Epigenetic alterations in the predisposition to and progression of melanoma

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General Introduction

MELANOMA

EPIDEMIOLOGY AND RISK FACTORS

Cutaneous melanoma is the most aggressive type of skin cancer and originates from melanocytes of the skin. It can present as four histopathological subtypes: superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, and acral lentiginous melanoma.[1] Over 50.000 melanoma-related deaths are registered worldwide annually and about 232.100 new cases are diagnosed. The incidence rates are generally increasing over the past 50 years.[2] The risk factors for melanoma include intermittent exposure to sunlight, sunburns and indoor tanning beds.[3-5] Besides the environmental factors, several phenotypic and genetic characteristics of an individual account for the risk of melanoma. They include fair skin, light eye and hair colour, propensity for freckles, presence of solar lentigines as a sign of actinic damage, the presence of melanocytic and dysplastic nevi and familial history of cutaneous melanoma.[6-9]

GENETIC ALTERATIONS IN MELANOMA

Cutaneous melanoma has one of the highest mutational burdens among all types of cancer, approximately 38.3 mutations/Mb with many ultraviolet signature mutations (C>T).[10,11] Melanomagenesis may follow a sequential genetic model from a visible pre-existing lesion but most melanomas develop *de novo*. *BRAF* V600E mutations are often already present in benign nevus, while the intermediate lesions harbour *NRAS* mutations and other additional mutations such as in the *TERT* gene promoter.[12] According to TCGA, 52% of melanomas harbour *BRAF* mutations, 28% present *RAS* mutations, 15% have mutation in the *NF1* gene and the remaining 10% are the triple-wild-type for the *BRAF, RAS* and *NF1* genes (triple-WT) melanomas. Low-frequency loss-of-function driver mutations in cell-cycle regulation and chromatin remodelling genes (*CDKN2A* and *ARID2)* as well as in *PTEN* or *TP53* genes are required for triple-WT advanced melanoma.[12-14] Also activating hotspot mutations in *KIT, CTNNB1* and *EZH2* genes were found in this subgroup.[11,13] *TERT*-promoter mutations occurred in 72-83% of the *BRAF, NRAS* and *NF1* mutant subtypes, but only 7% in triple-WT melanomas.[13]

Regarding copy number alterations, the *BRAF* mutant subtype shows amplifications of *BRAF, MITF, PD-L1* genes. As expected, for *NRAS* mutated subtype present the highest amplification of *NRAS*. The triple-WT subtype was significantly enriched for the amplification of 4q12 including *KIT*, co-amplification of *PDGFRA* and *KDR*, as well as *CDK4, CCND1, MDM2* and *TERT*.[13]

Overall, the mitogen-activated protein kinase (MAPK) and phosphoinositol 3-kinase (PI3K) pathways, RB1/CDKN2A cell-cycle pathways and MDM2/TP53 apoptosis pathways are the most affected in either *BRAF, NRAS* and *NF1* mutant melanomas or triple-WT subtype.[11,13]

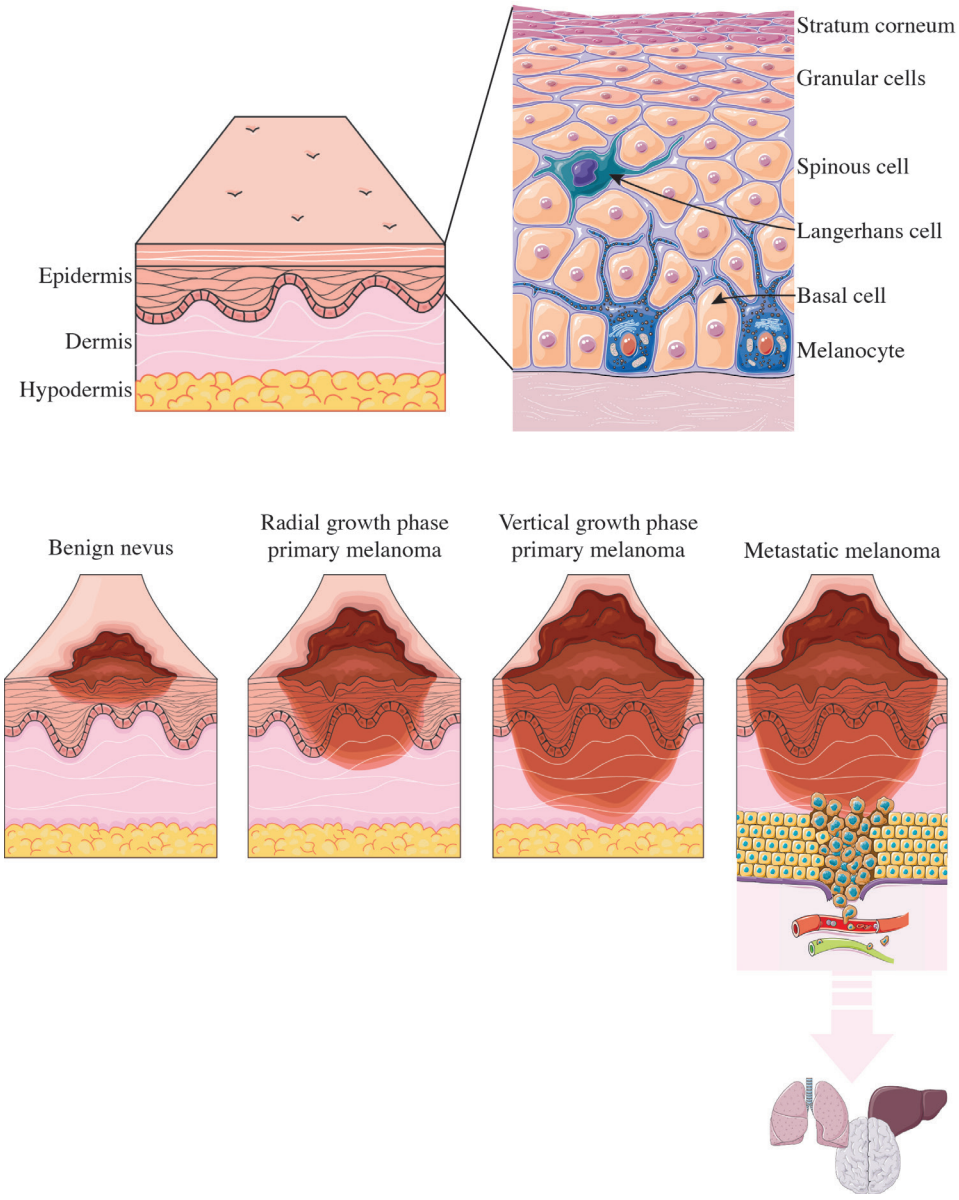

FIGURE 1. Schematic representation of the different layers and the cells that constitute the epidermis (top) and the stages in the development and progression of melanoma (bottom). Figure composed with Servier Medical Art images (https://smart.servier.com/).

PROGNOSIS AND TREATMENT

According to the AJCC classification, melanoma can be divided into 4 categories: stage

I for thin localized tumours, stage II a thick localized tumour, at stage III there are nodal metastases and stage IV for those with distant metastases.[15]

For stage I melanoma tumour thickness is the main criterium to define a precise prognosis, along with ulceration.[16,17] The first line of treatment for primary tumours consists of surgical excision with margins depending on Breslow thickness.[18] The complete lymph node dissection is performed when there is lymph node metastatic disease (macrometastases), but is no longer performed for sentinel node-positive melanoma (micrometastases, stage IIIa) since it does not significantly reduce the mortality rate.[19]

The prognostic value of *BRAF* and *NRAS* mutations remains quite unclear.[20,21] In general the *NRAS* mutant subset of melanomas are more aggressive and associated with poorer outcomes.[20,22] However, no efficient targeted therapy has emerged so far for this group of patients.[23] *BRAF* inhibitors are approved for advanced and metastatic *BRAF*-mutated melanomas, alone or in combination with MEK inhibitors (vemurafenib plus cobimetinib, dabrafenib plus trametinib and encorafenib plus binimetinib).[24,25] *BRAF* amplifications and *MEK1/2* mutations are the best described mechanisms that reactivate *MAPK* signaling pathway or activate *PI3K-AKT* pathway and resistance to targeted therapy remains a major issue.[26] *KIT*-mutant melanomas are commonly treated with imatinib or nilotinib. Immunotherapy in melanoma, especially the immune checkpoint inhibitors anti-*CTLA4* (ipilimumab) and anti-*PD-1* (pembrolizumab and nivolumab), has shown to be highly effective, also in NRAS-mutant tumours.[20,22] However, only a subset of patients has a complete response to this therapy and many of them show disease progression during treatment.[18]

FAMILIAL MELANOMA

Approximately 10% of patients diagnosed with melanoma mention a positive family history for this malignancy. Familial melanoma is arbitrarily defined as the occurrence of three or more melanomas in multiple members of a family, with at least two diagnosed in first-degree relatives.[27] Only approximately 50% of melanoma families can currently be attributed to pathogenic variants in high and medium penetrance melanoma genes such as *CDKN2A*, *CDK4*, *BAP1, TERT, POT1, ACD*, *TERF2IP* and *MITF*.[28] *CDKN2A* gene is the major contributor to melanoma susceptibility with ~39% of the families being affected by genetic alterations in this gene. Some years later alterations in CDK4 gene were also discovered as a causative event in some melanoma families. Over the last few years, new approaches using genomic sequencing technologies gave rise to identification of new pathways dysregulated in melanoma. The *CDK4* gene along with alterations in *BAP1, MITF, TERT, POT1*, *ACD* and *TERF2IP* constitute the genetic cause in about 10% of all familial clustering of melanoma.[18,28] Therefore, the genetic cause remains to be resolved in almost

half of the affected melanoma families.

The diagnostic testing to melanoma still relies on *CDKN2A* and *CDK4* susceptibility genes.[29] Clarifying the genetic basis of familial melanoma is clinically relevant as it would allow for genetic testing, risk estimation, counselling and targeted clinical surveillance of patients at high risk of melanoma. The genetic basis of familial melanoma might be uncovered by applying next generation sequencing methodology in families with a history of melanoma and by exploring different mechanisms of inheritance including heritable epigenetic alterations.

EPIGENETICS

The term epigenetics refers to the changes in the genome, affecting gene function and expression, that do not affect the DNA sequence.[30]

EPIGENETIC REGULATION OF GENE EXPRESSION

The epigenetic regulation of gene expression relies on three distinct levels: DNA (hydroxy) methylation (covalent modifications of DNA bases), histone modifications (post-translational modifications on the amino-terminal tail of histones) and chromatin remodelling.[31]

In the nucleus of eukaryotic cells, genomic DNA and histones form the nucleosomes, the basic complexes of chromatin. Different levels in chromatin organization have been described, from nucleosome array, an 11-nm “beads-on-a-string fiber” conformation, to a more condensed 30-nm chromatin fiber, after binding of H1 and H5 linker histones.[32,33] Chromatin plasticity and dynamics has an essential role in regulation of gene expression by influencing the binding of transcription factors.[34] Active gene transcription takes place when chromatin is in the loose form, euchromatin, as this conformation enables the binding of transcription factors. However, when chromatin acquires a very condensed form, called heterochromatin, transcription is repressed.[34]

Cancer genomes are characterized by hypermethylation of promoter CpG islands that interferes with transcription factors binding and enhances heterochromatin formation which therefore impair gene expression.[35,36] Otherwise, methylation of CpG sites located in the gene body are correlated with active gene expression.[37]

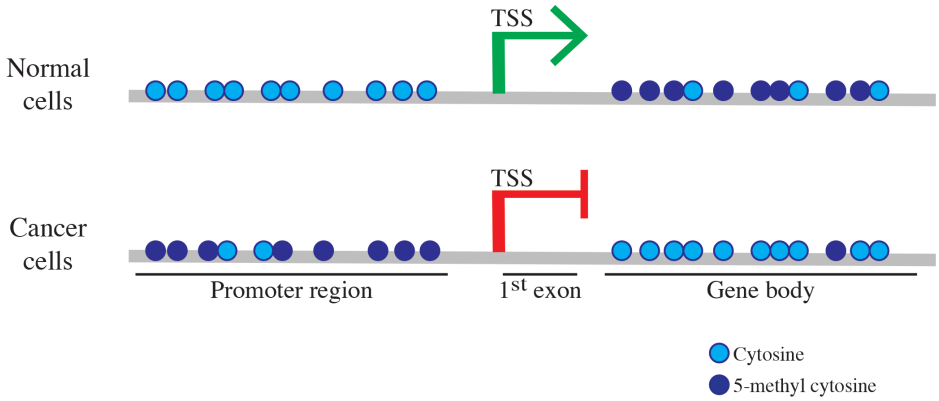

FIGURE 2. Schematic representation of a gene promoter region in a normal cell and a cancer cell. The aberrant increase of methylation (hypermethylation) in the cancer cell often leads to transcriptional silencing of the gene.

DNA METHYLATION AND HYDROXYMETHYLATION IN CANCER

Epigenetic mechanisms are also involved with progression of melanoma. As mentioned, DNA methylation, histone modifications and chromatin remodelling complexes regulate gene expression programs. DNA methylation can be divided into *de novo* or maintenance of methylation upon cell division and is mainly regulated by DNA methyltransferases (DNMTs).[38] However, DNA methylation at CpG dinucleotides is not only mediated by DNMTs but additionally governed by DNA demethylation. DNA demethylation is the reverse process of methylation, thus consists in discarding the methyl groups from the CpG dinucleotides. This process can occur in a passive or an active way. Passive demethylation occurs when there is insufficient methyltransferase activity during replication. Active demethylation involves three consecutive steps of oxidation from 5-methylcytosine (mC) into 5-hydroxymethylcytosine (hmC), 5-formylcytosine (fC) and 5-carboxylcytosine (caC) performed by the Ten Eleven Translocase (TET) family of dioxygenase enzymes.[39,40] 5-fC and 5-caC are then recognized by the thymine DNA glycosylase (TDG) that activates the base excision repair pathway responsible for the replacement of the altered cytosine by a 'regular' cytosine.[41] TET1, TET2 and TET3 constitute the TET protein family and require α-ketoglutarate (αKG) as a co-substrate. In its turn, αKG is produced by isocitrate dehydrogenase (IDH)1, 2 and 3 proteins. While approximately 4% of all cytosines are methylated, only 0.1% - 0.7% of cytosine bases are hydroxymethylated in mammalian cells.[42] At first, hmC was assigned as a mere intermediate in the demethylation. Nowadays, different studies have pinpointed strong arguments in favour of a proper hmC role in the cell: the stability of hmC in the genome of *in vivo* and cultured cells, specific hmC-binding proteins as well as the abundance of hmC in neuronal cells.[43-46]

Although the low fraction of cytosines affected by this epigenetic modification throughout the genome, reduced levels of hmC in different cancer types, such as breast, liver, lung, pancreatic, colon, prostate, compared to precursor lesions were recently reported.[47] Namely in melanoma it has been reported that low levels of hmC were associated with worse survival.[48] The reasons behind hmC reduction have been explored by different approaches and targets. Some studies report that mutational inactivation and/or downregulation of *TET2* might explain the loss of hmC.[48,49] Also IDH proteins seem to have a role in this depletion. While wild-type IDH protein produces αKG, the co-substrate of TET enzymes, the mutant IDH transforms αKG into R-2-hydroxyglutarate, a oncometabolite that is a competitive inhibitor of TET. However, only 10% of melanomas harbour mutations in *IDH* genes.[50] Resetting the differential mC and hmC levels towards a functional demethylation pathway might be an interesting target to cancer therapy.[51]

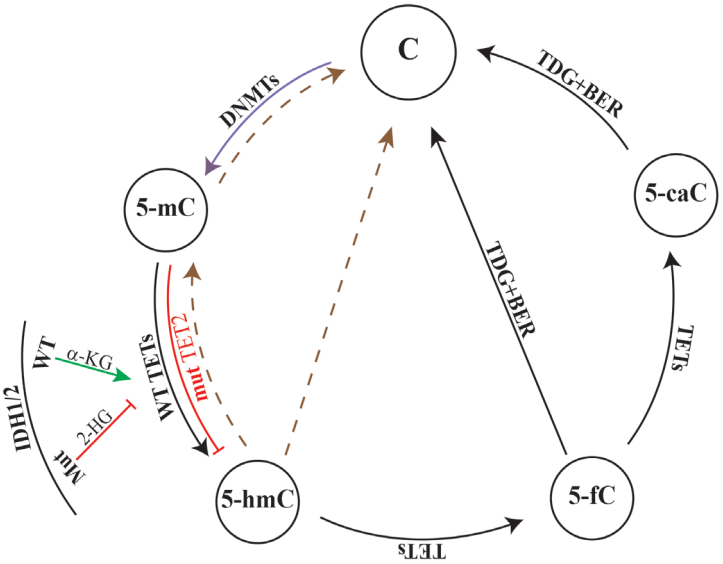

FIGURE 3. DNA methylation and demethylation pathways. Purple arrow: The enzyme DNA methyltransferase (DNMT3A/B and DNMT1) catalyzes the addition of a methyl group to the fifth carbon atom within the pyrimidine ring of the cytosine base to yield 5-methylcytosine (5-mC); Brown dashed arrows: passive demethylation during cell division. Black arrows: active demethylation pathway in which TET (TET1/2/3) proteins convert 5-mC into 5-hmC, 5-fC and 5-caC through three consecutive oxidation reactions. Then, 5-fC and 5-caC are recognized by thymine DNA glycosylase (TDG) proteins which activate the base excision repair (BER) pathway responsible for the replacement of the altered cytosine by a 'regular' cytosine. TET protein family require α-ketoglutarate (α-KG) as a co-substrate that is produced by wild-type isocitrate dehydrogenase (IDH) proteins. The mutant IDH transform it into R-2-hydroxyglutarate (2-HG), a oncometabolite that is a competitive inhibitor of TET.

EPIMUTATIONS

Throughout the last decade, the application of next generation sequencing methodology has identified new germline mutations, however it did not reveal all causative genetic alterations that might explain the predisposition to melanoma in families.[52] For this reason, the attention has turned to different causes of inheritance including heritable epigenetic alterations.

Epimutations have been defined as heritable changes in gene activity due to DNA modifications, not encompassing changes in the DNA sequence itself.[53] It has been postulated to constitute an alternative mechanism to genetic mutation for cancer predisposition and commonly refers to constitutional promoter CpG island hypermethylation in all somatic cells of an individual.[54] This mechanism of an inherited epigenetic alteration was firstly seen in patients of hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome), who were not affected by the inactivating mutations in DNA mismatch repair genes but by heritable promoter hypermethylation of the *MLH1* gene.[55-57]

Epimutations have been classified as primary, occurring in the absence of an underlying DNA sequence alteration, and secondary, when a genetic mutation triggers the occurrence of an epigenetic modification. Epimutations found in *MSH2* and *DAPK1* genes in HNPCC and familial chronic lymphocytic leukemia, respectively, are examples of secondary epimutations.[58-60]

EPIGENETIC REGULATION OF AN ESSENTIAL GENE IN MELANOMA, *TERT*

In the past few years, we have witnessed the growing importance of non-coding mutations in cancer.[61] The telomerase reverse transcriptase (*TERT*) gene promoter (*TERT*p) mutations (chr5:1,295,228 C>T and chr5:1,295,250 C>T in hg19) are a recognized example.

Approximately 90% of all human cancers share the transcriptional reactivation of the *TERT* gene.[62,63] *TERT* encodes the catalytic subunit of the ribonucleoprotein telomerase and is capable of extending the repetitive, non-coding DNA sequence on terminal ends of chromosomes, the telomeres. Telomeres become shorter at each cell division, but through *TERT* reactivation, the cells keep the ability of extending their telomeres or prevent their shortening. This telomere maintenance is one of the hallmarks of cancer.[64-66]

By creating new binding motifs for the transcription factor E26 transformation-specific/ternary complex factor (ETS/TCF), the two point mutations in the *TERT*p lead to a two-fold increase in *TERT* expression, resulting in maintenance of telomere length and ultimately in immortalization.[67-69] These mutations were first identified in melanoma and are

mutually exclusive. They are located at –124 bp and –146 bp from the translation start site (chr5:1,295,228 C>T and chr5:1,295,250 C>T in hg19, respectively).[68]

Aside from *TERT*p mutations, *TERT*p methylation has been widely explored. Remarkably, *TERT*p hypermethylation performs an opposite role enhancing gene expression, as transcriptional repressors rely on unmethylated promoter CpGs, such as CCCTC-binding factor (CTCF)/cohesin complex or MAZ.[70-72] In combination with transcription factor binding, dissociation of the repressor may result in *TERT* expression.[64,73-75]

The methylation of a specific CpG in the *TERT*p region was found to be correlated with progression and poor prognosis in paediatric brain tumours and later with *TERT* expression in tumour samples with no somatic alterations.[64,74]

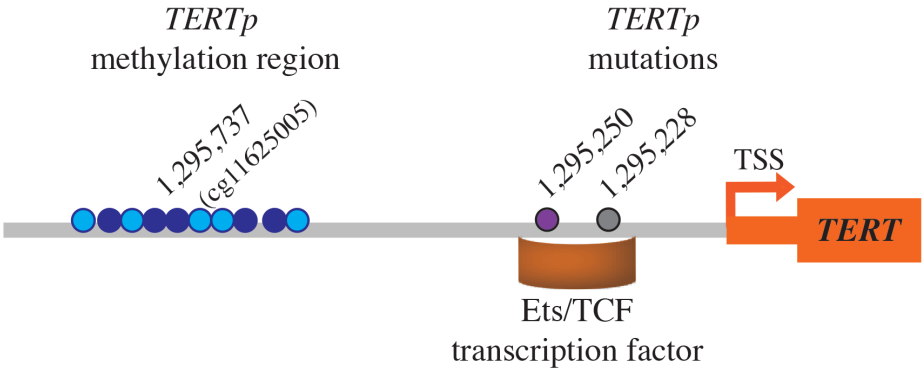

FIGURE 4. Schematic representation of *TERT* promoter region with the relative positions of cg11625005 (position 1,295,737 in hg19) to the *TERT*p mutations (position 1,295,228 and 1,295,250) and the transcription start site (TSS).

THESIS OUTLINE

With the present thesis we aim to reveal new melanoma susceptibility genes and heritable epigenetic alterations that could explain the melanoma predisposition in a significant proportion of melanoma-affected families in which the cause is still unknown. Moreover, we also intend to explore at which extent epigenetic alterations, namely the hydroxymethylation, are involved in the progression from benign lesions to melanoma. Finally we wonder what is the role of genetic and epigenetic mechanisms and their interplay in regulating *TERT* gene expression in both healthy skin and in melanoma cell lines.

In **Chapter 2**, we aim to identify a new melanoma susceptibility gene, the genetic cause that co-segregates with melanoma in a family with multiple melanoma-affected members.

In **Chapter 3**, we investigate whether inherited epigenetic events are potential explanation for melanoma predisposition in families with history of melanoma.

In **Chapter 4**, we aim to find new diagnostic and prognostic markers based on differential hydroxymethylation patterns by comparing nevus and melanoma.

In **Chapter 5**, we address how *TERT*p mutations and *TERT*p methylation along with chromatin accessibility are able to trigger *TERT* expression in healthy skin and melanoma cell lines.

Finally in **Chapter 6**, a summary of Chapters 2 to 5 is presented in light to previous literature. We discuss to which extent the aforementioned aims have been met and what additional experiments are needed to answer remaining questions.

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