construct transcriptionally silent heterochromatin and cause direct downregulation of genes involved in various pathways. C: histone post-transcriptional modifications alter gene expression of invasion and proliferation related molecules

added to a cytosine residue, methyl-CpG-binding proteins can attach to methylated regions. Therefore, a specific signature is constructed in assembling transcriptionally silent heterochromatin. It is widely accepted that DNA methylation of promoter regions, which are the main site of CpGs, can cause direct inactivation of specific genes [Howell et al., 2009; Sigalotti et al., 2010].

Because DNA methylation is described as a reversible mechanism, inhibition of promoter hypermethylation might represent the most promising therapeutic target for the treatment of melanoma. DNMT inhibitors are compounds that are able to demethylate 5-methyl-cytosines by the direct obstruction of DNMT enzymes. To date, 3 types of DNMT inhibitors have been characterised. 1. Nucleoside analogues were the first of these inhibitors to be developed and include 5-azacitidine (Vidaza) and 5-aza-2'-deoxycytidine (Decitabin). The common feature of these two drugs is their incorporation into DNA strands; because they contain an amino group instead of carbon at the 5 position of cytosine, they cannot be methylated. Currently, both molecules are undergoing phase I or phase II clinical trials in combination with either interferon α-2b or temozolomide. 2. Zebularine, a new type of nucleoside analogue, is less toxic compared to others because it does not contain an amino group. To date, only one study conducted on murine B16 melanoma has suggested a positive effect of Zebularine resulting in an increase in life span. 3. Non-nucleoside analogue DNMT inhibitors currently include procainamide and procaine. However, there are no data available related to their effect on melanoma. 4. Antisense oligonucleotides are substitute molecules for the DNMT1 enzyme (DNMT1 ASO), and they are currently undergoing preclinical drug testing [Howell et al., 2009; Sigalotti et al., 2010].

5.1.2 Localised hypermethylation

Given the existence of relatively easy approaches that require even minute amounts of tumour DNA, there are currently substantial amounts of data available that refer to gene silencing associated with the localised CpG hypermethylation of a specific gene promoter. There are two options for investigating this epigenetic phenomenon: it can be estimated indirectly or measured directly. Indirect assessment consists of three steps: first, measuring mRNA or protein expression; next, treating samples with a specific drug that acts against the process of methylation, mainly by inactivating DNMT3a (see above); and finally, measuring gene expression again. Powerful arguments have been presented in the literature that support direct experiments as being less ambiguous; additionally, because treatment is only possible in cell lines, tumour tissues are not appropriate for this purpose. In spite of the existence of a large dataset that has revealed more than 80 genes downregulated by promoter methylation, to allow their clinical utilisation, the detected elements must be distinguished based on the number of primary tumour samples involved in the study and the frequency of positive results as eligibility criteria for diagnosis or to determine whether they are a candidate therapeutic target. Promoter hypermethylation of two molecules involved in the cell cycle, Ras association domain-containing protein 1 (*RASSF1A*) and the cyclin-dependent kinase inhibitor 2A-coding gene (*CDKN2A*), has been confirmed by multiple, substantive experiments; these findings have also been confirmed in melanoma cell lines [Furuta et al., 2004; Marini et al., 2006; Sigalotti et al., 2010]. A higher level of methylation of oestrogen receptor alpha (*ERa*) compared to normal tissues has also been revealed in both tumour specimens and cell lines [Furuta et al., 2004; Mori et al., 2006]. Gaining 5-methyl-cytosines in the promoter regions of suppressor of cytokine signalling

molecules (*SOCS1* and *SOCS2*) has been demonstrated simultaneously with *MGMT*, which plays an essential role in DNA repair, and with *TIMP3*, which encodes a protein that protects the extracellular matrix from enzymatic degradation [Liu et al., 2008].
Downregulation of *RARB2* (retinoic acid receptor B2) has been validated in repeated studies: 6 groups have investigated the methylation level of the *RARB2* promoter, but their results are still inconsistent: some of these investigators have recorded high levels methylation in most of the examined specimens, while others have reported promoter methylation in only a few melanomas [Furuta et al., 2004; Liu et al., 2008]. One of the most remarkable studies concluded that *PTEN* methylation was a predictor for patient survival, even though it had not been associated with other clinical records. This study involved 230 primary melanomas [Lahtz et al., 2009].
In addition to the rapid progress that has been made in studying promoter hypermethylation at the single-gene level, only one group has attempted to conduct an array-based experiment, having chosen the most powerful and high-throughput bead array technology, to provide valuable information on the methylation pattern of the 1505 gene promoter. It is important to note that previous studies have given irrefutable proof of the reproducibility of this approach. It is regrettable that researchers have focused on only comparing the methylation level of primary invasive melanomas with benign melanocytes; therefore, no data are available on the methylation markers of diverse melanomas with different clinical behaviours. However, the findings of these investigators support the claim that a covalent change from cytosine to 5-methyl-cytosine in the promoter region occurs as an early aberration event in melanomas. The group's results have clearly identified a group of genes in a statistically powerful interpretation that can be used to discriminate nevi from melanomas considering their methylation signature. Furthermore, they adapted this high-throughput methylation profiling to FFPE samples, which are generally prepared by pathologists, abundantly available and appropriate for further routine screening [Conway et al., 2011].

5.1.3 Methylation patterns of cell-free DNA

It is notable that efforts are now under way to develop methods to measure the methylation of genes in body fluids that are easy to obtain. Additionally, it is firmly believed that the methylation pattern of a specific gene in cell-free DNA (serum or urine) should resemble the markings of that gene in tumour tissue. Regarding early diagnosis, this represents a rapidly growing field, and a blood test aimed at quantifying tumour suppressor genes has already been made commercially available for colon cancer samples. Similar attempts have also been made in melanomas: hypermethylation of *RASSF1A* in serum has been demonstrated, although 3 conflicting publications have reported that it occurs to a lesser (19%) or a greater (63%) extent in melanomas [Furuta et al., 2004; Liu et al., 2008]. In serum, *CDKN2A* has also exhibited hypermethylation to a considerably higher level than in tumour tissues [Marini et al., 2006].
Because one of the most notable previous investigations demonstrated the prognostic relevance of *PTEN* methylation (as described above), studies were extended to quantify its methylation pattern in serum. A significant correlation was found between the methylation level of tumour tissue specimens and blood serum. Moreover, *PTEN* methylation might function as an early event in carcinogenesis, rather than a progression-related mechanism, based on experiments that compared the methylation levels of tumours and melanocytes

[Mirmohammadsadegh et al., 2006]. As it has been firmly demonstrated that *PTEN* silencing is due to an epigenetic event [Furuta et al., 2004], although it may have little impact on tumour progression, *PTEN* appears to be the most relevant candidate for the early diagnosis of melanoma by non-invasive tools.

5.1.4 Genome-wide hypomethylation

While most groups are studying extensively promoter-related hypermethylation, the importance of genome-wide demethylation or hypomethylation remains underestimated. However, these phenomena might also reflect important epigenetic alterations due to their ability to cause genetic instability. Genome-wide hypomethylation is characterised by the overall loss of 5-methyl-cytosines, which is believed to correspond to the loss of methylcytosines of repetitive transposable elements. During evolution, these elements integrated into the human genome and became protected from transcription due to their higher levels of methylcytosines. Considering the abundance of repetitive elements, it is clear why scientists generalise the loss of their 5-methyl-cytosine content to the 'whole genome'. Repetitive elements constitute 40% of the human genome and mainly consist of two different types: among *SINE* (short interspersed nucleotide element) sequences, only the Alu family is known, ranging from 400 to 500 bp, and it can be found in 10^5 copies across the human genome; *LINE* (long interspersed nucleotide element) sequences are longer than 5000 to 6000 bp, and 10^4 copies of these elements are spread throughout the genome [Howell et al., 2009].

Investigations of demethylation in carcinogenesis have raised a crucial question: how can genome-wide hypomethylation contribute to genetic instability? As described above, the overall loss of 5-methyl-cytosines can be explained by the modification of repetitive elements exhibiting strong homology. As these elements are reactivated by hypomethylation, they can recombine with each other, causing karyotypic instability [Wild &Flanagan, 2010].

The cause of their demethylation remains in question, as current evidence is divided between two hypotheses: hypomethylation could be an important early cancer-causing aberration, or it might occur as a passive inconsequential side effect of carcinogenesis. Both theories have gathered supporting data. Based on animal experiments, DNMTs have a higher affinity for recognising and affecting damaged DNA; therefore, their normal function of maintaining methylation patterns during replication might fail, suggesting passive demethylation as a subsequent genetic alteration. For many years, it has been hypothesised that indirect proof of active demethylation is provided by the imprinting of gametes and early embryos, which undergo massive epigenetic reprogramming involving demethylation followed by remethylation [James et al., 2003; Wild & Flanagan, 2010]. Regrettably, studies on melanomas have been unable to give evidence of active demethylation. However, results from breast cancer studies are promising: oestrogen receptor-alpha-responsive genes show remarkable demethylation followed by remethylation under the influence of oestrogen treatment. These encouraging results might prompt scientists to extend their experiments to investigate the active demethylation of *ERa*-responsive elements in melanoma cell lines. As previous studies in melanoma have detected promoter hypermethylation of *ERa*, suggesting an important role of *ERa* epigenetics, in addition to hypermethylation, *ERa* might provide a significant contribution to the field of active demethylation [Méteiver et al., 2008].

To date the existence of genome-wide hypermethylation has been demonstrated in 16 melanoma cell lines compared to melanocytes via repetitive elements. We are unable to conclude what the actual clinical effects of genome-wide methylation as the above-

mentioned study focused only on comparing cell lines to controls, instead of drawing a demethylation-based distinction between cell lines with different characteristics. Our unpublished data also suggest a significant difference between normal tissues (nevus and skin) and melanoma specimens. However, aberrant hypomethylation may be the best marker for early diagnosis, as the results of a number of studies agree on the relevance of *LINE* hypomethylation in both cell lines and tissues [Tellez et al., 2009].

It is important to note that single-copy DNA regions can also feature hypomethylation, accompanied by mRNA and protein upregulation, as has been shown in colorectal, hepatocellular and breast cancers, although there is currently no evidence to suggest the same characteristics for melanomas.

5.2 Histone modification and microRNA regulation of melanoma

Eukaryotic DNA is packed into chromatin consisting of nucleosome units, each of which contains two copies of H2A, H2B, H3 and H4, as well as 146 bp of DNA. Each histone contains flexible N-terminal tails that can be a target for various post-translational modifications that determine the electrostatic power between DNA and histones and, therefore, contribute to determining transcriptionally active euchromatin or inactive heterochromatin. Histone modifications uniquely define the "histone code" that is read by multi-protein chromatin remodelling complexes to finally determine the transcriptional status of a target gene by modulating the chromatin compaction grade. While most studies rely on cell lines, there are preliminary results that show the importance of histone methylation in advance staged melanomas: a type of histone methyl-transferase (H3K27 HMT EZH2) has been found to be upregulated in tumours, and as it has an inhibitory effect on the transcription of *E-cadherin*, it could contribute to invasion [O'Neill et al., 2001; Sigalotti et al., 2010]. In addition to histone methylation, losses of histone lysine acetylation have also been reported. The hypoacetylation-related suppression of CDKN1A/P21 and the pro-apoptotic proteins (APAF-1, BAX, BAK, BID, BIM, caspase 3 and caspase 8) have also been described in melanoma cell lines [Feinberg et al., 2006].

Another promising field of melanoma epigenetics is related to gene regulation by microRNAs (miRNAs). These are small, non-coding RNAs transcribed in the nucleus into primary transcripts, followed by further cleavage by a complex of Drosha and its cofactor into precursor miRNAs (pre-miRNAs). Pre-miRNAs are exported to the cytoplasm, where Dicer protein reduces them to short double strands, which are then incorporated into RNA-induced silencing complexes that recognise target mRNA sequences, causing sequence-specific degradation and thus impairing their translation.

Only a single high-throughput experiment involving a relatively large set of samples has been performed on this topic [Levati et al., 2009]. However, the results of this study demonstrated inconsistency between cell lines and tumour tissues; moreover, the microarray results could not be confirmed by qPCR. Despite the high discrepancy in many cases, this group found that miRNA-205 and miRNA-23b were notably reduced, whereas miR-146a and miR-155 were upregulated only in tumour specimens compared to melanocytes and melanoma cell lines. Further functional studies have demonstrated the role of miRNA-155 in cell proliferation and survival [Levati et al., 2011].

Although strenuous efforts have been made to characterise the epigenetic changes involved in melanoma, studies have more often focused on elucidating alterations in DNA methylation than on estimating the functions of microRNAs and the realignment of histone molecules in melanomas. Despite the availability of a huge amount of data regarding

promoter-related hypermethylation, the function of this phenomenon related to progression is still controversial. Some experiments have shown that tumours associated with different clinical stages exhibit a characteristic methylation signature, while others have found that the patterns of 5-methyl-cytosine gains in promoter regions are rather homogeneous in melanomas, or exhibit minimal differences between various clinical subgroups of tumours. However, some studies have suggested that differential methylation patterns have a greater impact in driving melanocytic lesions to melanoma. According to the literature, the current state of our knowledge about genome-wide hypomethylation seems to be clearly outlined. Studies have already shown that hypomethylation of repetitive elements occurs as an early event in melanomas. Therefore, hypomethylation can offer a new facility that emphasises early detection in the case of applying a technical approach that may make rapid, systematic screening possible.

Despite the strong progress in this field, it is still difficult to provide a concise summary about the importance of epigenetic changes in melanomas, although it can be asserted that promising findings will prompt further efforts in designing additional studies to obtain better insights into how melanoma progression is controlled by epigenetic events. Long-term follow-ups of epigenetic drug treatments will be indispensable to characterise possible adverse effects of general demethylation.

Finally, it should be noted that epigenetic mechanisms do not act separately but represent different utility in an integrated apparatus: the results of most recent studies indicate more challenges for the design of future experiments, as they have revealed that DNA methylation can influence histone modification to maintain altered gene expression, and miRNAs could also be a target of DNA methylation, besides representing a unique epigenetic mechanism.

Although the genomic methods described above are mainly used in research laboratories, they are also employed in the clinical field as methods for patient selection in clinical trials and as predictive tests for the selection of treatments for some malignancies (e.g., Oncotype DX; Genomic Health, Redwood City, CA; MammaPrint, Agendia, Amsterdam, Netherlands). Integration of genomic datasets from different platforms, such as gene copy number and expression profiling data, represents a powerful method for the identification of functionally relevant molecular aberrations. However, genomic techniques do not supplant more targeted analytical methods. The complementary use of both of these approaches will be essential to identify and exploit molecular changes in cancer for improved diagnosis and treatment.

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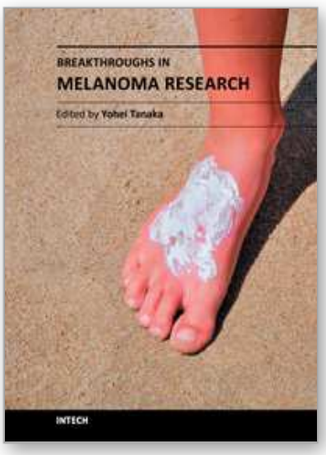

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Melanoma is considered to be one of the most aggressive forms of skin neoplasms. Despite aggressive researches towards finding treatments, no effective therapy exists to inhibit the metastatic spread of malignant melanoma. The 5-year survival rate of metastatic melanoma is still significantly low, and there has been an earnest need to develop more effective therapies with greater anti-melanoma activity. Through the accomplishment of over 100 distinguished and respected researchers from 19 different countries, this book covers a wide range of aspects from various standpoints and issues related to melanoma. These include the biology of melanoma, pigmentations, pathways, receptors and diagnosis, and the latest treatments and therapies to make potential new therapies. Not only will this be beneficial for readers, but it will also contribute to scientists making further breakthroughs in melanoma research.

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