Epigenetic alterations in the predisposition to and progression of melanoma

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I am among those who think
that science has great beauty.

Marie Curie, 1867-1934

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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]

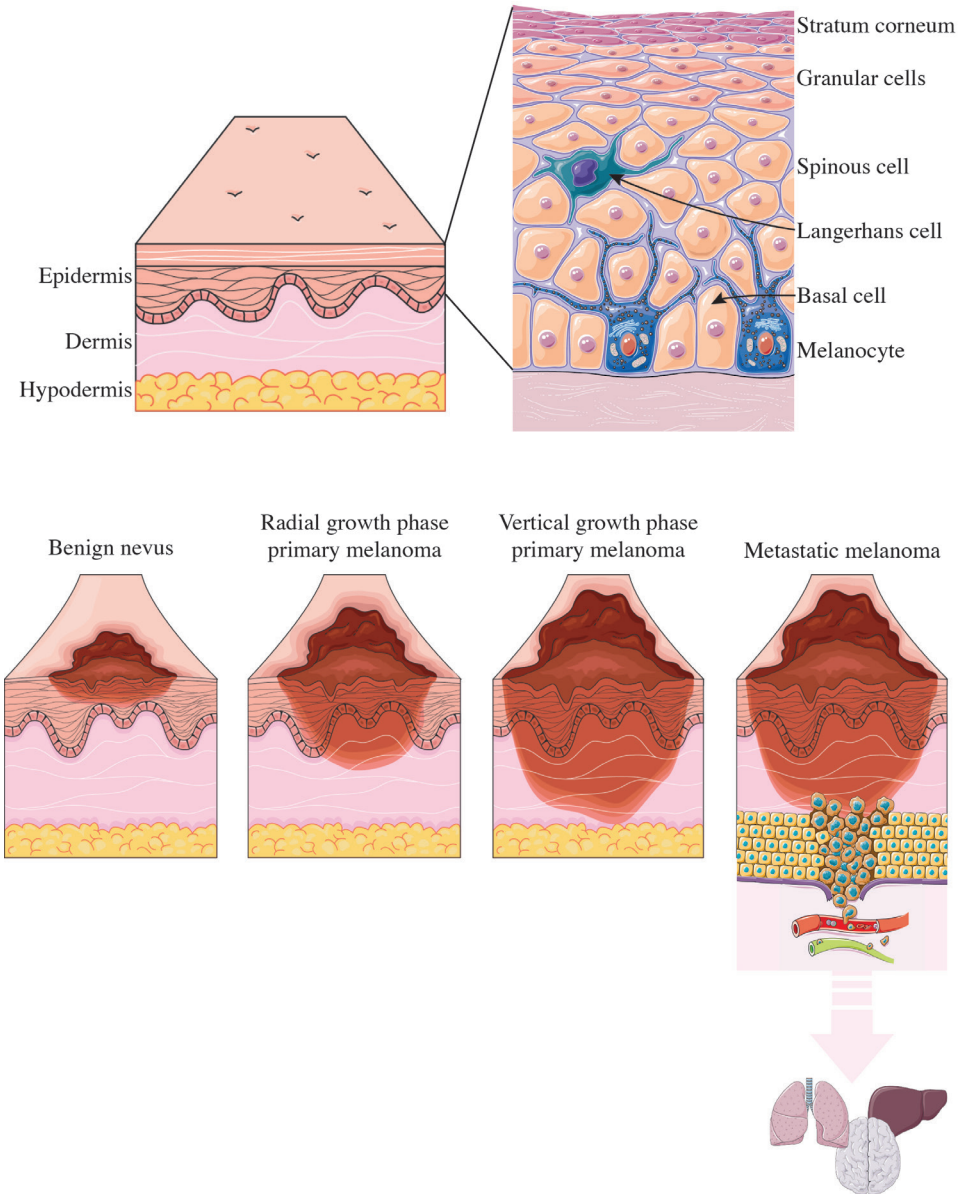

FIGURE 1. Schematic representation of the different layers and the cells that constitute the epidermis (top) and the stages in the development and progression of melanoma (bottom). Figure composed with Servier Medical Art images (https://smart.servier.com/).

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]

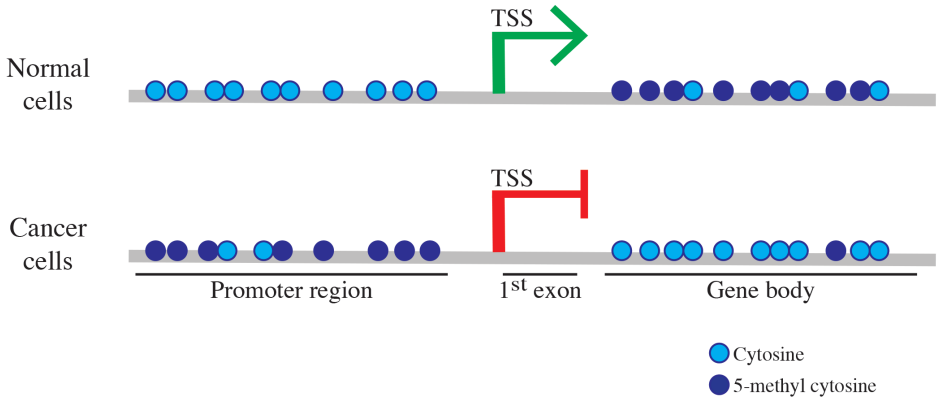

FIGURE 2. Schematic representation of a gene promoter region in a normal cell and a cancer cell. The aberrant increase of methylation (hypermethylation) in the cancer cell often leads to transcriptional silencing of the gene.

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]

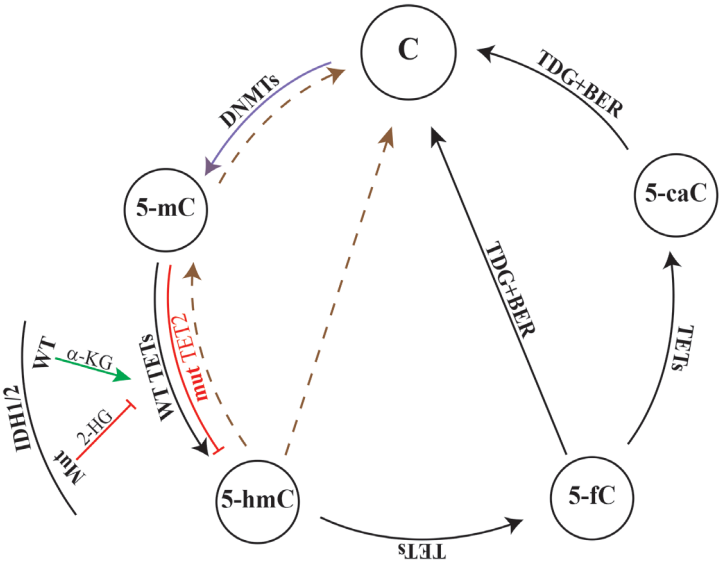

FIGURE 3. DNA methylation and demethylation pathways. Purple arrow: The enzyme DNA methyltransferase (DNMT3A/B and DNMT1) catalyzes the addition of a methyl group to the fifth carbon atom within the pyrimidine ring of the cytosine base to yield 5-methylcytosine (5-mC); Brown dashed arrows: passive demethylation 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]

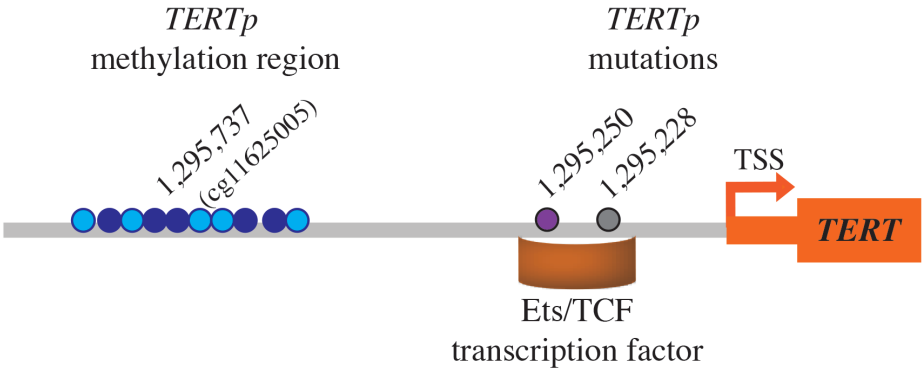

FIGURE 4. Schematic representation of *TERT* promoter region with the relative positions of cg11625005 (position 1,295,737 in hg19) to the *TERT*p mutations (position 1,295,228 and 1,295,250) and the transcription start site (TSS).

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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2

A novel germline variant in the *DOT1L* gene co-segregating in a Dutch family with a history of melanoma

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ABSTRACT

A proportion of patients diagnosed with melanoma has a positive family history. Despite increasing knowledge on the genes responsible for familial clustering, the genetic basis in the majority of the families with an inherited predisposition to melanoma remains to be clarified. To identify novel melanoma-susceptibility genes we applied whole exome sequencing (WES) on DNA from two members of a family with four melanoma cases, not explained by established high penetrance melanoma-susceptibility genes. WES identified 10 rare, co-segregating, predicted deleterious missense gene variants. Subsequent co-segregation analysis revealed that only variants in the *DOT1L* (R409H) and the *SLCO4C1* (P597A) genes were present in the other two affected members of this family. DOT1L is a methyltransferase that methylates histone H3 lysine 79 (H3K79). It is involved in maintenance of genomic stability, since mutations in the *DOT1L* gene have been previously reported to compromise the removal of UV photoproducts in UV-irradiated melanocytes, thereby enhancing malignant transformation. We hypothesized that the presence of *DOT1L* R409H variant might be associated with an increased risk of melanoma, since we found co-segregation of the *DOT1L* mutation in all four melanoma-affected family members. However, this missense variant did neither lead to detectable loss-of-heterozygosity nor reduction of histone methyltransferase activity in melanoma samples from mutation carriers nor altered UV-survival of mouse embryonic stem cells containing an engineered homozygous *DOT1L* R409H mutation. Although functional analysis of this rare co-segregating variant did not reveal compromised histone methyltransferase activity and UV exposure sensitivity, the role of *DOT1L* as melanoma susceptibility gene deserves further study.

KEYWORDS

DOT1L, familial melanoma, histone methyltransferase, whole-exome sequencing

INTRODUCTION

Cutaneous melanoma is an aggressive form of skin cancer and the leading cause of death among all skin cancer patients.[1] Approximately 10% of melanoma cases present familial clustering. In Europe, familial melanoma is defined as the occurrence of three or more melanomas in multiple members of a family, at least two of which are diagnosed in first-degree relatives. Thus far only in ~50% of melanoma families the melanoma susceptibility can be attributed to a genetic defect in the high and medium penetrance melanoma genes such as *CDKN2A*, *CDK4*, *BAP1, TERT, POT1, ACD*, *MITF* and *TERF2IP*.[2] Clarifying the genetic basis of melanoma predisposition is of major clinical importance since new genetic testing can be approved and more personalized surveillance offered to the patients.[3] Exome-wide sequencing approaches can be valuable in the identification of putative new-melanoma susceptibility genes.[4]

In the present study, we describe a Dutch family of which four family members were diagnosed with melanoma. Whole-exome sequencing (WES) of DNA from two family members identified a new germline missense variant c.G1226A:p.R409H in the *DOT1L* gene, that co-segregated with melanoma in all 4 affected family members. DOT1L is the unique histone methyltransferase responsible for methylating the nucleosome core on lysine 79 of histone H3 (H3K79).[5,6] The observed *DOT1L* variant appeared to be the most promising pathogenic variant since recently loss of *DOT1L* (by silencing or mutation) has been reported to promote melanomagenesis in a pre-clinical mouse model upon UV radiation.[7] The role of DOT1L in DNA damage repair pathway involves the transcriptional recovery through reactivation of RNA Pol II in mouse-derived cell lines[8] and the recruitment of *XPC* for an efficient nucleotide excision repair in melanocytes and cell lines derived from human melanoma, thereby protecting melanocytes from the UV-induced transition to melanoma.[7]

We hypothesized that in the family under study, the R409H *DOT1L* variant represents a loss of function mutation which diminishes the protective role of DOT1L enhancing melanoma development.

METHODS

PATIENTS

DNA from members of a Dutch family with four family members affected with melanoma was isolated from whole blood samples, a primary tumor and a brain metastasis. The study was approved by the Leiden University Medical Center institutional ethical committee

(LUMC, P00.117). The affected family members were tested negative for variants in the high penetrance genes *CDKN2A* and *CDK4* and cases II.2 and III.3 were subjected to WES (Figure 1).

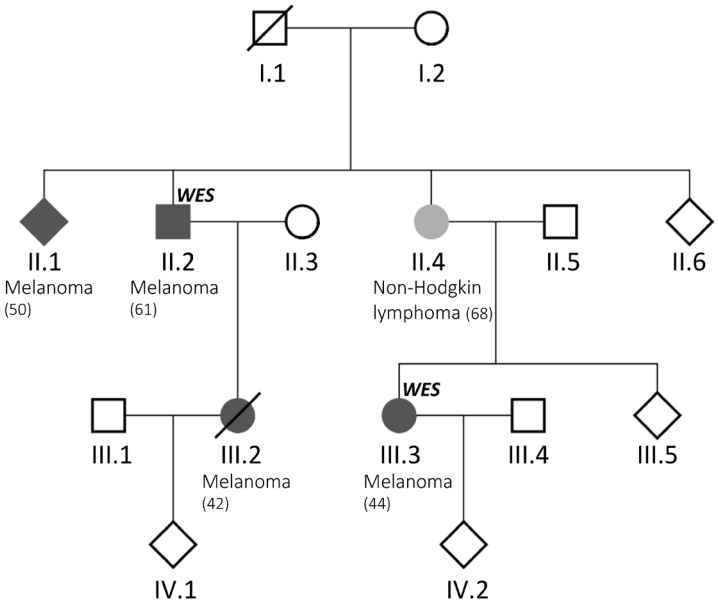

FIGURE 1. Family pedigree. The melanoma affected members are in dark grey color (II.1, II.2, III.2, III.3). The age of diagnosis is indicated in brackets. The melanoma cases subjected to whole-exome sequencing are indicated by an 'WES'.

WHOLE EXOME SEQUENCING

Whole-exome sequencing was performed using Agilent All-exon capture baits (Agilent, California) and sequenced on the Illumina platform at Sanger Institute, Cambridge, UK. The bioinformatics analysis and subsequent filtering steps were performed at Sanger Institute and later confirmed by our in-house bioinformatics pipeline. Briefly, the reads were aligned to the human genome build hg19 using BWA.[9] To pass the filtering steps the variants needed to have a high-quality score (>30), to have high coverage (>40X), to be a nonsynonymous single nucleotide variant (SNV), to be heterozygous present in both samples, to have low ExAC frequency (<0.001), and needed to be predicted as deleterious and damaging by Polyphen and SIFT. Variants which did not fulfil all the filtering criteria were excluded, resulting in a list of 10 variants of interest (Table 1).

TABLE 1. Germline variants identified by whole exome sequencing shared by two affected family members of a 4 case Dutch melanoma family.

Gene	Location	Variant	Amino acid substitution
SLCO4C1	Chr5: 101582978-101582978	G>C	P597A
PEX6	Chr6: 42932102-42932102	G>A	R972C; R884C
FBXL13	Chr7: 102462622-102462622	G>A	S583L; S600L; S628L
NAIF1	Chr9: 130825802-130825802	G>A	R297C
LAMC3	Chr9: 133914340-133914340	C>T	R356C
CIT	Chr12: 120152035-120152035	C>T	V1383M; V1425M
FREM2	Chr13: 39433637-39433637	C>T	R2477W
DOT1L	Chr19: 2210729-2210729	G>A	R409H
FUT1	Chr19: 49253896-49253896	C>A	V215F
UMODL1	Chr21: 43508479-43508479	G>A	V155M; V227M

CO-SEGREGATION ANALYSIS

All 10 variants were confirmed by Sanger sequencing using DNA from the 2 family members subjected to WES (II.2 and III.3, see Supplementary Figure S1). Briefly, 20-100 ng of DNA was amplified through a touchdown PCR using the Platinum Taq DNA Polymerase following the manufacturer's instructions (Invitrogen, California, USA). The PCR product was cleaned-up using the NucleoSpin Gel and PCR Clean-Up (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to manufacturer's instructions. Then, Sanger sequencing was performed using 20-50 ng of purified DNA mixed with 1 μL of 10 μM of sequencing primer and nuclease-free water (B. Braun, Melsungen, Germany) up to 10 μL. Later on, we used the same approach to evaluate the co-segregation of these variants with melanoma in all affected family members (see Supplementary Figure S2 and Figure 2)

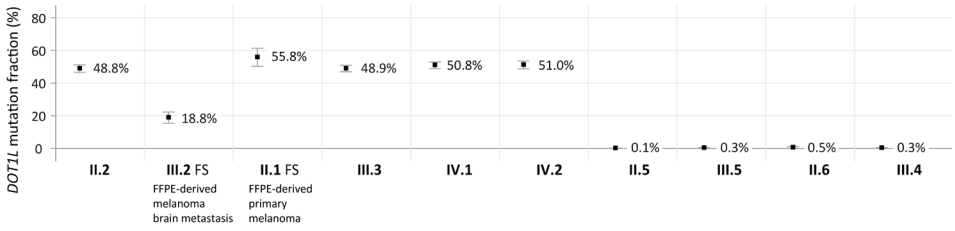

FIGURE 2. Droplet digital PCR results showing the *DOT1L* mutation fraction in DNA samples extracted from whole blood samples [II.2, III.3 (both subjected to WES) and IV.1 and IV.2], a primary melanoma (II.1 FS) and a brain metastasis (III.2 FS). The mutation fraction is around 50% in mutation-carriers and around 0% in wild-type family members (II.5, II.6, III.4, III.5), as control samples. In a full section (III.2 FS) of the brain metastasis, a mutation fraction of ~20% was found. The primary melanoma (II.1 FS) is mutated for *DOT1L*, however, no LOH was observed.

LOSS OF HETEROZYGOSITY (LOH) ANALYSIS

Loss of heterozygosity was assessed by droplet digital PCR (ddPCR). Tumors from two family members were examined: a FFPE-derived primary melanoma biopsy from II.1 and a brain metastasis from III.2 (Figure 2). The DNA extraction was performed using Tissue Preparation System (Siemens, Germany) at the department of pathology, LUMC. Briefly, 10 ng of DNA was combined with 1X ddPCR Mut Assay *DOT1L* R409H (dHsaMDS130625855; Bio-Rad Laboratories, Inc., Hercules, California, USA), 1X ddPCR supermix for probes (no dUTP) (Bio-Rad), 1U/μL MseI restriction enzyme [New England Biolabs, Inc. (NEB), Ipswich, MA, USA] diluted in its own buffer CutSmart (NEB) and nuclease-free water (B. Braun) up to 22 μL. To generate droplets the Automated Droplet Generator (Bio-Rad) was used, followed by the PCR using the cycling conditions for Bio-Rad's C1000 Touch Thermal Cycler (Bio-Rad) with an annealing temperature of 55°C. The number of droplets was determined by the QX200 Droplet Reader (Bio-Rad) and analysed using QuantaSoft version 1.7.4.0917 (Bio-Rad).

IMMUNOHISTOCHEMISTRY

For immunohistochemistry (IHC, see Supplementary Figure S3), sections from FFPE-derived primary melanomas from II.1 and brain metastasis from III.2, along with EAF (ethanol, acetic acid, formol saline) fixed and paraffin embedded thymus tissues from mice with conditional deletion of Dot1L as described in [10] were pre-incubated with goat serum (Dako, Agilent Technologies, California, USA) for 30 minutes and then incubated overnight with H3K79me2 antibody (1:8000 dilution, RRID:AB_1587126[11]) followed by incubation with Dako EnVision+ System HRP labeled polymer anti-rabbit (Agilent Technologies) for 30 minutes. The slides were washed with phosphate-buffered saline, incubated with Dako 3,3'-diaminobenzidine (DAB) substrate chromogen system (dilution 1:50, Agilent Technologies), and counterstained with hematoxylin (Merck KGaA, Darmstadt, Germany).

CELL LINES GENERATION

IB10 wild-type mouse embryonic stem cells (mESCs) were used to engineer site specific mutations using CRISPR/Cas9 to generate mESC lines expressing *DOT1L* R409H or the catalytic site mutant *DOT1L* G165R according to the protocol described by Harmsen *et al.*[12] IB10 mESCs were cultured on a feeder layer of irradiated murine embryonic fibroblasts (MEFs) in complete medium containing GMEM-BHK12 (Gibco/Thermo Fisher Scientific, Massachusetts, USA), 100 mM Sodium Pyrovate (Gibco), non-essential amino acids (Gibco) and 10% ES cell certified serum (HyClone/Thermo Fisher Scientific). This was complemented with 0.1 μM β-mercapthoethanol (Sigma-Aldrich, Missouri, USA) and mouse recombinant leukemia inhibitor factor (Merck KGaA). For transfection, cells were grown on gelatin coated plates in 60% BRL medium (150 mL Buffalo Rat Liver conditioned medium + 100 mL complete medium). Cells were incubated at 5% CO_2, at 37°C.

Oligonucleotides encoding the gRNAs are in Supplementary Table S1. Single strand homology directed repair (HDR) templates are in Supplementary Table S2. The repair templates were purchased from Sigma-Aldrich, all other oligonucleotides from Integrated DNA Technologies, Inc., Illinois, USA. The gRNAs were cloned into the px330.pgkpuro vector (a gift from Hein te Riele). A mixture of 0.1 µg CRISPR/Cas9 vector and 0.4 µg homology directed repair template in optiMEM (Gibco) with 1.25 µL TransIT LT1 (Mirus Bio LLC, Wisconsin, USA) was incubated for 15-20 min at room temperature and added to the cells. The next day cells were replated in 60% BRL medium containing 3.6 µg/mL puromycin. Two days later the medium was replaced with medium without puromycin. Cells were then sparsely seeded to grow single clones. After 1 week, single clones were selected and genomic DNA was isolated to validate the mutations, which also introduced restriction sites. The regions containing the R409H and G165R mutations were amplified using MyTaq Redmix (GC-Biotech B.V., Alphen aan den Rijn, The Netherlands) and the following primers: R409H – 5'TGCCTCAGCCTATGGTCTTGT and 5'TGGCACATGGCAGAGTCCCATA, for G165R – 5'ACTACACAGCCCATGAAGCTGA and 5'TGGTTAAGCAGCCACAACCCA. The PCR product containing the R409H region was digested with MlcI (Thermo Fisher Scientific) directly after amplification. PCR products containing the G165R region were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturers protocol and then digested with BauI (Thermo Fisher Scientific). Clones that showed the expected digestion pattern were further validated using Sanger sequencing. For UV-survival assays three clones with the R409H mutation were selected and two clones that had the G165R mutation, of which one had a homozygous G165R and one clone with a heterozygous G165R mutation and one nucleotide deletion causing a frame-shift in *DOT1L*.

UV-SURVIVAL ASSAY

To assess UV sensitivity, we performed a colony formation assay upon UV-C exposure in wild-type and CRISPR/Cas9 engineered *DOT1L* mutant mESCs. For UV-survival assays mESCs were cultured in 60% BRL conditioned medium. One thousand cells were plated in a 10-cm dish and grown overnight. The next day cells were washed with PBS and exposed to UV-C irradiation (254 nm, UV-C irradiation chamber, Dr Gröbel UV-Elektronik, GmgH, Germany; dose range: 0.5, 1, 2, 4, 8 J/m^2). After eight days of incubation the colonies were fixed and stained using Leishman's eosin methylene blue solution modified (Merck KGaA). Colonies were counted with the ColCount (Oxford Optronix Ltd., Abingdon, UK).

WESTERN BLOT

Murine ESCs were grown in feeder-free conditions in serum free ES cell medium containing neurobasal medium (Thermo Fisher Scientific), DMEM/F12 (Thermo Fisher Scientific), N2 supplement (Thermo Fisher Scientific), B27 (Thermo Fisher Scientific) and BSA (Thermo

Fisher Scientific) supplemented with GSK inhibitor CHIR99021 (BioConnect B.V., Huissen, The Netherlands) and MEK1&2 inhibitor PD0325901 (BioConnect) and cell pellets were frozen. Lysates were made using 1X SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol) and sonicated. Samples were boiled for 5 min in 5X SDS-sample buffer (250 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 0.5 M DTT, 0.5% bromophenol blue) and separated on a 16% polyacrylamide gel. Separated proteins were transferred on a 0.45 µm nitrocellulose membrane for 1h. Membranes were blocked using 2% Nutrilon (Nutricia/ Danone, Schiphol, The Netherlands) in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 1h and incubated overnight with anti-H3K79me1 (RRID: AB_2631105), anti-H3K79me2 (RRID: AB_2631106) or anti-H3 (ab1791, Abcam, Cambridge, UK). All antibodies were diluted 1:100 in TBST containing 2% nutrilon. After incubation, the membranes were washed three times with TBST and incubated with IRDye 800CW goat anti-Rabbit igg (1:10 000, Li-COR (RRID: AB_621843)) for 45 min in TBST with 2% nutrilon. Then, the membranes were washed three times with TBST and once in PBS and scanned using an LI-COR Odyssey IR Imager (LI-COR Biosciences, Nebraska, USA). Images were analysed using Image studio 2.0 (LI-COR Biosciences).

RESULTS

R409H, A NOVEL GERMLINE VARIANT OF THE *DOT1L* GENE IDENTIFIED IN A 4-CASE MELANOMA FAMILY

Upon WES of the DNA from two melanoma cases (II.2 and III.3, Figure 1), 4892 heterozygote variants were found. The bioinformatics analysis encompassed the alignment to genome build hg19 and the above-mentioned filtering criteria. Frameshift and truncating variants were found but did not pass the ExAC filter. Only 10 rare, co-segregating, predicted deleterious missense variants in the genes *SLCO4C1, PEX6, FBXL13, NAIF1, LAMC3, CIT, FREM2, DOT1L, FUT1, UMODL1* met our criteria (Table 1). The presence of these 10 germline variants in the two cases (II.2 and III.3) subjected to WES was confirmed by Sanger sequencing in DNA derived from blood leukocytes (see Supplementary Figure S1). Subsequently, co-segregation of the variants was evaluated in other family members for whom DNA was available (II.1, 2, 5, 6; III. 2, 3, 4, 5; IV. 1, 2; data not shown).

Only 2 out of 10 variants co-segregated with melanoma in all 4 affected relatives: c.C1789G:p.P597A in *SLCO4C1* gene and c.G1226A:p.R409H in *DOT1L* gene (see Supplementary Figure S2 and Figure 2). The *SLCO4C1* gene encodes for a member of the organic anion transporting polypeptide (OATP) family. Human *SLCO4C1* is involved in the membrane transport of cardiac glycosides, thyroid hormones, bile acids and many other compounds.[13] However,

a putative function for *SLCO4C1* in cancer development is unclear. Only 2 studies describe *SLCO4C1* mutation or silencing in head and neck cancers, affecting the platinum uptake and clearance.[14,15] *SLCO4C1* is not expressed in melanocytes and melanomas according to publicly available databases.[16,17] Taken together, these reasons appear to exclude *SLCO4C1* as a candidate susceptibility gene for the family under investigation.

DOT1L is the unique histone methyltransferase responsible for methylating the nucleosome core on H3K79. Based on the function of the *DOT1L* gene in UV-induced DNA damage repair and its reported role in melanoma development we considered the *DOT1L* gene variant a strong candidate responsible for melanoma susceptibility in this family. Additional rare and possibly deleterious variants were found in four sporadic and familial melanoma cases from the UK (Table 2). Moreover, the 19p13.3 locus, containing *DOT1L* gene, has been shown to be frequently deleted in metastatic melanoma cases.[18]

TABLE 2. Additional *DOT1L* variants found in familial and sporadic melanoma cases from the UK.

Location	Variant	Amino acid substitution	Polyphen	SIFT	Allele frequency	Familial vs. sporadic
Chr19: 2226478	G>A	G1320R	Possibly damaging	Deleterious	1.918e-5	Melanoma family with 2 cases of melanoma and multiple primaries
Chr19: 2191090	A>T	Y115F	Probably damaging	Deleterious	0	
Chr19: 2226839	G>C	S1440T	Benign	Deleterious	0	Sporadic case, with early onset
Chr19: 2213960	C>T	A591V	Benign	Tolerated	0	Melanoma family with 3 cases of melanoma and multiple primaries
Chr19: 2217838	T>C	L871P	Probably damaging	Tolerated	0	Sporadic case, with early onset

ABSENCE OF LOH AND ALTERED METHYLTRANSFERASE CAPACITY IN TUMOR SAMPLES FROM MELANOMA-AFFECTED FAMILY MEMBERS

First, we assessed LOH of *DOT1L* p.R409H by Sanger sequencing and ddPCR analysis in a FFPE-derived primary melanoma biopsy from individual II.1 and melanoma brain metastasis from individual III.2. In the ddPCR result, the mutation fraction is about 50% in mutation-carriers [II.2, III.3 (both subjected to WES) and IV.1 and IV.2, the youngest family members who did not develop melanoma yet] and close to 0% in wild-type family members (II.5, II.6, III.4 and III.5). We observed a low mutation fraction in the metastasis from III.2 (~20%) and a mutation fraction of about 56% in the primary tumor from II.1 (Figure 2). These numbers show an absence of LOH in the primary tumor and the brain metastasis.

Then, we checked whether this variant might be involved in a generation of a new splice site. Through Human Splicing Finder[19], the splicing motif is not altered due to the nucleotide substitution. Therefore, there is no indication that this variant might have an impact on splicing. Since three previously reported somatic loss-of-function *DOT1L* mutations in melanoma affect the methyltransferase activity[7], we aimed to assess whether the *DOT1L* mutation identified in our family disturbs DOT1L protein function by assessing H3K79 methylation via immunohistochemistry. A positive and negative controls derived from mouse thymus tissue were included to demonstrate the sensitivity of H3k79me antibody. A high percentage (~80%) of positive staining nuclei in primary melanoma from II.1 and brain metastasis from III.2 could be observed (see Supplementary Figure S3), indicating that the methyltransferase activity is only marginally, if at all, affected in the tumors.

R409H VARIANT DOES NOT SIGNIFICANTLY AFFECT THE UV SENSITIVITY

We next determined cell survival upon treatment with UV-C radiation through a clonogenic assay with the use of wild-type and CRISPR/Cas9-engineered homozygous *DOT1L* p.R409H-mutant mESCs. No significant difference could be observed in survival after UV-irradiation between *DOT1L* p.R409H-mutant and wild-type mESCs while *DOT1L* p.G165R mutant mESCs, expressing a catalytically inactive DOT1L protein showed reduced survival (Figure 3a). In mESCs, *DOT1L* p.R409H mutation did not lead to detectable loss of H3K79 methylation, while methylation was completely lost in the G165R mutant (Figure 3b).

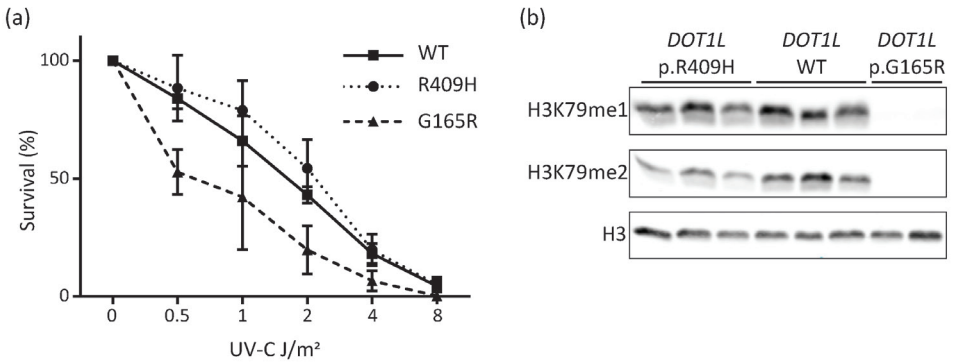

FIGURE 3. UV-survival assay of WT and *DOT1L*-mutant mouse embryonic stem cells (mESCs). (a) Colony formation capacity upon UV-C irradiation (dose range: 0.5, 1, 2, 4, 8 J/m^2) in wild-type, *DOT1L*-R409H and *DOT1L*-G165R mutated mESCs (n = 3 independent replicates, error bars represent s.d.) (b) Immunoblot analysis of H3K79me levels in the ESC clones used for UV-survival assay. Each lane shows one independently generated clone as described in materials and methods.

DISCUSSION

Here, we report a novel missense germline mutation in the *DOT1L* gene shared by four first-degree family members diagnosed with melanoma with an early age of onset. Another variant in *SLCO4C1* gene was found to co-segregate with melanoma in the family. However, the lack of evidence in association with cancer or expression in melanocytes did not encourage us to explore it further. On the other hand, DOT1L is a highly evolutionary conserved protein and is the unique histone methyltransferase responsible for mono-, di- and trimethylating the core of histone H3 on lysine 79 (H3K79).[5,6,20] In addition, DOT1L regulates transcription elongation, establishes cell cycle checkpoints, and maintains genomic stability.[21,22] Dysregulation of DOT1L has been associated with a number of cancers either as an oncogene or tumor suppressor gene.[20]

The DOT1L protein has been reported to interact with mixed lineage leukemia (MLL) fusion partners, such as *AF4*, *AF9, AF10* and *ENL*, leading to H3K79 hypermethylation and transcriptional activation of target genes favoring leukemic transformation.[23] Furthermore, DOT1L was described to interact with c-Myc-p300 complex to activate the epithelial–mesenchymal transition (EMT) regulators in breast cancer progression.[24] In addition, IL22/STAT3 signaling was reported to increase *DOT1L* expression, which subsequently increased the transcription of core stem cell genes, enhancing the cancer stemness and colorectal carcinogenesis, correlating with poor patient outcome.[25] In all these studies, DOT1L functions as an oncoprotein.

Recently, DOT1L has been described in colorectal cancer as an important player in DNA double-stand break repair via homologous recombination through γH2AX phosphorylation.[26] Also in melanoma a role for DOT1L in DNA damage repair has been envisioned. Three new mutations (M55L, P271L, and P505L) in the *DOT1L* gene that negatively affect the catalytic activity of the methyltransferase were identified.[7] Loss of *DOT1L* (by silencing or mutation) impaired the DNA damage repair induced by UV-B radiation, thereby promoting melanoma development *in vivo*. The authors show that DOT1L promotes the assembly of the NER repair complex on chromatin by interacting with XPC and stimulating its recruitment to the DNA lesion but DOT1L is not involved in transcriptional regulation of the DNA repair genes.[7] Therefore, in human melanoma *DOT1L* seems to behave as a tumor suppressor gene. In mESCs carrying a catalytically inactive G165R mutant we also observed a protective role of DOT1L against UV radiation.

In our study, the R409H variant in *DOT1L* gene, which protects melanocytes from the UV-induced transition to melanoma, was identified upon WES of two members of a family with a

family history of melanoma. The R409H was confirmed in other two affected family members, therefore co-segregating with melanoma in all four first-degree melanoma-affected family members. Then, we functionally explored this variant but we could neither detect histone methyltransferase activity reduction in melanoma and mESCs nor an effect on UV-induced survival in mESCs. However, it is possible that dynamic changes in or alternative functions of H3K79me were missed in the assays used or that the role of R409 in melanocytes is not recapitulated in the cell model used here. Accordingly, two previously reported *DOT1L* variants (V135A and F243A) hardly showed a decrease of the DOT1L methyltransferase activity.[27] R409 is located in a part of the DOT1L protein that is enriched for positively charged residues.[28] This region contains a nuclear localization signal[29] and is part of a C-terminal extension of the catalytic core of DOT1L that is required for nucleosome binding and DOT1L activity [28]. Furthermore, lysine 410, adjacent to R409, was identified as a site that can be methylated by SUV39H1, suggesting that the function of this part of DOT1L may be subject to post-translational modifications. SUV39H1 targets RK sites[30] and the R409H mutation disrupts this RK motif. However, very little is known about the interactions between the DOT1L C-terminal extension and the nucleosome. Recent efforts to elucidate the mechanisms of these interactions by determining the structure of DOT1L bound to nucleosomes have not yet revealed the molecular details.[31-33] It has been reported that DOT1L only binds the ubiquinated nucleosome, which is dependent on H2BK120 monoubiquitination and H2A-H2B acidic path, that subsequently enhances the catalytic function of the methyltransferase DOT1L.[27] However, the lack of unequivocal structural information is most likely caused by the dynamic nature of the interactions between the DOT1L C-terminal extension of the catalytic core and the nucleosome. It could also be possible that the R409H mutation affects a methyltransferase-independent function of DOT1L. For example, budding yeast DOT1L functions as a transcription de-repressor, a histone chaperone and enhances H2B ubiquitination all independent of its methyltransferase activity.[34-36] However, in mammalian cells this activity of DOT1L has been shown to be required for several critical functions, including reactivation of repressed genes upon targeting, cycle progression in lung cancer cell lines, and leukaemic transformation in CALM-AF10 MLL-rearranged leukemia.[37-39] Taken together, methyltransferase-independent functions of DOT1L have been reported, but not in mammalian cells. Despite a lack of evidence for a direct functional effect of the R409H variant, several variants in *DOT1L* have been observed in independent familial and sporadic melanoma cases. Therefore, our finding reinforces the ones by Zhu *et al.*[7] and we consider that is worthwhile to investigate the *DOT1L* variants in future WES and WGS studies involving large familial melanoma cohorts, albeit that further functional and structural analyses are required in order to confirm *DOT1L* to be a melanoma-susceptibility gene.

ACKNOWLEDGEMENTS

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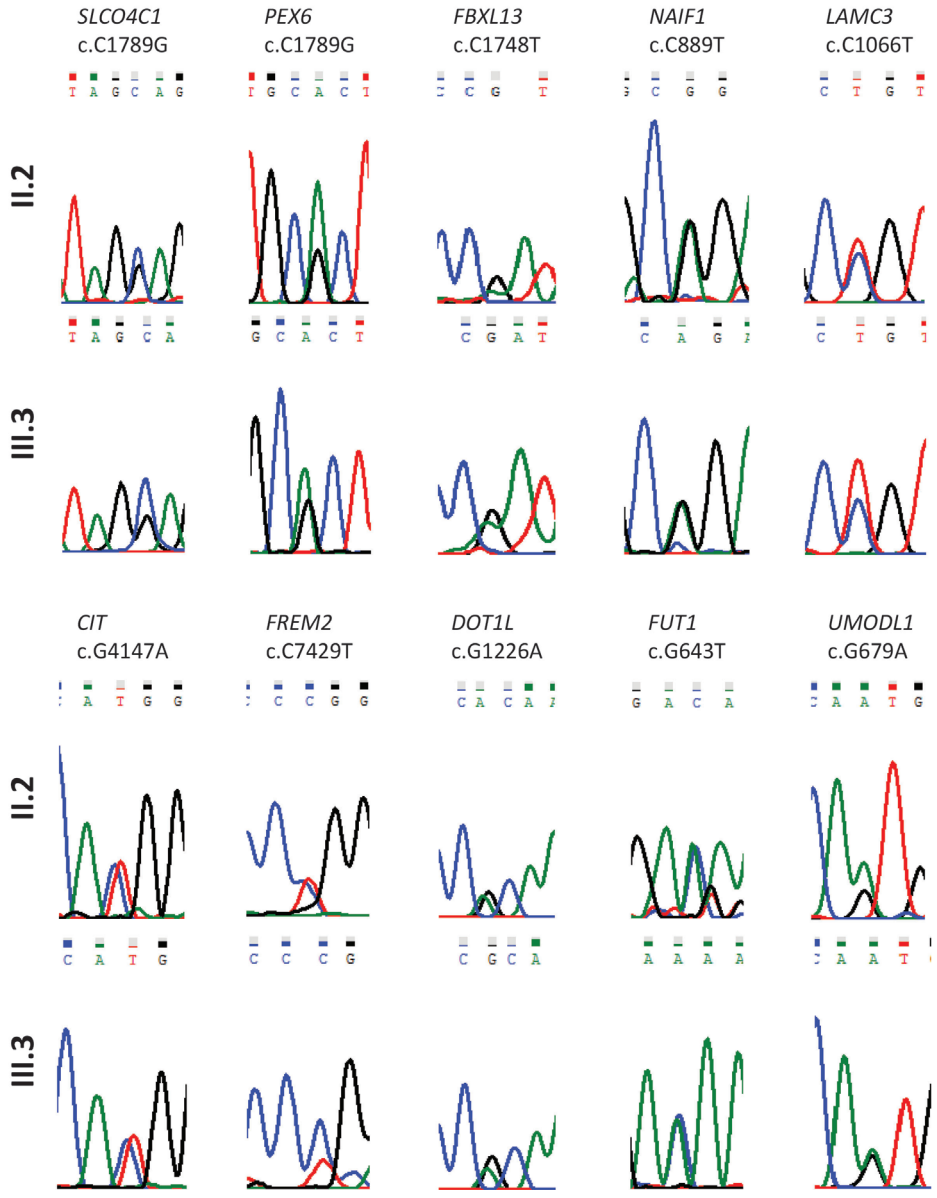
SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Table reporting the oligonucleotides used to encode the gRNAs for CRISPR/Cas9 in the generation of mESC lines.

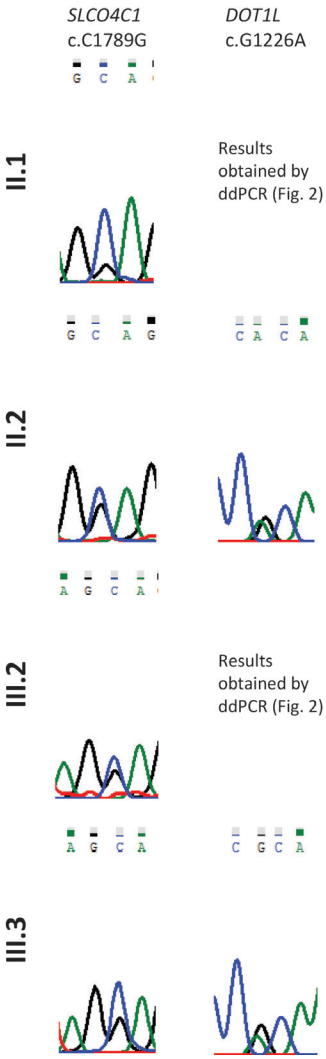
Oligonucleotide	Sense	Antisense
R409H guide 1	CACCGCTTGGGCCGCCCACGTTTC	AAACGAAACGTGGGCGGCCCAAGC
R409H guide 2	CACCGGATGGCTGGCCGGAAACGT	AAACACGTTTCCGGCCAGCCATCC
R409H guide 3	CACCGAGATGGCTGGCCGGAAACG	AAACCGTTTCCGGCCAGCCATCTC
R409H guide 4	CACCGGGCTGGCCGGAAACGTGGG	AAACCCCACGTTTCCGGCCAGCCC
G165R guide 1	CACCGTGTTTGTCGACCTGGGCAG	AAACCTGCCCAGGTCGACAAACAC
G165R guide 2	CACCGTGGTGAGTGTCTTGCAGCA	AAACTGCTGCAAGACACTCACCAC

SUPPLEMENTARY TABLE S2. Table with the single strand homology directed repair (HDR) templates used for generation of mESC lines.

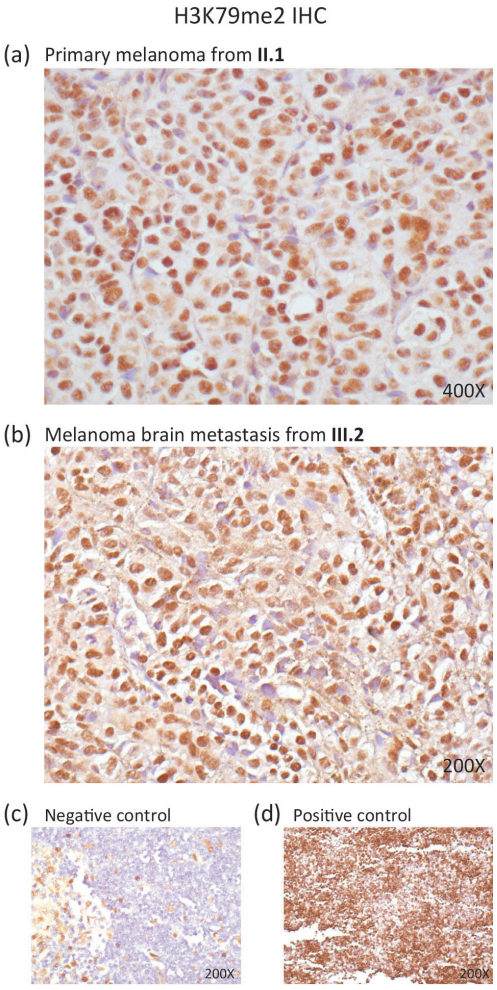
HDR template	
R409H	TCCCTCCAAAGCCCGGAAGAAGAAACTGAACAAGAAAGGGAGAAAGATGGCTGGCCACAAGAGGG GGCGGCCCAAGAAAATGAGTGCTGCCAGTGCCGAACGCAAGTCTAAGAAGAGCCA
G165R	AGCCTTGACTTTTTTTGGGACTCAAGGTCGGCTCAAAGACCATGCTGCAAGACACTCACGAGAGCCC AGGTCGACAAACAGGTCATCCTCTGTCATCTTGATCTCATCAATCATCTGGGC



SUPPLEMENTARY FIGURE S1. Sanger Sequencing validation of the 10 germline variants found by whole-exome sequencing in two affected family members (II.2 and III.3).



SUPPLEMENTARY FIGURE S2. Co-segregation of the variants c.C1789G in *SLCO4C1* gene and c.G1226A in *DOT1L* gene with melanoma in all 4 affected family members.



SUPPLEMENTARY FIGURE S3. Immunohistochemistry to evaluate the histone methyltransferase activity using an anti-H3K79me2 antibody (1:8000 dilution), that works cross-species. (a) Primary melanoma biopsy from family member II.1; (b) Brain metastasis from family member III.2; (c) Negative control – thymus tissue from a conditional *DOT1L*-knockout mouse; (d) Positive control – wild-type mouse thymus tissue.

3

Genome-wide analysis of constitutional DNA methylation in familial melanoma

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Clin Epigenet. 2020 Mar 6;12(1):43

ABSTRACT

BACKGROUND

Heritable epigenetic alterations have been proposed as an explanation for familial clustering of melanoma. Here we performed genome-wide DNA methylation analysis on affected family members not carrying pathogenic variants in established melanoma susceptibility genes, comparing with healthy volunteers.

RESULTS

All melanoma susceptibility genes showed absence of epimutations in familial melanoma patients, and no loss of imprinting was detected. Unbiased genome-wide DNA methylation analysis revealed significantly different levels of methylation in single CpG sites. The methylation level differences were small and did not affect reported tumour predisposition genes.

CONCLUSION

Our results provide no support for heritable epimutations as a cause of familial melanoma.

KEYWORDS

Epimutation, loss of imprinting, DNA methylation, familial melanoma

INTRODUCTION

Cutaneous melanoma is an aggressive form of skin cancer with a propensity to metastasize, causing significant mortality and health care expenditure. Approximately 10% of patients diagnosed with melanoma have a positive family history for this malignancy. In familial or hereditary melanoma, multiple melanoma cases aggregate in several generations of a family, consistent with an autosomal dominant inheritance pattern. A subset of familial melanoma cases is caused by germline mutations in the established high penetrance melanoma predisposition genes *CDKN2A* or *CDK4*. Recently, pathogenic variants in the *BAP1*, *TERT*, *POT1*, *TERF2IP*, *ACD* and *MITF* genes have been identified as a cause of familial melanoma. Several candidate melanoma susceptibility genes including *POLE*, *GOLM1* and *EBF3*, have been reported.[1-3] However, in more than half of affected families the cause of melanoma predisposition remains to be resolved despite much research effort. For this reason the attention has turned to different mechanisms of inheritance including heritable epigenetic alterations. Clarifying the genetic basis of familial melanoma is clinically relevant as it would allow for genetic testing, risk estimation and targeted clinical surveillance of patients at high risk of melanoma.

Epimutations have been defined as a heritable changes in gene activity due to DNA modifications, not encompassing changes in the DNA sequence itself.[4] 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.[5] The best-described example in cancer is hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) where cases not affected by inactivating mutations in DNA mismatch repair genes were found to be caused by heritable promoter hypermethylation of the *MLH1* gene.[6-8] 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.[9] Secondary epimutations in the *MSH2* and *DAPK1* gene have been identified in HNPCC and familial chronic lymphocytic leukemia, respectively.[10-12]

Genomic imprinting causes certain genes to be silenced by DNA and histone methylation in a parent of origin-specific manner, ensuring proper expression during development. Loss of imprinting is a distinct epigenetic mechanism of disease, associated with deregulated gene expression that can be implicated in cancer development.[13] The association between loss-of-imprinting at the *IGF2–H19* locus at chromosome 11p15.5 and predisposition to Wilms tumour is an example of this epigenetic mechanism.[14]

In familial melanoma we and others have shown absence of epimutation of the *CDKN2A* gene, the major high penetrance melanoma susceptibility gene.[15,16] A previous study analysed methylation of 14 cancer-related genes in blood DNA from melanoma-prone family members. This analysis revealed no constitutional promoter hypermethylation, but reduced methylation of the *TNFRSF10C* promoter.[17]

In this study we aim to identify heritable epigenetic alterations that might account for familial clustering of melanoma in families where no genetic variants in established or candidate melanoma susceptibility genes were found. To this end a genome-wide methylation analysis of peripheral blood DNA from patients with familial melanoma was performed. We assessed promoter hypermethylation of recently identified melanoma susceptibility genes, loss of imprinting and performed an unbiased analysis of hypermethylated CpG sites and regions.

RESULTS AND DISCUSSION

Patients from 5 Dutch families with at least 3 melanoma cases in different generations were selected for this study (Table 1, pedigrees in Supplementary Figure S1). The presence of pathogenic gene variants in all currently established and candidate high penetrance melanoma susceptibility genes was assessed in all included cases using whole genome sequencing. No germline mutations were found in these genes. To examine DNA methylation, DNA from peripheral blood of 2 affected members of each family (n=10) was subjected to 450K Illumina arrays interrogating over 450,000 CpG sites (namely 483891 probes after quality control) covering 99% of human genes following bisulfite conversion.[18] For comparative analysis we could make use of DNA methylation data obtained from peripheral blood samples of a reference group of 1000 healthy Dutch individuals included in the Biobank-based Integrative Omics Studies (BIOS) consortium analysed using similar 450K arrays (raw data available from the European Genome-phenome Archive (EGA) under accession EGAS00001001077). All samples were compared individually to the reference group, while taking multiple testing into account using Bonferroni correction.

First, we analysed the presence of promoter hypermethylation in the *CDKN2A*, *CDK4*, *BAP1*, *TERT*, *POT1*, *TERF2IP*, *ACD, MBD4, POLH, MITF, MC1R*, *POLE, EBF3* and *GOLM1* melanoma susceptibility genes. All CpG sites designated by a probe located across the entire sequence of these melanoma susceptibility genes in the familial melanoma samples were compared with reference samples. No significant difference (≥ β-value average ± 5.65 SD) in methylation level, hypermethylation nor hypomethylation was found at any of these genes (Figure 1).

TABLE 1. Clinical characteristics of patients/families involved in the genome-wide analysis.

Family	Number of CMM affected members	Patient number	Degree of kinship	Age at melanoma diagnosis	Age at DNA collection
I	9	I_1	2nd	62	76
		I_2		34,34,46,48*	53
II	5	II_1	3rd	41	52
		II_2		52	56
III	4	III_1	2nd	51,57*	68
		III_2		28	33
IV	3	IV_1	3rd	34	56
		IV_2		35	43
V	4	V_1	1st	49	55
		V_2		30	25

*multiple primary melanomas diagnosed.

The methylation status of imprinted genes in familial melanoma patients was then investigated. We checked all CpG sites in the entire gene sequence of all imprinted genes in humans (http://www.geneimprint.com/site/genes-by-species, accessed August 2019) interrogated by the arrays. The methylation levels of 4790 interrogated CpG sites located at these imprinted gene loci did not differ significantly (≥ β-value average ± 5.65 SD) from the BIOS reference samples (Supplementary Figure S2). Since the regulation of imprinted genes is largely dependent on methylation levels, and there is no significant difference in any of the familial melanoma patients compared to BIOS, we conclude that there was no indication of loss of imprinting.

Following on analysis of candidate genes we performed an agnostic genome-wide analysis by comparing DNA methylation of all interrogated CpG sites in the familial melanoma patients with those in healthy subjects. We considered as potential epimutations CpG sites located in gene promoters (using probes assigned to promoter regions according to annotation provided by Illumina) with significantly aberrant methylation levels in both members of an affected family compared to BIOS control samples. Since all reported cancer predisposing epimutations were cases of constitutional promoter hypermethylation, we focused our analysis on this type of epigenetic event. All CpGs in promoters were assessed. Probes interrogating CpG sites lost due to single nucleotide variants, as identified using whole genome sequence data, were not included in the analysis of the affected samples. We identified 6 single CpGs in gene promoters with significantly higher β-values in both affected members of a melanoma family compared to healthy controls (Figure 2, Table 2, Supplementary Table S1). In healthy controls these CpG sites showed low average β-values consistent with absence of methylation. The CpG sites in the *RABGGTB, SND1, SCAF11, ZNF638, THAP1* and *SFSWAP* genes showed significantly higher Δβ-values in both members of multiple families.

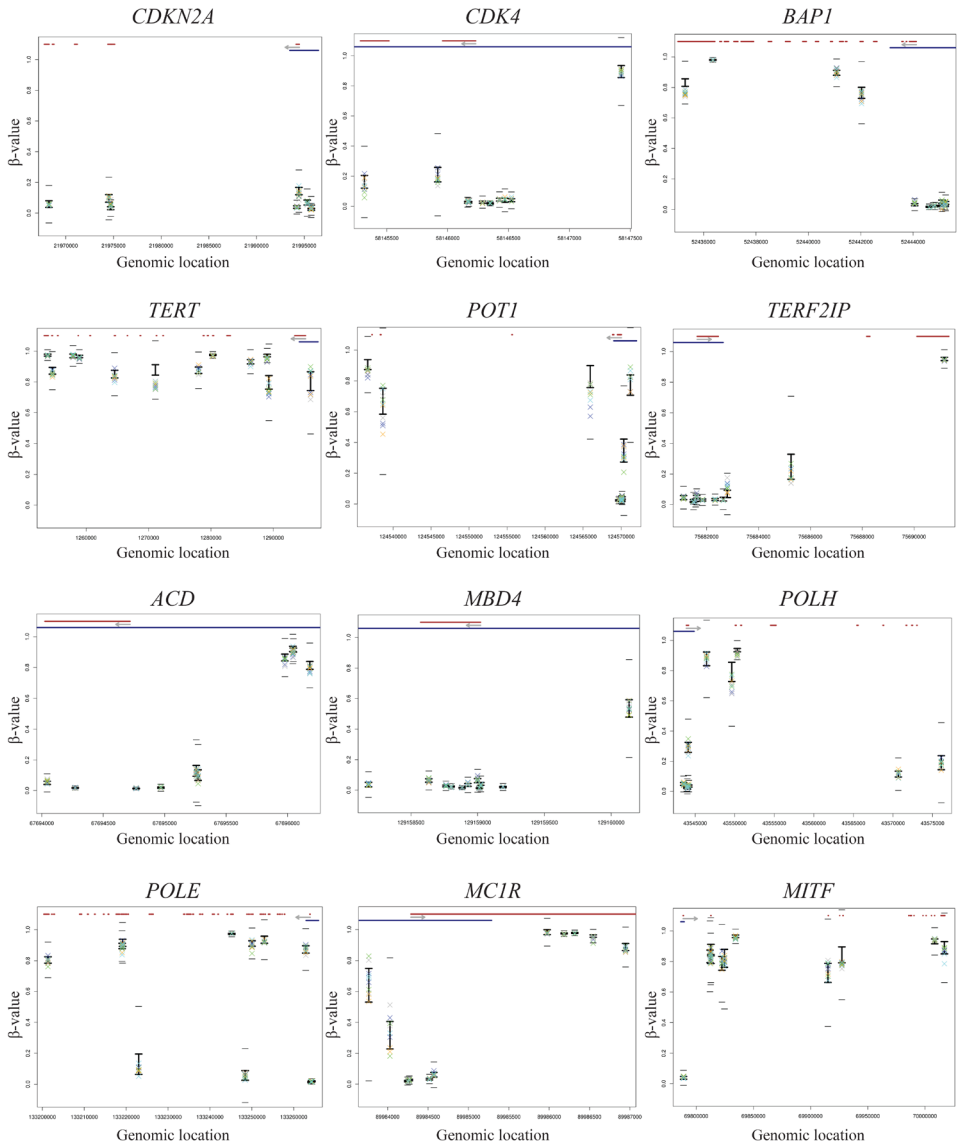

FIGURE 1. Methylation levels (β-value) across the entire sequence of all established melanoma predisposition genes. In the upper part of each plot, the gene structure is represented in dark red and promoter region ("Promoter_associated" feature retrieved from Illumina annotation) in blue. The light grey arrow represents the transcription direction of the gene. For each CpG, the BIOS values are represented by the black vertical line with upper (average + 1 SD) and lower limits (average – 1SD). The families are represented as a X of different colours (Family I – green, Family II – blue, Family III – yellow, Family IV – light purple, Family V – dark blue). To be considered as significantly different from the BIOS, the families symbols must go beyond the small black horizontal line (average ± 5.65 SD). Genes with more than 10 CpG sites assessed by 450K array, were represented by 10 randomly selected CpGs.

TABLE 2. Methylation levels (Δβ-value) in all 6 significant upregulated CpGs in all subjects (n=10).

		BIOS control cases	Family I		Family II		Family III		Family IV		Family V	
			I_1	I_2	II_1	II_2	III_1	III_2	IV_1	IV_2	V_1	V_2
CpG ID	Gene ID	β-value[a]	Δβ-value[b]									
cg21812670	RABGGTB	0.06	0.30	0.38	0.34	0.30	0.36	0.26	0.39	0.32	0.39	0.30
cg26642667	SND1	0.06	0.25	0.30	0.33	0.22	0.35	0.16	0.37	0.29	0.34	0.27
cg04385631	SCAF11	0.02	0.14	0.00	0.00	0.22	0.00	0.22	0.23	0.00	0.22	0.28
cg21843755	ZNF638	0.11	0.16	0.21	0.18	0.07	0.20	0.10	0.21	0.16	0.23	0.21
cg03301282	THAP1	0.08	0.26	0.23	0.20	0.27	0.25	0.12	0.29	0.27	0.30	0.24
cg02470959	SFSWAP	0.05	0.18	0.20	0.18	0.16	0.19	0.17	0.21	0.21	0.20	0.17

[a] Represents the average β-value for the 1000 BIOS controls at each CpG site.

[b] Represents the difference between BIOS average β-value and patient β-value at each CpG site

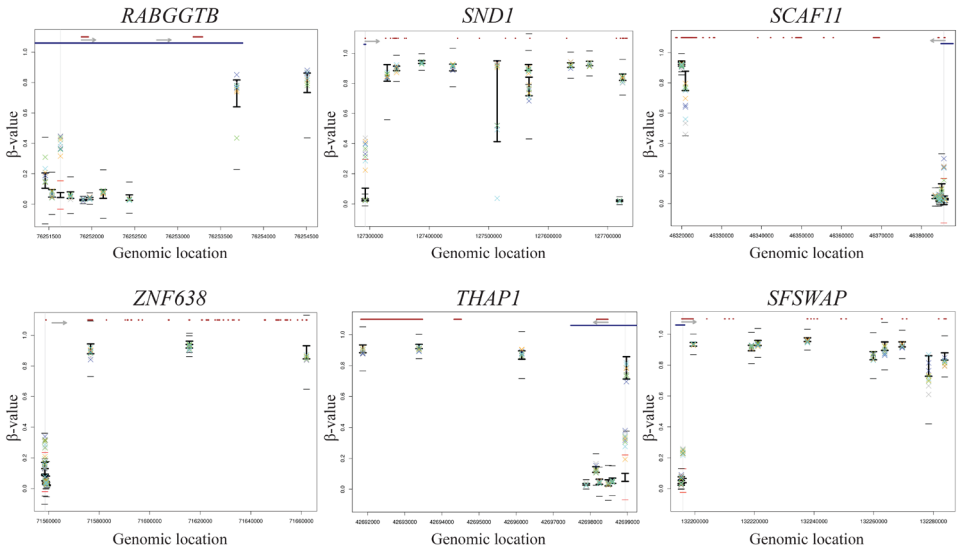

FIGURE 2. Methylation levels (β-value) in all 6 significant upregulated CpGs located in the promoter regions of the genes. In the upper part of each plot, the gene structure is represented in dark red and promoter region ("Promoter_associated" feature retrieved from Illumina annotation) in blue. The light grey arrow represents the transcription direction of the gene. For each CpG, the BIOS values are represented by the black vertical line with upper (average + 1 SD) and lower limits (average – 1SD). The families are represented as a X of different colours (Family I – green, Family II – blue, Family III – yellow, Family IV – light purple, Family V – dark blue). To be considered as significantly different from the BIOS, the families symbols must go beyond the small black horizontal line (average ± 5.65 SD). Genes with more than 10 CpG sites assessed by 450K array, were represented by 10 randomly selected CpGs. The upregulated CpG in each plot is aligned with a vertical light grey line and, in this case, the little horizontal lines become red since the families' symbols exceeded these limits.

We assessed the methylation of contiguous interrogated CpG sites and for all 6 cases the hypermethylation was observed exclusively in the single identified CpG site, with neighbouring CpGs not showing significantly higher methylation levels. None of these genes have been reported as cancer predisposition genes by Rahman *et al.*[19] Only one of the CpGs is located in a cancer-related gene, *SND1*, according to Cancer Gene Census (http://cancer.sanger.ac.uk/census, accessed August 2019). This gene has no reported role in melanoma and functions as a gene fusion partner in certain malignancies. Given the established genetic heterogeneity, it is unlikely that the same epimutation would cause melanoma susceptibility in all 5 families. Together with the information about the function of the genes (Supplementary Table S1), we conclude that the identified hypermethylated CpG sites in these families do not appear to constitute plausible pathogenic high penetrance epimutation events.

Additionally we evaluated CpG sites with significantly lower methylation levels in familial melanoma than in healthy control samples and found 35 hypomethylated CpGs in both members of a family. Fifteen CpG sites showed hypomethylation in all 5 families, suggestive of a batch effect as has been described for 450k methylation arrays (Supplementary Table S2).[20] Of the 35 hypomethylated CpG sites only 2 were located in established cancer-related genes: *BRCA1*, an established breast and ovarian cancer susceptibility gene, and *ROS1*, encoding a receptor tyrosine kinase with a possible oncogenic role in melanoma.[21] For both genes a single CpG site in the promoter demonstrated significantly lower methylation levels, with normal methylation of neighbouring CpG sites assessed by 450K array. For *BRCA1* and for *ROS1*, the CpG site was not part of a predicted transcription factor binding motif.[22] Hypomethylation of the *BRCA1* gene promoter has never been associated with transcriptional downregulation and therefore reduced methylation of this single CpG site in the *BRCA1* gene promoter is unlikely to have pathogenic significance. Expression of the *ROS1* oncogene is not known to be regulated by promoter methylation, but high expression has been associated with histone modifications and *EZH2* repression.[23] β-values for the single CpG site in the distal promoter of the *ROS1* gene were 0.87 in control samples and approximately 0.65 in familial melanoma DNA samples. We consider it possible, but unlikely that lower methylation levels of the single CpG site in the distal promoter *ROS1* would cause familial melanoma. Similar to the finding of *TNFRSF10C* hypomethylation in familial melanoma patients from the US, that we could not detect in our patients, this finding might be analysed in a large number of melanoma families.[17]

Since regions containing multiple CpG sites in promoters commonly work as units of transcriptional regulation we additionally tried to identify differentially methylated regions. For this we evaluated the average of all probes assigned to promoters for each gene comparing familial melanoma and healthy control samples. The annotation of the 450K

array contains 13,715 genes with CpG probes assigned to promoters. The promoter of one gene (*CCNI*) showed significant higher methylation levels in 4 families, while promoters of the *CD47* and *USP46* genes had slightly higher methylation levels in 1 family each. Although statistically significant the averaged promoter methylation level (β-value) differences were minor, which does not support a relevant effect.

In this study we analysed the possible occurrence of epimutations and loss of imprinting in familial melanoma using a genome-wide approach. A strength of the study is the selection of DNA samples from families with many affected members in multiple generations where no genetic cause could be identified and the availability of methylation data from a large cohort of 1000 Dutch healthy individuals for comparative analysis. There are some limitations to this study; the number of analysed families is small and our results do not exclude the possibility that pathogenic epimutations might occur in a small proportion of melanoma families. Secondly, the 450K arrays interrogate CpG sites in almost all gene promoters, but do not cover all potentially regulatory sequences. In addition, we analysed blood DNA for the occurrence of epimutations, but certain epimutations might occur only in specific cell types in a mosaic state. In these patients with familial melanoma we have not identified promoter hypermethylation of any melanoma predisposition gene, cancer predisposition gene or tumour suppressor gene. We have been able to determine several DNA methylation events that are candidate epimutations, methylation events shared by multiple members of a family that were not identified in healthy volunteers. However, it is not clear if the observed methylation alterations in these single CpG sites impact on expression of the respective genes. Based on the function of the genes and the fact that we did not identify a differentially methylated region, but only a single CpG site, we consider it is not plausible that any of the DNA methylation alterations that were detected constitute the cause of melanoma predisposition in these families. Moreover, given the established genetic heterogeneity, it is unlikely that the same epimutation would cause melanoma susceptibility in all 5 families. Therefore we consider the observed CpG sites with higher and lower detected methylation levels to represent rare variations with no pathogenic significance or possibly the result of batch array effects. Summarizing, our results of genome-wide analysis provide little or no support for a role of heritable DNA methylation alterations as a cause of familial melanoma.

MATERIALS & METHODS

We selected 5 unrelated Dutch families with 3 or more melanoma cases in multiple generations and tested negative for germline mutations in the established high penetrance

melanoma susceptibility genes *CDKN2A*, *CDK4*, *BAP1*, *TERT*, *POT1*, *TERF2IP*, *ACD* and *MITF* by next generation sequencing (Figure 1). Some patients had developed multiple melanomas. The majority of the melanomas was of the superficial spreading or nodular subtypes. The study was approved by the Leiden University Medical Center institutional ethical committee and was conducted according to the Declaration of Helsinki Principles. DNA from 2 affected members from 5 families was isolated from whole blood samples. DNA was bisulfite-converted using the EZ DNA methylation kit (Zymo Research, D5001) and hybridized to Illumina 450K arrays (Illumina). The reference group encompassed 1000 whole blood DNA samples of healthy individuals included in the Biobank-based Integrative Omics Studies (BIOS) Consortium analysed with Illumina 450K arrays under similar conditions.[24] The median age of patients during blood sampling was 54 years and for the 1000 healthy controls it was 55 years. Sample quality control was performed using *MethylAid*[25], probes with a high detection *P* value (>0.01), probes with a low bead count (<3 beads), and probes with a low success rate (missing in >95% of the samples) were set to missing. Subsequently imputation[26] was performed to impute the missing values. Functional normalization, as implemented in the *minfi* package, was used on a random subset of 1000 samples together with the melanoma samples.[27] Detailed description of the 450K DNA methylation pre-processing steps are available from the https://molepi.github.io/DNAmArray_workflow/. Sample specific aberrant melanoma CpGs were detected using a t-test for comparing a single melanoma case to the 1000 BIOS controls.[28] In order to control for the number of tests a very stringent cut-off, 1.03×10^{-9}(0.01/(number of probes on array*2)), was used. After the bioinformatic analysis a set of 13 hypermethylated CpGs and 164 hypomethylated CpG sites was obtained. The list of significant CpGs was further reduced by only considering significant co-segregating CpGs with an absolute β-value difference of 0.2 when compared to BIOS controls (Δβ-value ≥0.2 in 2 members of at least one family). To be considered as a putative epimutation a CpG should meet the following criteria. CpG probes on chromosome X were excluded (as they reflect X-chromosome inactivation in females). Only CpGs in promoter regions (retrieved from Illumina annotation for gene promoters, "promoter_associated in regulatory_feature_group field") of the genes were selected. Both members of a family were required to harbor the hypo/hypermethylation, since we are looking at high penetrance epigenetic events. A single nucleotide variant (SNV) must be within a window of 100 bp around the CpG (that can either influence/impair the probe binding or reveal the presence of a genetic variant around the epigenetically altered CpG, that in this case would be the so called "second epimutation"). The SNV data were retrieved from dbSNP Release 153. This resulted in 6 hypermethylated CpGs and 35 hypomethylated CpGs, which were compared with lists of cancer-related genes according to Cancer Gene Census (http://cancer.sanger.ac.uk/census, accessed August 2019) and cancer predisposition genes suggested by Rahman *et al*.[19] We have checked whether some CpG sites of interest were

part of predicted transcription factor binding motifs using the TFBIND tool.[22] We also aimed at identifying differentially methylated regions. For that we assessed the probes assigned for promoter regions according to the annotation of 450K array. There were 13,715 genes with probes assigned to promoters. On average each of these promoters contained 6.7 probes. We averaged all the probes assigned for each gene promoter and compared with the average of the same promoter in BIOS controls.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Methylation levels (β-value) and cancer genes information of all 6 significant upregulated CpGs in all subjects (n=10).

							Family I		Family II	
				In CpG	BIOS controls		I_1	I_2	II_1	II_2
Position	CpG ID	Gene ID	Location	island?	β-value	SD	β-value			
chr1: 76251636	cg21812670	RABGGTB	Promoter	No	0.06	0.02	0.36	0.44	0.40	0.36
chr7: 127292389	cg26642667	SND1	5'UTR	Yes	0.06	0.04	0.31	0.37	0.39	0.29
chr12: 46385625	cg04385631	SCAF11	Promoter	Yes	0.02	0.03	0.15	0.02	0.02	0.24
chr2: 71558577	cg21843755	ZNF638	Promoter	No	0.11	0.02	0.26	0.32	0.29	0.17
chr8: 42698936	cg03301282	THAP1	Promoter	No	0.08	0.03	0.34	0.31	0.28	0.35
chr12: 132196036	cg02470959	SFSWAP	Promoter	Yes	0.05	0.01	0.23	0.25	0.23	0.22

[a] Data retrieved from GeneCard (https://www.genecards.org/)

[b] Data retrieved from cancer predisposition genes reported by Rahman et al.[19]

[c] Data retrieved from Cancer Gene Census - COSMIC (https://cancer.sanger.ac.uk/census)

SUPPLEMENTARY TABLE S2. Methylation levels (β-value) and cancer genes information of all 35 significant downregulated CpGs in all subjects (n=10).

							Family I	
				In CpG	BIOS controls		I_1	I_2
Position	CpG ID	Gene ID	Location	island?	β-value	SD	β-value	
chr1: 28416532	cg24509398	EYA3	proximal promoter	No	0.86	0.05	0.52	0.45
chr1: 43752185	cg19086488	C1orf210	proximal promoter	No	0.83	0.03	0.65	0.63
chr2: 237412985	cg21740507	IQCA1	distal promoter	No	0.85	0.03	0.56	0.53
chr2: 39003850	cg25348336	GEMIN6	distal promoter	No	0.86	0.04	0.44	0.63
chr2: 230933934	cg25621735	SLC16A14	proximal promoter	No	0.87	0.03	0.62	0.59
chr3: 155860278	cg25924827	KCNAB1	proximal promoter	No	0.86	0.04	0.62	0.61
chr4: 39186234	cg16401578	WDR19	distal promoter	No	0.86	0.04	0.61	0.63
chr5: 39220260	cg18740872	FYB	proximal promoter	No	0.81	0.05	0.51	0.49
chr5: 36149258	cg25013978	LMBRD2	distal promoter	No	0.88	0.04	0.60	0.52
chr6: 137366545	cg08823985	IL20RA	proximal promoter	No	0.79	0.07	0.37	0.36
chr6: 167704188	cg08904369	UNC93A	proximal promoter	No	0.85	0.03	0.62	0.62
chr6: 117748486	cg12631085	ROS1	distal promoter	No	0.87	0.03	0.63	0.62
chr6: 32785740	cg19811863	HLA-DOB	proximal promoter	No	0.88	0.03	0.60	0.54
chr7: 93534693	cg07547788	GNGT1	proximal promoter	No	0.84	0.03	0.63	0.61
chr7: 123294503	cg15313859	LMOD2	distal promoter	No	0.77	0.07	0.32	0.30

Family III		Family IV		Family V				
III_1	III_2	IV_1	IV_2	V_1	V_2			
β-value						Gene function[a]	Cancer predisposition genes[b]	Cancer-related genes?[c]
0.42	0.32	0.45	0.38	0.45	0.37	Beta-subunit of the enzyme that catalyzes the transfer of a geranylgeranyl groups to cysteine residues of Rab proteins.	NA	NA
0.41	0.22	0.44	0.35	0.40	0.33	Transcriptional activator involved with Epstein-Barr virus and B-lymphocyte transformation.	NA	oncogene fusion partner in pancreatic carcinoma
0.02	0.24	0.25	0.02	0.24	0.30	Splicing regulatory protein. It regulates the spliceosome assembly.	NA	NA
0.31	0.21	0.32	0.27	0.34	0.32	Nucleoplasmic protein associated with packaging, transferring, or processing transcripts.	NA	NA
0.33	0.19	0.37	0.34	0.38	0.32	DNA-binding transcription regulator and proapoptotic factor.	NA	NA
0.24	0.23	0.26	0.26	0.25	0.23	Splicing regulatory protein. It regulates the splicing of fibronectin and CD45 genes.	NA	NA

Family II		Family III		Family IV		Family V		Cancer predisposition genes[a]	Cancer-related genes[b]
II_1	II_2	III_1	III_2	IV_1	IV_2	V_1	V_2		
β-value									
0.49	0.46	0.42	0.50	0.44	0.48	0.42	0.47	NA	NA
0.64	0.65	0.62	0.68	0.60	0.63	0.62	0.67	NA	NA
0.56	0.60	0.50	0.64	0.48	0.57	0.56	0.66	NA	NA
0.60	0.63	0.57	0.57	0.62	0.61	0.56	0.59	NA	NA
0.62	0.65	0.62	0.69	0.58	0.62	0.57	0.64	NA	NA
0.58	0.64	0.56	0.64	0.59	0.62	0.67	0.69	NA	NA
0.56	0.64	0.63	0.69	0.67	0.64	0.63	0.62	NA	NA
0.45	0.54	0.47	0.57	0.47	0.50	0.46	0.49	NA	NA
0.52	0.60	0.56	0.63	0.49	0.61	0.50	0.59	NA	NA
0.34	0.39	0.33	0.37	0.32	0.36	0.32	0.39	NA	NA
0.56	0.61	0.57	0.69	0.60	0.63	0.59	0.61	NA	NA
0.64	0.67	0.57	0.75	0.61	0.70	0.64	0.66	NA	oncogene fusion partner in multiple cancers
0.56	0.62	0.58	0.69	0.55	0.59	0.57	0.66	NA	NA
0.68	0.71	0.63	0.77	0.60	0.65	0.68	0.72	NA	NA
0.35	0.33	0.28	0.35	0.28	0.28	0.26	0.32	NA	NA

SUPPLEMENTARY TABLE S2 CONTINUED.

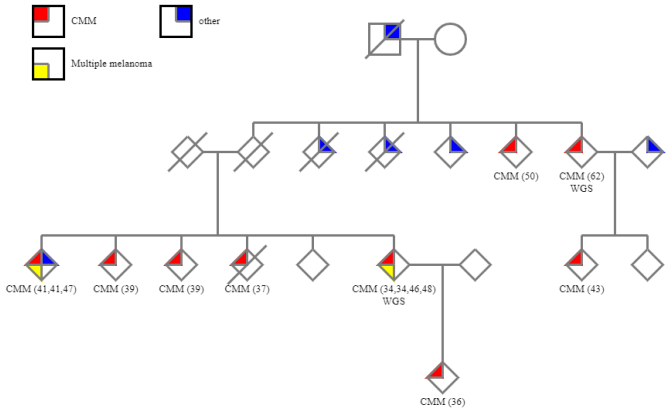
							Family I	
				In CpG	BIOS controls		I_1	I_2
Position	CpG ID	Gene ID	Location	island?	β-value	SD	β-value	
chr7: 123564016	cg24641201	SPAM1	distal promoter	No	0.87	0.02	0.65	0.63
chr10: 123748615	cg22562363	TACC2	proximal promoter	No	0.87	0.04	0.61	0.60
chr11: 47585888	cg00214780	PTPMT1	proximal promoter	No	0.83	0.03	0.59	0.59
chr11: 119054921	cg00395990	PDZD3	proximal promoter	No	0.91	0.02	0.64	0.58
chr11: 57436966	cg13582500	ZDHHC5	distal promoter	No	0.83	0.04	0.59	0.54
chr11: 125649040	cg15229773	PATE2	proximal promoter	No	0.88	0.03	0.59	0.57
chr12: 117348108	cg26332016	FBXW8	proximal promoter	No	0.90	0.02	0.63	0.62
chr13: 39564046	cg05380910	STOML3	distal promoter	No	0.89	0.03	0.77	0.81
chr14: 20710881	cg24137472	OR11H4	proximal promoter	No	0.91	0.03	0.91	0.91
chr15: 42750798	cg09240555	ZNF106	proximal promoter	No	0.84	0.04	0.63	0.64
chr15: 54052305	cg13131111	WDR72	proximal promoter	No	0.82	0.04	0.58	0.51
chr16: 31270808	cg09736526	ITGAM	proximal promoter	No	0.83	0.03	0.62	0.60
chr17: 41278712	cg20185525	BRCA1	proximal promoter	No	0.86	0.04	0.57	0.56
chr19: 9360855	cg11396122	OR7E24	proximal promoter	No	0.85	0.04	0.70	0.64
chr19: 57678865	cg13472369	DUXA	proximal promoter	No	0.81	0.05	0.63	0.68
chr19: 45174671	cg20559736	CEACAM19	proximal promoter	No	0.83	0.03	0.54	0.50
chr19: 12986352	cg21908038	DNASE2	5'UTR	No	0.85	0.05	0.52	0.50
chr20: 33758526	cg23101469	PROCR	proximal promoter	No	0.83	0.04	0.49	0.51
chr22: 27016806	cg15559737	CRYBA4	proximal promoter	No	0.83	0.03	0.62	0.59
chr22: 25594918	cg19288514	CRYBB3	proximal promoter	No	0.81	0.06	0.42	0.43

[a] Data retrieved from cancer predisposition genes reported by Rahman et al. [19]

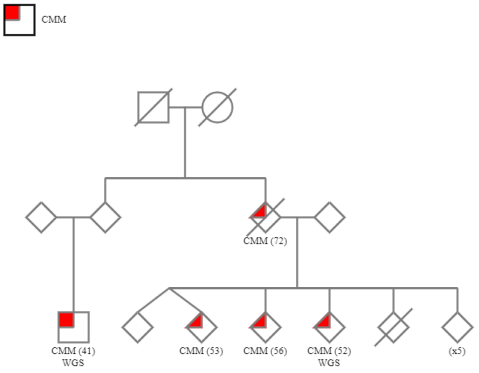
[b] Data retrieved from Cancer Gene Census - COSMIC (https://cancer.sanger.ac.uk/census)

Family II		Family III		Family IV		Family V		Cancer predisposition genes[a]	Cancer-related genes[b]
II_1	II_2	III_1	III_2	IV_1	IV_2	V_1	V_2		
β-value									
0.66	0.75	0.63	0.73	0.63	0.65	0.68	0.69	NA	NA
0.59	0.65	0.59	0.68	0.61	0.61	0.60	0.66	NA	NA
0.59	0.61	0.58	0.70	0.56	0.60	0.57	0.64	NA	NA
0.57	0.70	0.56	0.69	0.56	0.63	0.60	0.69	NA	NA
0.52	0.55	0.54	0.55	0.52	0.55	0.51	0.55	NA	NA
0.62	0.66	0.56	0.67	0.58	0.56	0.59	0.62	NA	NA
0.64	0.67	0.60	0.71	0.67	0.69	0.62	0.73	NA	NA
0.76	0.74	0.78	0.81	0.78	0.83	0.68	0.68	NA	NA
0.92	0.89	0.90	0.93	0.89	0.92	0.52	0.64	NA	NA
0.60	0.62	0.64	0.71	0.64	0.64	0.64	0.64	NA	NA
0.54	0.62	0.51	0.64	0.49	0.56	0.55	0.53	NA	NA
0.61	0.63	0.60	0.69	0.61	0.65	0.60	0.67	NA	NA
0.56	0.57	0.54	0.60	0.59	0.57	0.54	0.60	breast and ovarian cancer	tumour suppressor gene in breast and ovarian cancer
0.65	0.70	0.58	0.66	0.64	0.62	0.57	0.63	NA	NA
0.57	0.48	0.51	0.63	0.43	0.56	0.45	0.41	NA	NA
0.53	0.59	0.52	0.55	0.51	0.56	0.53	0.62	NA	NA
0.48	0.56	0.44	0.56	0.46	0.52	0.47	0.53	NA	NA
0.54	0.52	0.48	0.52	0.49	0.48	0.46	0.58	NA	NA
0.60	0.68	0.58	0.70	0.58	0.67	0.58	0.65	NA	NA
0.41	0.39	0.45	0.52	0.40	0.39	0.38	0.46	NA	NA

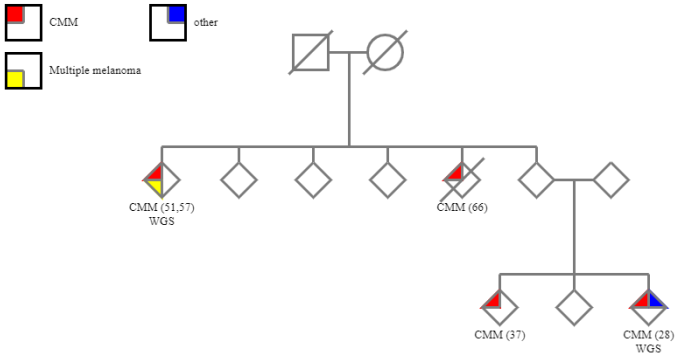
A. Family I



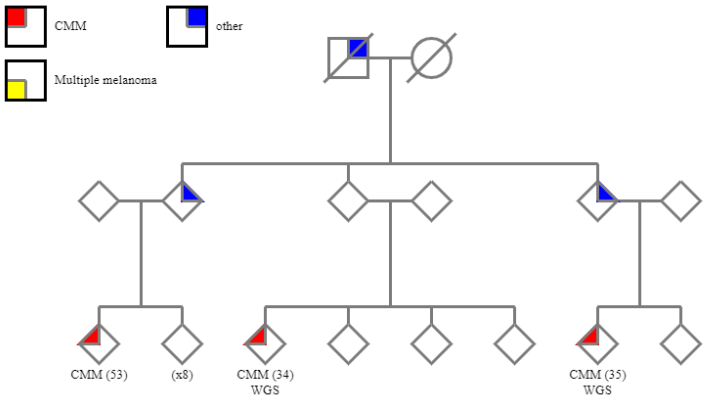
B. Family II



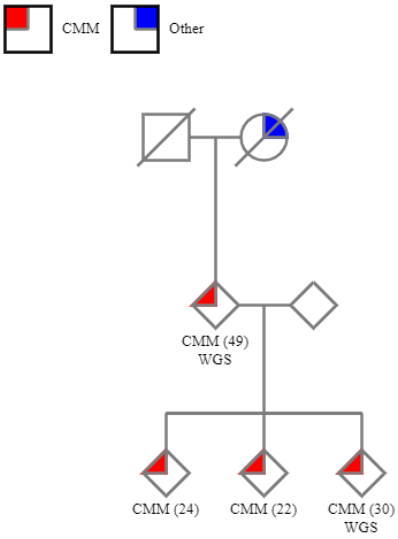
C. Family III



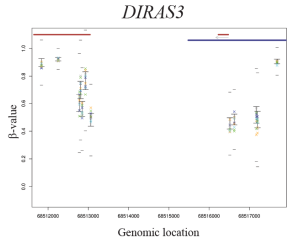
D. Family IV

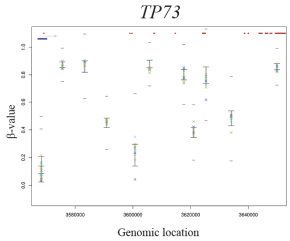


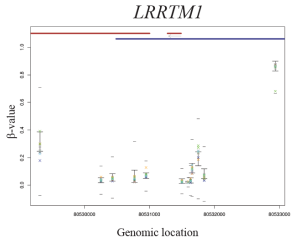
E. Family V



SUPPLEMENTARY FIGURE S1. Dutch melanoma families included in the whole-genome sequencing analysis. Left quarter red panel: cutaneous malignant melanoma (CMM) only; left quarter yellow panel: multiple melanoma (patient number I_2 and III_1 included in our study, see Table 1); right quarter blue panel: other cancer(s). The melanoma cases subjected to whole-genome sequencing included in this study are indicated by 'WGS'. Age at CMM diagnosis is given between brackets. A. Family I B. Family II C. Family III D. Family IV and E. Family V.


DIRAS3
β-value
Genomic location


TP73
β-value
Genomic location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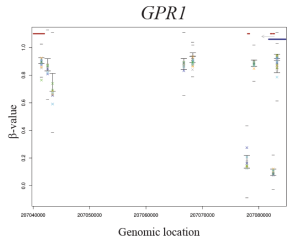
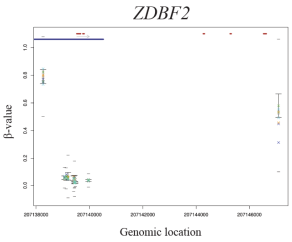
β-value
Genomic location


GPR1
β-value
Genomic location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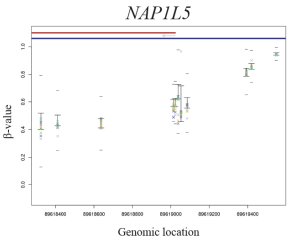
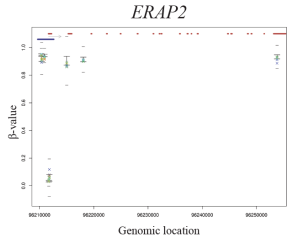
β-value
Genomic location


NAP1L5
β-value
Genomic location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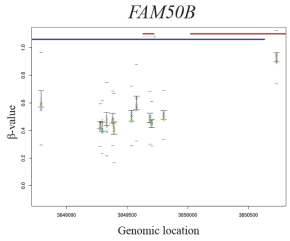
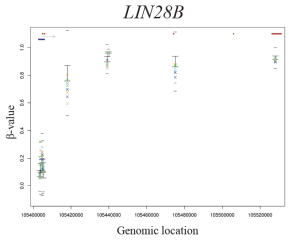
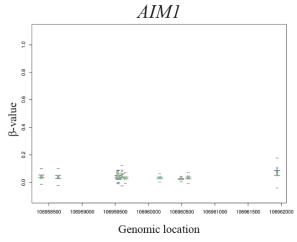
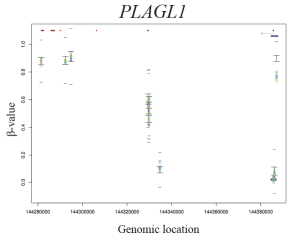
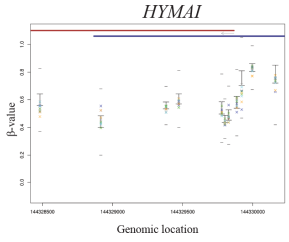
β-value
Genomic location

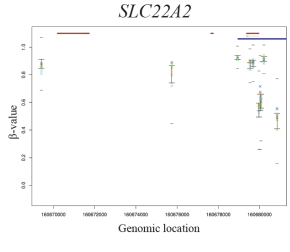

FAM50B
β-value
Genomic location

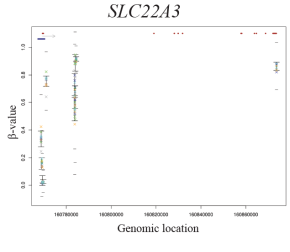

LIN28B
β-value
Genomic location

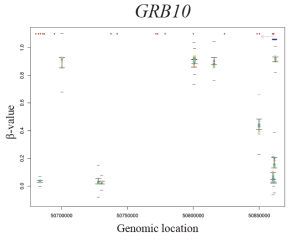

AIM1
β-value
Genomic location

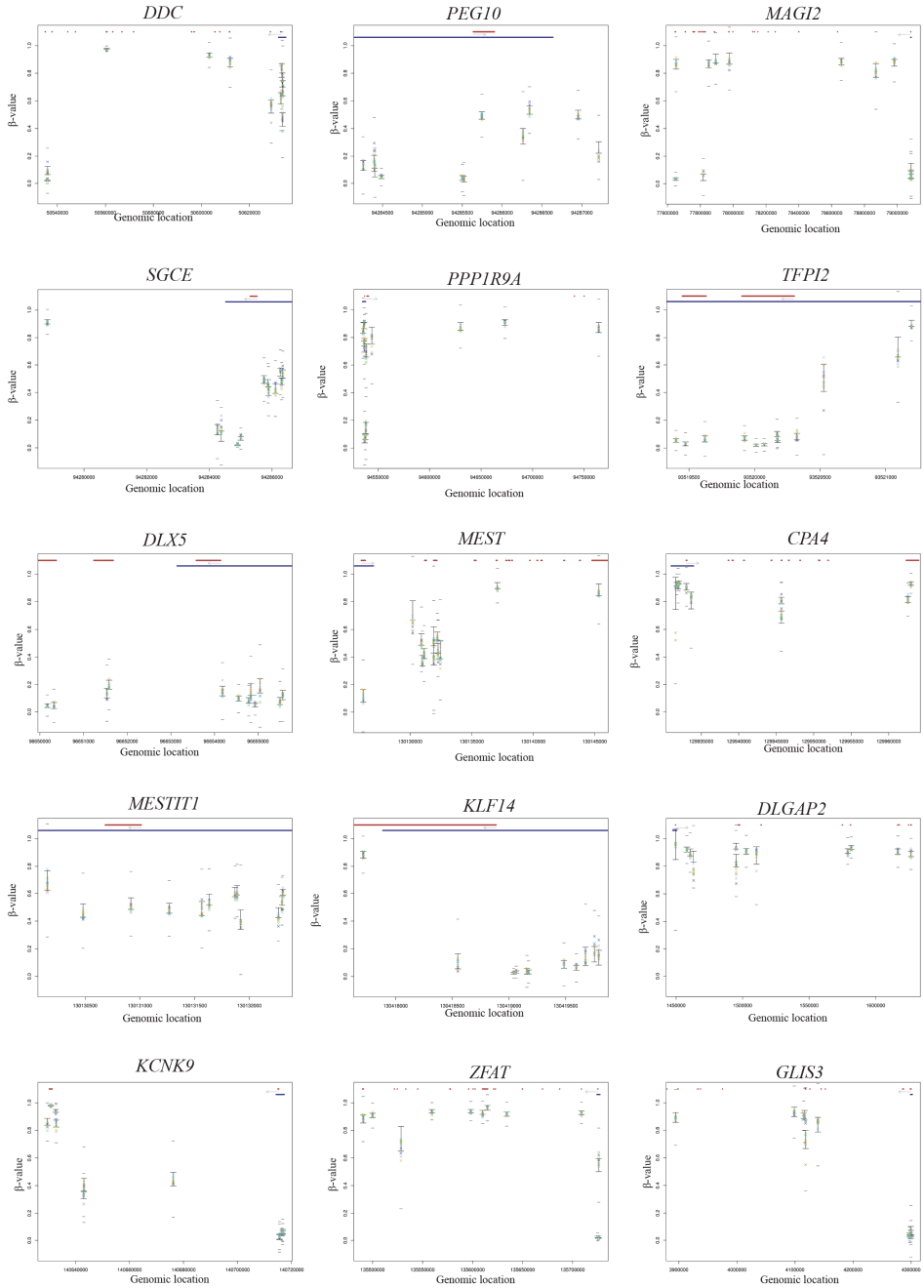

PLAGL1
β-value
Genomic location

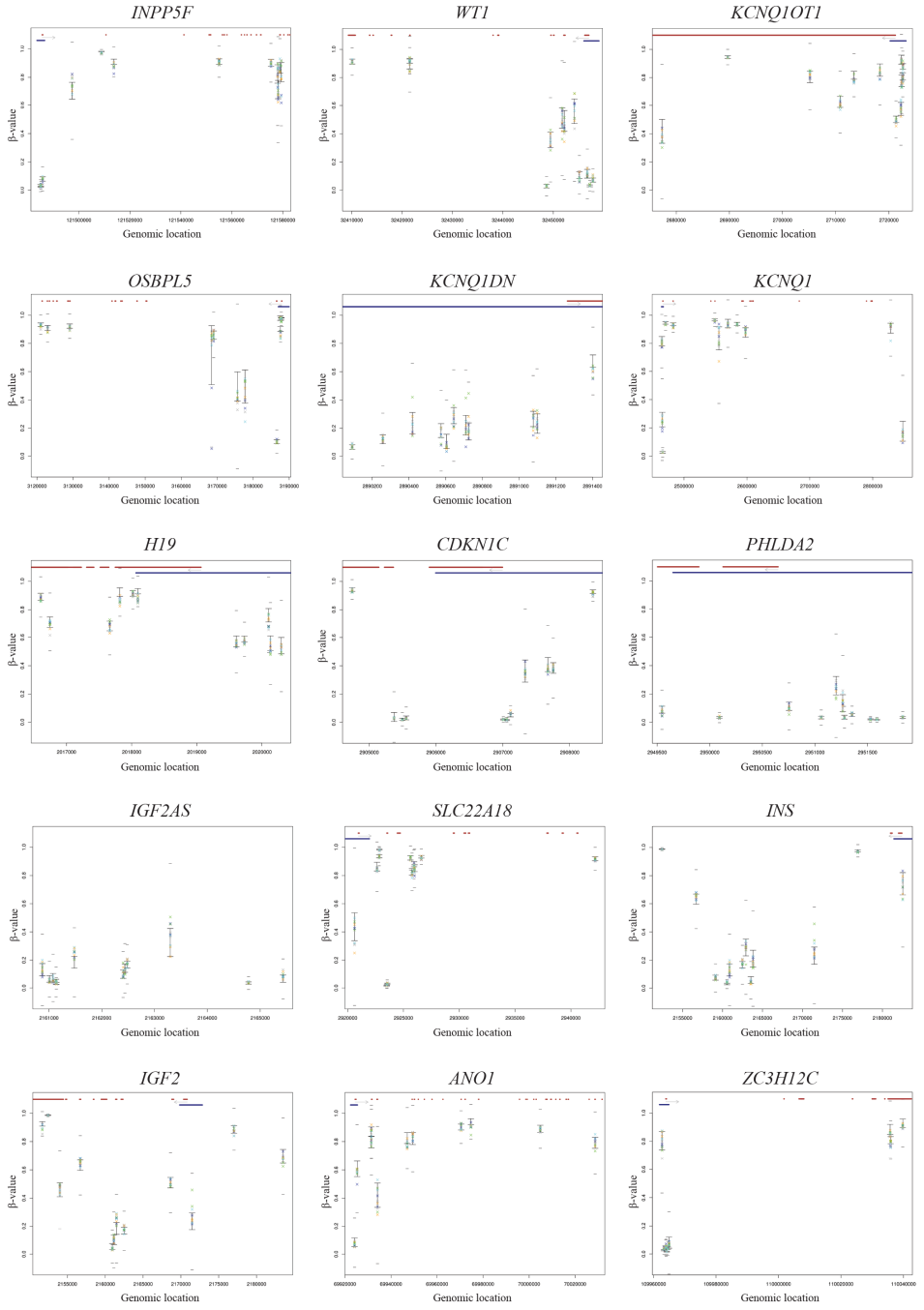

HYMAI
β-value
Genomic location


SLC22A2
β-value
Genomic location


SLC22A3
β-value
Genomic location


GRB10
β-value
Genomic location


DDC
PEG10
MAGI2
SGCE
PPP1R9A
TFPI2
DLX5
MEST
CPA4
MESTIT1
KLF14
DLGAP2
KCNK9
ZFAT
GLIS3
β-value
Genomic location


INPP5F
WT1
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OSBPL5
KCNQ1DN
KCNQ1
H19
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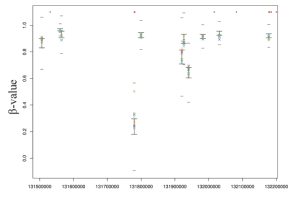
SLC22A18
INS
IGF2
ANO1
ZC3H12C
β-value
Genomic location

NTM



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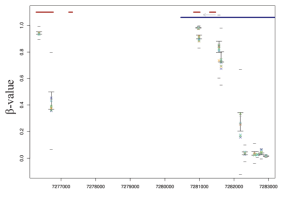

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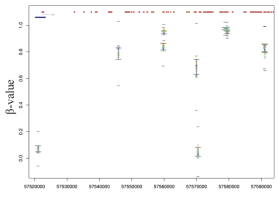

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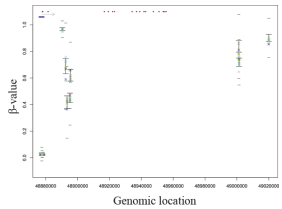

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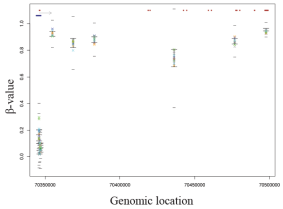

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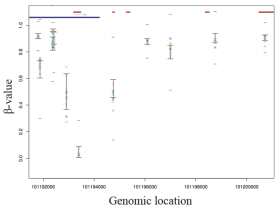

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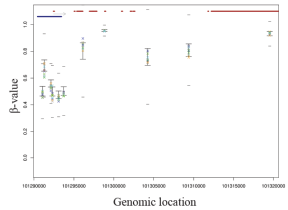

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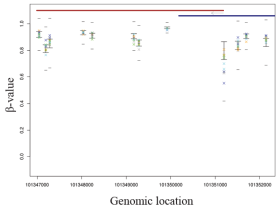

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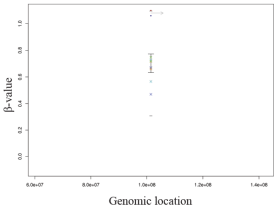

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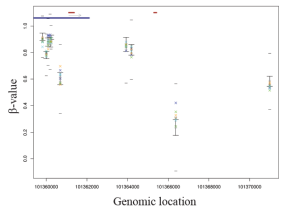

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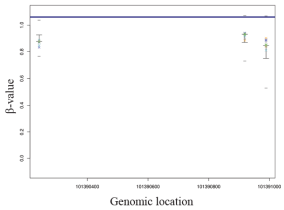

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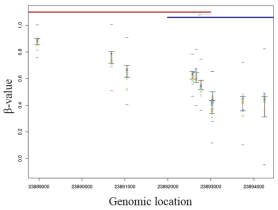

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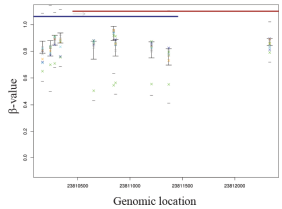

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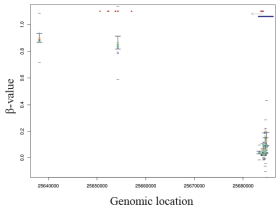

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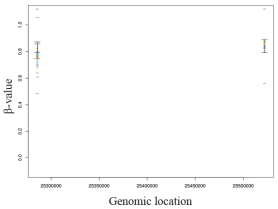

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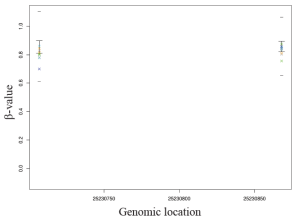

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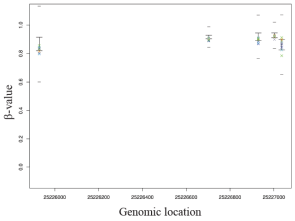

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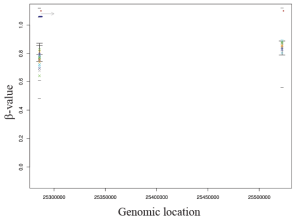

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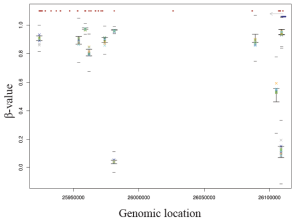

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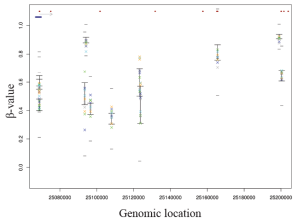

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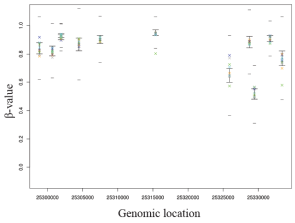

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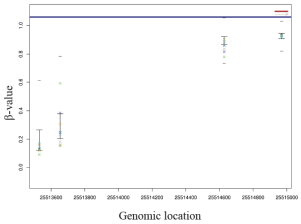

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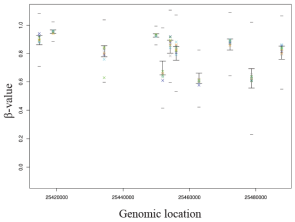

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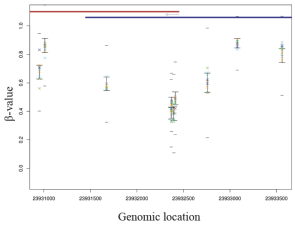

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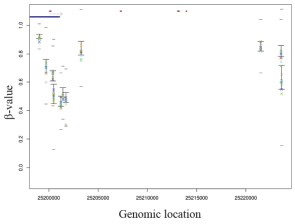

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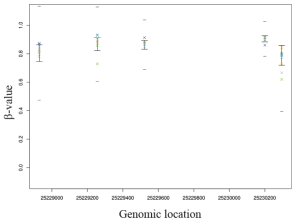

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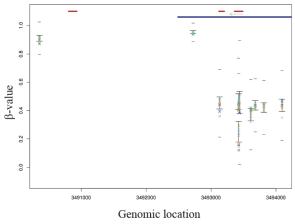
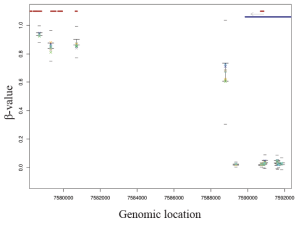
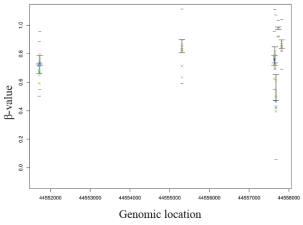


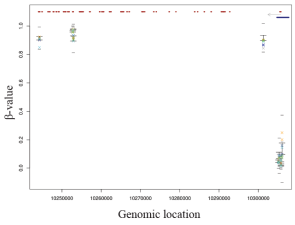
TP53

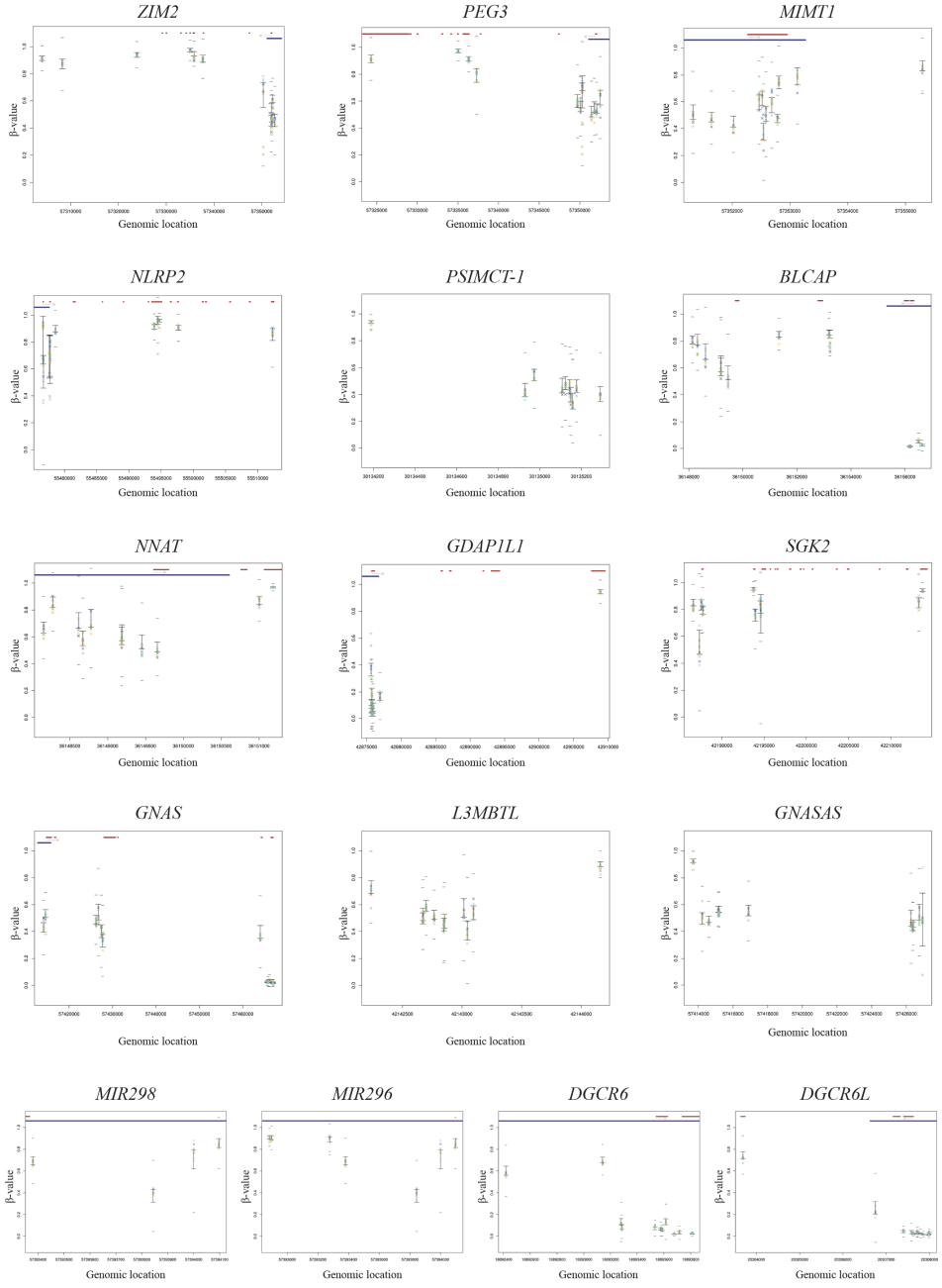


TCEB3C



DNMT1





SUPPLEMENTARY FIGURE S2. Methylation levels (β-value) across the entire sequence of all imprinted genes (http://www.geneimprint.com/site/genes-by-species, accessed August 2019) assessed by 450K array. In the upper part of each plot, the gene structure is represented in dark red and promoter region ("Promoter_associated" feature retrieved from Illumina annotation) in blue. The light grey arrow

represents the transcription direction of the gene. For each CpG, the BIOS values are represented by the black vertical line with upper (average + 1 SD) and lower limits (average – 1SD). The families are represented as a X of different colours (Family I – green, Family II – blue, Family III – yellow, Family IV – light purple, Family V – dark blue). To be considered as significantly different from the BIOS, the families symbols must go beyond the small black horizontal line (average ± 5.65 SD). Genes with more than 10 CpG sites assessed by 450K array, were represented by 10 randomly selected CpGs.

4

Genome-wide characterization of 5-hydroxymethylcytosine in melanoma reveals major differences with nevus

Catarina Salgado, Jan Oosting, Bart Janssen, Rajiv Kumar, Nelleke Gruis, Remco van Doorn

Genes Chromosomes Cancer. 2020 Jun;59(6):366-374.

ABSTRACT

Melanoma demonstrates altered patterns of DNA methylation that are associated with genetic instability and transcriptional repression of numerous genes. Active DNA demethylation is mediated by TET enzymes that catalyze conversion of 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC). Loss of hmC occurs in melanoma and correlates with disease progression. Here we analysed the genomic distribution of hmC along with mC in nevus and melanoma using oxidative bisulfite chemistry combined with high-density arrays. HmC was enriched relative to mC at enhancers, 5'UTR regions and CpG shores in nevus and melanoma samples, pointing to specific TET enzyme activity. The proportion of interrogated CpG sites with high hmC levels was lower in melanoma (0.54%) than in nevus (2.0%). Depletion of hmC in melanoma was evident across all chromosomes and intragenic regions, being more pronounced in metastatic than in non-metastatic tumours. The patterns of hmC distribution in melanoma samples differed significantly from those in nevus samples, exceeding differences in mC patterns. We identified specific CpG sites and regions with significantly lower hmC levels in melanoma than in nevus that might serve as diagnostic markers. Differentially hydroxymethylated regions localized to cancer-related genes, including the *PTEN* gene promoter, suggesting that deregulated DNA hydroxymethylation may contribute to melanoma pathogenesis.

KEYWORDS

5-hydroxymethylcytosine; DNA hydroxymethylation; DNA methylation; melanoma; *PTEN* gene

INTRODUCTION

Cutaneous melanoma is a malignant tumour derived from melanocytes residing in the skin. Clinically melanoma needs to be distinguished from melanocytic nevus, a benign lesion composed of melanocytes in a stable growth arrest.[1] Integrative genomic and transcriptomic analysis has identified common mutations and recurrent signaling perturbations yielding insight into melanoma biology.[2] In addition to accumulated genetic alterations, epigenetic mechanisms drive the development and evolution of melanoma.[3,4] DNA methylation, histone modifications and chromatin remodeling complexes regulate chromatin accessibility to transcription factors, thereby controlling gene expression programs. DNA methylation at CpG dinucleotides is mediated by DNA methyltransferases and additionally governed by DNA demethylation. Passive DNA demethylation can occur through insufficient methyltransferase activity during replication. Active demethylation involves the oxidation of 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC) performed by the Ten Eleven Translocase (TET) family of dioxygenase enzymes.[5] In mammalian cells approximately 4% of all cytosines are methylated, and depending on cell type 0.1% - 0.7% of cytosine bases are hydroxymethylated.[6] Epigenetic deregulation is a universal characteristic of malignant tumours implicated in tumourigenesis. Cancer genomes are characterized by widespread loss of DNA methylation that contribute to genomic instability, and gain of DNA methylation at promoter CpG islands is associated with transcriptional repression.[7] In melanoma, selected tumour suppressor genes with a critical role in malignant transformation and metastatic behaviour, including *CDKN2A*, *PTEN* and *CDH11*, show frequent promoter hypermethylation and associated transcriptional silencing.[8] In addition, variation of methylation density at enhancer regions contributes to melanoma cell plasticity and correlates with patient survival.[9]

Different tumour types demonstrate loss of DNA hydroxymethylation and in certain instances this epigenetic event can be attributed to mutations in *TET* or *IDH* genes. Although the functional relevance of hmC loss remains to be resolved, studies in melanoma suggest its involvement in tumour progression.[10] Accordingly, low hmC levels were associated with worse survival from melanoma. Thus, in melanoma and other tumour types hmC loss might have diagnostic as well as prognostic significance. Hydroxymethylation mapping of melanoma samples using hydroxymethylated DNA immunoprecipitation showed hmC clusters in gene-rich regions and loss at specific loci.[10] In glioblastoma hmC depletion was shown to be most pronounced at enhancer regions.[11] To understand the functional consequences of aberrant hydroxymethylation and to apply it in the diagnosis and prognosis of melanoma, it is essential to obtain precise maps of the distribution of this epigenetic mark. Here we characterized the genomic distribution of hmC and mC in nevus and melanoma using oxidative bisulfite chemistry combined with arrays that simultaneously interrogate hmC

and mC at 850,000 CpG sites. This methodology is not affected by bias associated with antibody-based DNA capture methods and provides robust estimates of hmC and mC.[12] We sought to identify differentially hydroxymethylated CpG sites and regions by comparing nevus and melanoma hmC patterns. In addition, we compared the hmC patterns between primary melanoma samples that differ with respect to metastatic behavior. The genomic landscapes of hmC show depletion of hydroxymethylation in melanoma across various intragenic and intergenic regions compared to nevus. The hydroxymethylation patterns show more differences between nevus and melanoma than the methylation patterns, which has potential implications for biomarker discovery.

MATERIAL & METHODS

PATIENT SAMPLES

Fresh-frozen biopsy samples were obtained from patients diagnosed with common nevus (n=8), non-metastatic primary melanoma (n=8), and metastatic primary melanoma (n=8) (Supplementary Table S1). Only tissue samples containing at least 50% nevus or melanoma cells were included. Genomic DNA from all samples was extracted using the Genomic-tip kit (Qiagen, Hilden, Germany). The study was approved by the Leiden University Medical Center institutional ethical committee (05-036) and was conducted according to the Declaration of Helsinki Principles.

BISULFITE AND OXIDATIVE BISULFITE CONVERSION AND HYBRIDIZATION

Genomic DNA (1µg) was subjected to BS and OxBS conversion using the TrueMethyl 96 Kit (CEGX, Cambridge, UK) and applied to the Infinium MethylationEPIC BeadChip Kit (Illumina, San Diego, USA) at GenomeScan (Leiden, The Netherlands). The BeadChip images were scanned on the iScan system and the data quality was assessed using the R script MethylAid.[13]

850K BEADCHIP DATA ANALYSIS

Data were processed using the ChAMP package,[14,15] normalized using the default BMIQ algorithm and analysed as described previously with genome build GRCh37/hg19.[12] The ratio of the signal for the cytosine sequence to the combined intensity is the β-value, reflecting the methylation level on a scale from 0 (unmethylated) to 1 (fully methylated). To obtain the hydroxymethylation fraction oxBS β-values are subtracted from BS beta values, generating Δβ-values.[12,16] To define CpGs with high hmC we established a cut-off based on the average of absolute Δβ-value for all probes (0.008 plus 3 standard deviations, 0.166). To compare groups (nevus vs. melanoma; non-metastatic vs. metastatic melanoma) a statistical

test using the Limma R package[17] was used with multiple testing corrections applying a stringent p-value <0.005.[18] The Bump Hunting Algorithm was used to identify differentially hydroxymethylated regions with closely positioned probes.[19] The rate of hmC, the average of Δβ-values for a specific group of CpGs, was calculated according to intragenic location, to CpG-context regions and at enhancer regions (melanocytic cell-specific and general) retrieved from FANTOM5 project (http://FANTOM5.gsc.riken.jp/5/).[20]

VALIDATION OF CANDIDATE LOCI

Validation of hydroxymethylation at the *PTEN* promoter was performed in an independent sample group (4 nevi and 4 melanoma metastases). Genomic DNA (1μg) was subjected to BS and OxBS conversion using TrueMethyl oxBS Module (NuGEN Technologies, Redwood City, USA). DNA was amplified using the PCR_X Enhancer System (Thermo Fisher Scientific, Waltham, USA) and subjected to capillary sequenced (primers: GGGGTTGTAAATAGATTTGATAGG and AAAAATATCTCCTACTACAACCCAAAA) and deep paired-end sequencing (tailed primers: GATGTGTATAAGAGACAGGGGGTTGTAAATAGATTTGATAGG and CGTGTGCTCTTCCGATCTAAAAATATCTCCTACTACAACCCAAAA) using a MiSeq system (Illumina).

RESULTS

OBTAINING GENOME-WIDE 5-HYDROXYMETHYLCYTOSINE PATTERNS

Twenty-four DNA samples were analysed, including 8 aggressive primary melanomas with metastatic behaviour (M+), 8 primary melanomas with no metastatic behaviour (M-) during long-term follow-up and 8 benign nevi (N) (Table 1, Supplementary Table S1). To detect methylcytosine (mC) and hydroxymethylcytosine (hmC), different states of the CpG sites, we applied oxidative bisulfite (oxBS) chemistry, calculating hmC levels based on differences between bisulfite (BS) and oxBS-treated samples, using arrays as described previously.[11,12,21] Bisulfite (BS) converts unmethylated cytosines to uracil, while methylated and hydroxymethylated cytosines are protected. The prior oxidative step in oxBS conversion allows the distinction between methylated and hydroxymethylated cytosines. Only hydroxymethylated but not methylated cytosines are oxidated into formylcytosines (5fC), which are converted to uracil. Arrays that interrogate over 850,000 CpG sites representing 99% of the RefSeq genes, encompassing more than 90% of interrogated sites of 450K arrays plus 333,265 CpGs located at enhancer regions were used.[22] After quality control and exclusion of X-chromosomal CpGs 743,016 CpGs were analysed. As a measure of DNA methylation, the fluorescence ratio (β-value, ranging from 0 to 1) for each CpG of the bisulfite-treated DNA sample was used. Subtraction of the normalized β-value of the oxBS-treated sample from that of the BS-treated replicate analysed in parallel (Δβ-value)

was used as a measure of hydroxymethylation (Supplementary Figure S1). The average Δβ-value for CpGs at different genomic locations (hmC rate) was calculated. In addition, we considered as CpGs with high hmC levels those having a Δβ-value exceeding the average plus 3 standard deviations (Δβ>0.166).

TABLE 1. Clinical characteristics of nevus and melanoma samples subjected to genome-wide DNA (hydroxy)methylation analysis.

	Melanocytic Nevi n=8	Non-metastatic primary melanomas n=8	Metastatic primary melanomas n=8
Gender			
Female	6	4	4
Male	2	4	4
Age at diagnosis in years, median (range)	42 (29-57)	39 (34-68)	63 (45-79)
Location			
Head/neck	4	1	3
Trunk	2	4	2
Extremities	2	3	3
Breslow depth in mm, median (range)		1.0 (0.73-4)	9.7 (1.9-17)

First, we compared the number of hydroxymethylated CpGs in the nevus, non-metastatic and metastatic melanoma sample groups. The number of CpGs with high hmC levels was significantly higher in nevus (2.0% of interrogated CpGs) than in melanoma (0.54%) samples as was the average Δβ-value for the sample groups (0.017 vs 0.004), consistent with earlier reports of hmC loss in melanoma (Figure 1a)[10] Comparative analysis of melanoma and nevus samples revealed 21,767 CpGs with significantly lower hydroxymethylation in melanoma than in nevus samples, whereas 397 CpGs showed higher levels of hmC in melanoma (FDR <0.005). However, the variation of hmC levels of these CpGs within sample groups was high (Supplementary Figure S2). In spite of heterogeneity certain CpG sites showed consistent hmC loss in melanoma. The 50 most differentially hydroxymethylated CpGs are presented in a heatmap in Supplementary Figure S3. When comparing metastatic and non-metastatic primary melanoma samples there were no interrogated CpGs with statistically significant different hmC level.

To capture the distribution of hmC, principal component analysis revealed that the hmC patterns of melanoma samples were distinct from those of nevus samples (Figure 1b). The differences between the sample groups were more pronounced for hmC than for mC patterns. The hmC patterns of metastatic and non-metastatic melanoma samples were not distinct in this analysis. The hmC levels at different chromosomal regions were almost uniformly higher in nevus than in melanoma samples, with no evident clustering of aberrant hmC at specific chromosomal regions (Figure 1c).

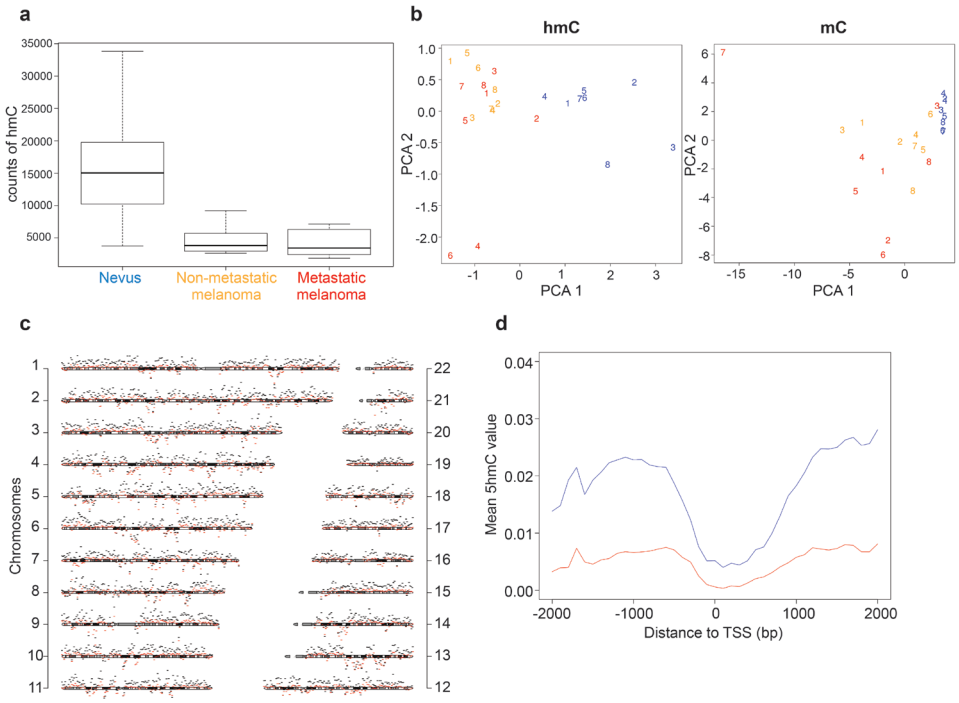


FIGURE 1. Genome-wide distribution of DNA hydroxymethylation in nevus, non-metastatic and metastatic melanoma. (a) Boxplot showing the counts of CpGs with high hmC (Δβ>0.166) for each group. (b) Principal component analysis of hmC and mC for 1% of probes with highest variation across samples. Numbers refer to individual samples. Blue – nevi; yellow – non-metastatic melanomas; red – metastatic melanomas. (c) Chromosomal distribution of hmC in nevi (black) and melanomas (red). The scheme of each chromosome represents the measurement baseline (null hmC level), the vertical distance between chromosomes is 10%, bin size is 1 Mb. (d) Mean of hmC level over 4 Kb around the transcription start sites for nevi (blue) and melanomas (red).

DEPLETION OF HMC IN DIFFERENT GENOMIC REGIONS

Since methylation of promoter, intragenic and intergenic regions has distinct associations with gene transcription, we determined the location of hmC and mC within these regions. First, we assessed the average hmC rate across 4Kb at promoter regions around the canonical transcription start site of all genes and observed slightly lower hmC levels in melanoma throughout the entire region compared to benign nevus (Figure 1d). TET proteins generate hmC as an intermediate from mC in active DNA demethylation; hmC levels tend to follow mC levels therefore. Accordingly, both mC and hmC levels were considerably lower at CpGs in the proximal promoter and first exon. However, the distal promoter (200-1500 bp upstream of transcription start site) and 5'UTR regions are exceptions that show high hmC

in spite of moderate mC levels in all sample groups (Figure 2a, b). Whereas the mC levels were only marginally lower in melanoma than in nevus, we observed a striking loss of hmC not only in promoters but across all gene regions. The levels of hmC were also significantly lower in the metastatic than in the non-metastatic melanomas in most gene regions.

Higher variation of hmC at enhancer regions in tumour has been reported in glioblastoma.[11] Therefore, we analysed the average rate of hmC at melanocyte-specific and at general enhancer regions retrieved from the FANTOM5 project.[20] We found higher hmC levels in enhancer compared to non-enhancer regions among the different sample groups (Supplementary Figure S4). The depletion of hmC at enhancer regions in melanoma compared with nevus was proportional to that at non-enhancer regions.

CpG islands, particularly located at promoter regions, are mostly protected from methylation. The regions adjacent to CpG islands, termed shores and shelves have also been found to demonstrate specific methylation patterns associated with transcriptional states.[23,24] Subsequently we calculated the hmC and mC levels of cytosines located in these regions and found that the mC levels were lower in CpG islands and shores than in shelves and open sea (Figure 2c, d). Again the loss of hmC in melanoma compared to nevus was much larger than the difference in mC across the CpG islands, shores, shelves and open sea. Whereas generally the hmC levels follow the mC levels, the CpG shores are another exception demonstrating high hmC in spite of moderate mC levels, especially in nevus samples.

Taken together, in nevus and melanoma hmC levels differ markedly across genomic regions and not following mC levels, which points to specific enzymatic activity in shaping hmC patterns. The hmC levels are substantially lower in melanoma than in nevus across all intragenic regions. This is in line with dilution through replication and insufficient active TET-mediated hydroxymethylation. Differences of hmC levels and distribution are much more pronounced than of mC levels.

DIFFERENTIALLY HYDROXYMETHYLATED REGIONS IN MELANOMA

Although the modification of a single CpG site may impact on gene expression, regions containing multiple CpG sites in promoters and enhancers commonly work as units of transcriptional regulation. Therefore, we sought to identify and examine regions with differential hydroxymethylation (DhMRs). When comparing melanoma and nevus samples, 68 regions were statistically significant differentially hydroxymethylated ($p<0.005$). In all 68 DhMR hmC levels were lower in melanoma compared to nevus (Figure 3, Supplementary Table S2). No significantly differentially hydroxymethylated regions were identified when comparing metastatic and non-metastatic melanoma samples. Five of these regions are located within

established cancer-related genes (http://cancer.sanger.ac.uk/census, accessed October 2019), namely in the *GNAS*, *GAS7*, *PTEN*, *TPM4* and *DAXX* (Supplementary Table S2). Notably, for the *PTEN* and *TPM4* tumour suppressor genes the DhMR is located in the promoter region.

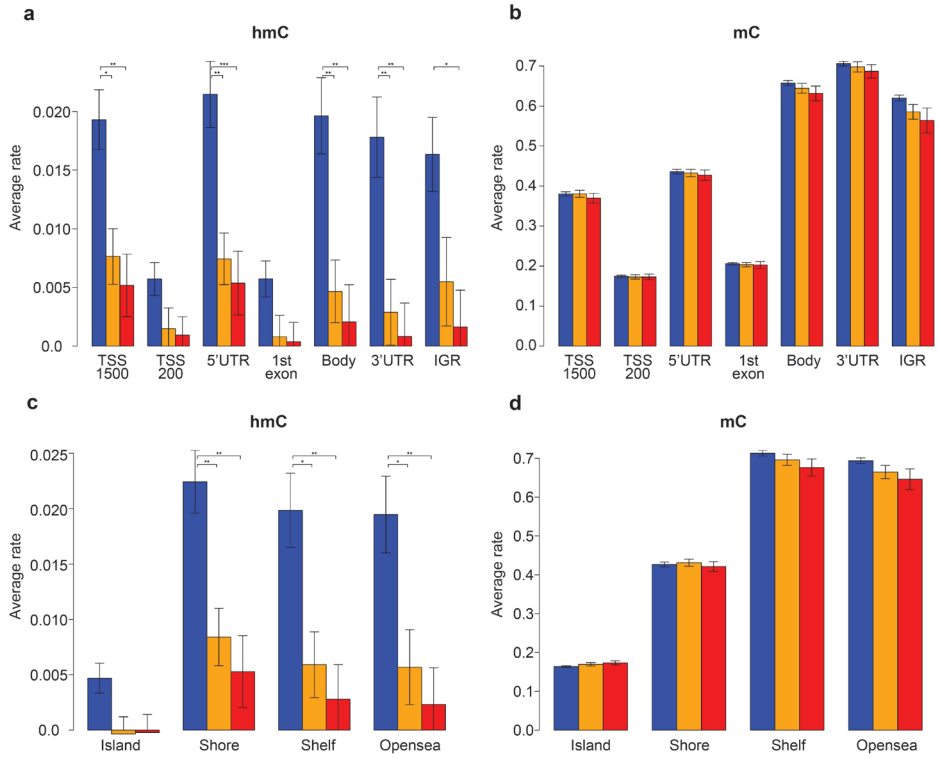


FIGURE 2. Average rate of hmC and mC at intragenic locations and CpG-context regions. (a) hmC levels in the N, M- and M+ sample groups at intragenic regions presented as average Δβ-values. (b) mC level presented as average β-values. Untranslated regions (3'UTR and 5'UTR), proximal promoter (TSS-200bp and 1stexon), distal promoter (TSS-1500bp), gene body, and intergenic region (IGR). (c) hmC levels in the N, M- and M+ sample groups at CpG-context regions presented as average Δβ-values. (d) mC level presented as average β-values. CpG island, shore (<2Kb flanking CpG Islands), shelves (<2Kb flanking outwards from CpG shore) and open sea (>4Kb from CpG island). Blue – nevi; yellow – non-metastatic melanomas; red – metastatic melanomas. The error bars represent standard errors among samples.

PTEN PROMOTER HYDROXYMETHYLATION IN NEVUS AND MELANOMA

PTEN is an established tumour suppressor gene, inactivated in melanoma and other tumour types through genetic and epigenetic mechanisms. Therefore, we further analysed hydroxymethylation at this locus in nevus and melanoma. In our study, a region in the *PTEN*

promoter (chr10:89621419-89622084) was found to show hydroxymethylation in all nevus samples, but higher methylation levels in the melanoma samples (Figure 4a).

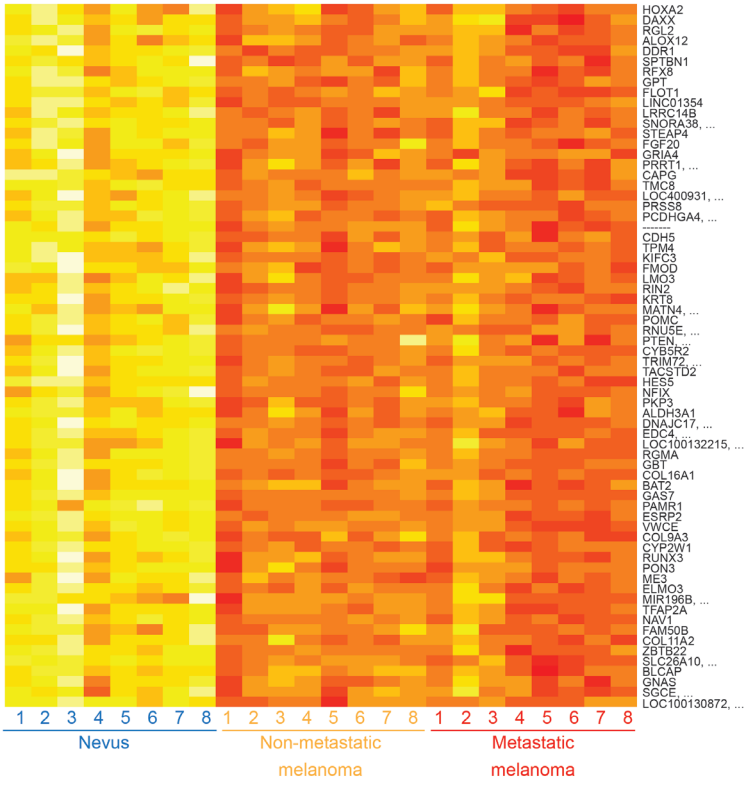


FIGURE 3. Heatmap depicting hmC levels for 68 significantly differentially hydroxymethylated regions in nevus and melanoma samples. Each row represents a DhMR with the associated gene and each column represents a different sample. Average hmC level, measured as Δβ-value, is indicated by variable colour (low hmC – red, high hmC – yellow).

Methylation of this specific region in the *PTEN* promoter, located from -1400 to -800bp upstream of the transcription start site, has been reported as being associated with transcriptional repression of *PTEN* in various malignancies and worse survival in melanoma patients (Supplementary Figure S5).[25-27] Capillary sequencing of the region following BS and oxBS conversion of DNA from nevus and melanoma samples subjected to hmC profiling, along with a normal skin sample, confirmed the presence of hydroxymethylation in nevus and normal skin samples (higher T peak upon oxBS) and methylation in a melanoma sample (maintenance of higher C peak after Bs and oxBS) (Figure 4b). Next, we analysed this DhMR

in the *PTEN* promoter using an independent quantitative BS/oxBS deep sequencing method in an independent set of 4 nevi and 4 metastatic melanoma samples. The 6 CpGs analysed using BS/oxBS NGS (chr10:89621419-89621537) confirmed the hydroxymethylation profile in nevi and a predominant methylation status in melanomas (Figure 4c).

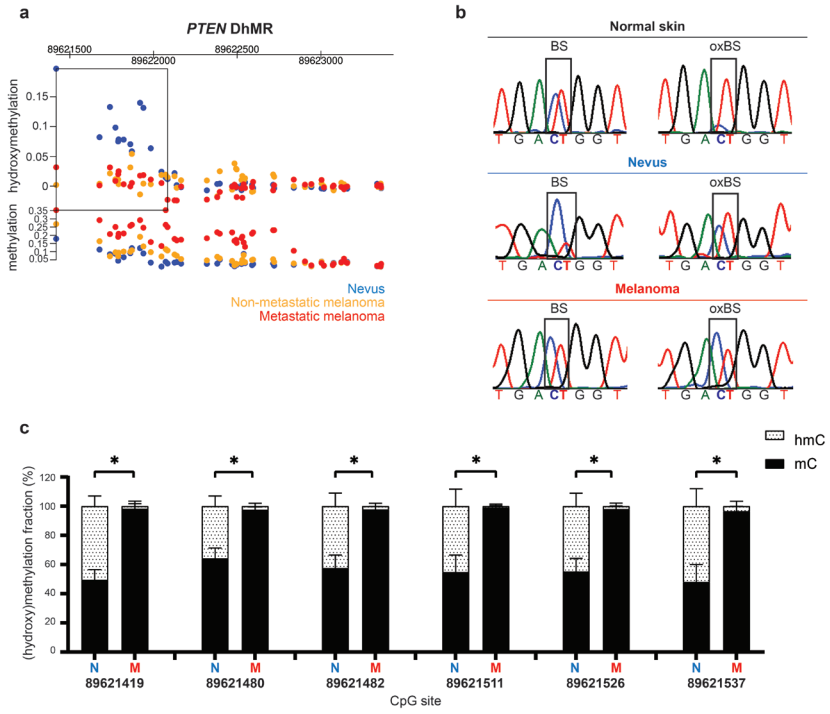


FIGURE 4. Differentially hydroxymethylated region in the *PTEN* promoter region. (a) DhMR (rectangle; chr10:89621419-89622084) located within the promoter region of *PTEN* gene with hydroxymethylation and methylation levels for the three sample groups. (b) Validation of a selected CpG site from *PTEN* DhMR by capillary sequencing upon BS and oxBS conversion. After oxBS a higher T peak appears in normal skin and nevus samples, while in melanoma sample there is a higher C peak. (c) BS/OxBS deep sequencing of 6 CpG sites at the *PTEN* DhMR (chr10:89621419-89621537) in 4 independent nevi and 4 melanomas (mean ± SD, *p<0.05, two-tailed Mann-Whitney U test). Blue – nevi; yellow – non-metastatic melanomas; red – metastatic melanomas.

DISCUSSION

Loss of hmC is an established feature of melanoma and other tumour types, with potential diagnostic and prognostic significance.[10,28] Here we provide a genome-wide landscape of hmC

and mC in nevus and melanoma by applying robust oxidative bisulfite chemistry combined with high-density arrays. Unsupervised analysis revealed significant differences in the global hmC patterns of melanoma and nevus samples, exceeding those of mC patterns. Numerous published studies have aimed to identify diagnostic and prognostic DNA methylation markers for melanoma.[29-31] Our study shows that analysis of hmC levels and distribution can equally be used to aid in distinguishing melanoma from benign melanocytic lesions. Accordingly, determination of hmC levels using immunohistochemistry in the diagnosis of melanoma has been proposed.[32] We identified thousands of single differentially hydroxymethylated CpG sites and 68 regions that might be used as specific diagnostic markers for melanoma. Although the levels of hmC were uniformly lower in metastatic than in non-metastatic melanoma, the patterns of distribution were not significantly different.

We observed a striking loss of hmC in melanoma relative to nevus, consistent with findings in other tumour types, across all autosomes, intragenic and intergenic regions, within and outside of CpG islands.[11,33] This phenomenon may be explained by passive dilution of the hmC mark due to DNA replication in proliferating melanoma cells and by insufficient active demethylation. Downregulation of IDH and TET family enzymes in melanoma has been shown previously, involving deregulation of active TET-mediated DNA demethylation in shaping the melanoma epigenome.[10] Within the pattern of global hmC depletion, specific CpG sites and regions could be identified with significantly lower hydroxymethylation in melanoma than in nevus, pointing to epigenetic deregulation at specific loci. In nevus and melanoma the hydroxymethylation levels were particularly low at the proximal promoter (TSS200) and first exon, corresponding with lower levels of methylation at promoter CpG islands. However, at CpG shores we observed high levels of hydroxymethylation disproportionate to the methylation levels at these sites in nevus and melanoma. Enrichment of hmC at CpG shores, regions that regulate gene expression, has been reported in non-small cell lung cancer and liver cancer previously.[24]

In melanoma and other tumour types, the methylation landscape demonstrates marked alterations at enhancer regions, which can impact on gene expression programmes and tumour aggressiveness.[9] Oxidation of mC into hmC is associated with enhancer activation.[34] Hydroxymethylation at these critical regulatory regions in tumours could induce functional demethylation and activation. In this study, we observed enrichment of hmC at enhancer regions in nevus and melanoma, as was reported for glioblastoma, but no excess depletion of hmC at enhancers in melanoma.[11]

The hmC mark is associated with an open chromatin configuration, affecting gene expression regulation.[34] Active demethylation can protect promoter and enhancer regions

from methylation-associated silencing. Loss of hmC might therefore contribute to malignant progression. Among the 68 DhMRs, 5 localized to the cancer-related genes *PTEN, DAXX*, *GAS7*, *GNAS* and *TPM4*. *PTEN* is an essential tumour suppressor gene in melanoma. Here, we demonstrate the presence of hydroxymethylation in the promoter region of the *PTEN* gene (chr10:89621419-89622084) in nevi and its absence in melanomas. It has been reported that *PTEN* expression is uniformly high in nevus and markedly lower in melanoma samples.[10,35,36] In melanomas, *PTEN* is functionally inactivated through genetic and epigenetic mechanisms, including promoter hypermethylation.[25,37] Loss of *PTEN* expression in murine nevi accelerates melanoma formation by allowing escape from oncogene-induced senescence.[38] It is tempting to speculate that hmC depletion at the *PTEN* regulatory region in melanoma has functional significance by affecting expression of this tumour suppressor gene. Accordingly, it was recently found that ablation of the *TET2* gene, resulting in genomic hmC loss, drives malignant transformation and melanoma progression.[39] In the genetically engineered mouse models studied deregulated expression of *CDKN2A* was observed. Even partial *PTEN* loss due to epigenetic mechanisms has biological relevance in melanoma.[36] Of note, the CpG sites showing hypermethylation in the study by Giles *et al.*[36] are located within the DhMR that we identified. The potential role of depletion of hmC at the *PTEN* promoter as an epigenetic mechanism driving melanoma progression requires further investigation.

In conclusion, we have resolved the genome-wide hmC and mC distribution in melanoma and nevus, of potential relevance for biomarker discovery and understanding of epigenetic deregulation in melanoma. We identified specific CpG sites and regions with significantly lower hydroxymethylation in melanoma than in nevus. Our results merit further investigation into the functional relevance of hydroxymethylation at the *PTEN* promoter in nevus and depletion at this locus in melanoma. Methods used in previous studies to analyse DNA methylation that rely on bisulfite conversion may have overestimated methylation, since part of the observed protection from conversion to uracil is caused by hydroxymethylation. However, we can assume that this potential error on melanoma is minor. Following on this genome-wide analysis of hmC, the value of the identified differentially hydroxymethylated CpG sites and regions should be tested in a large cohort of dysplastic melanocytic nevi and melanomas.

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Tumor samples characteristics.

Nr	Tumour type	Site	Breslow (mm)	Ulceration	Mitoses	Gender	Age	Color code
1	Naevus	leg				F	29	blue
2	Naevus	head & neck				F	52	blue
3	Naevus	trunk				F	43	blue
4	Naevus	upper leg				M	55	blue
5	Naevus	trunk				F	40	blue
6	Naevus	head & neck				F	39	blue
7	Naevus	head & neck				M	57	blue
8	Naevus	head & neck				F	30	blue
1	Met- primary melanoma	lower leg	2.08	yes	yes	M	68	orange
2	Met- primary melanoma	back	0.91	no	no	F	42	orange
3	Met- primary melanoma	foot	4	yes	yes	M		orange
4	Met- primary melanoma	buttock	0.73	no	no	F	35	orange
5	Met- primary melanoma	trunk	2.05	no	yes	F		orange
6	Met- primary melanoma	ear	0.81	no	yes	M	58	orange
7	Met- primary melanoma	shoulder	0.94	yes	yes	F	34	orange
8	Met- primary melanoma	back	1.06	no	yes	M	65	orange
1	Met+ primary melanoma	scalp	3.46	no	yes	M		red
2	Met+ primary melanoma	foot	1.9	no	yes	F		red
3	Met+ primary melanoma	back	3.09	no	yes	F	45	red
4	Met+ primary melanoma	neck, desmoplastic melanoma	15.6	no	yes	M		red
5	Met+ primary melanoma	thigh	9.2	yes	yes	F		red
6	Met+ primary melanoma	vulva	10.2	yes	yes	F	56	red
7	Met+ primary melanoma	scalp	17	yes	yes	M	79	red
8	Met+ primary melanoma	axilla	12	no	yes	M	70	red

Met- primary melanoma – non-metastatic primary melanoma

Met+ primary melanoma – metastatic primary melanoma

F – female

M – Male

SUPPLEMENTARY TABLE S2. 68 differentially hydroxymethylated regions (DhMRs).

Chr	start	end	p.valueArea	fwerArea	Gene ID
chr4	1201588	1203168	0.00E+00	0.00E+00	LOC100130872 LOC100130872-SPON2
chr7	94285642	94287211	0.00E+00	0.00E+00	SGCE PEG10
chr20	57462798	57464129	0.00E+00	0.00E+00	GNAS
chr20	36148133	36149455	0.00E+00	0.00E+00	BLCAP
chr12	58012960	58013942	0.00E+00	0.00E+00	SLC26A10 LOC101927583
chr6	33282885	33283317	0.00E+00	0.00E+00	ZBTB22
chr6	33130696	33132442	0.00E+00	0.00E+00	COL11A2
chr6	3848634	3849818	0.00E+00	0.00E+00	FAM50B
chr1	201708419	201709675	9.23E-06	8.00E-03	NAV1
chr6	10419016	10421069	1.85E-05	1.60E-02	TFAP2A
chr7	27208285	27209828	1.85E-05	1.60E-02	MIR196B HOXA10-AS
chr16	67232921	67234167	1.85E-05	1.60E-02	ELMO3
chr11	86382900	86383940	1.85E-05	1.60E-02	ME3
chr7	95025194	95027158	1.85E-05	1.60E-02	PON3
chr1	25257505	25258332	1.85E-05	1.60E-02	RUNX3
chr7	1022643	1023156	1.85E-05	1.60E-02	CYP2W1
chr20	61446962	61447623	1.85E-05	1.60E-02	COL9A3
chr11	61062665	61063378	1.85E-05	1.60E-02	VWCE
chr16	68270129	68271177	2.77E-05	2.40E-02	ESRP2
chr11	35546824	35548139	3.23E-05	2.80E-02	PAMR1
chr17	9862752	9863293	3.23E-05	2.80E-02	GAS7
chr6	31598379	31599955	3.23E-05	2.80E-02	BAT2
chr1	32169701	32170433	3.69E-05	3.20E-02	COL16A1
chr8	145728138	145728630	4.15E-05	3.60E-02	GPT
chr15	93616894	93617402	4.62E-05	4.00E-02	RGMA
chr2	63275509	63276833	5.08E-05	4.40E-02	LOC100132215 OTX1
chr16	67918001	67918965	5.08E-05	4.40E-02	EDC4 NRN1L
chr15	41061384	41062224	5.08E-05	4.40E-02	DNAJC17 C15orf62
chr17	19648846	19649293	5.08E-05	4.40E-02	ALDH3A1
chr11	392903	394545	5.08E-05	4.40E-02	PKP3
chr19	13135318	13135808	5.08E-05	4.40E-02	NFIX
chr1	2461278	2461929	5.08E-05	4.40E-02	HES5
chr1	59042931	59044110	5.08E-05	4.40E-02	TACSTD2
chr16	31227800	31228720	5.08E-05	4.40E-02	TRIM72 PYDC1
chr11	7695165	7695809	5.08E-05	4.40E-02	CYB5R2
chr10	89621419	89622084	5.08E-05	4.40E-02	PTEN KILLIN
chr5	80528581	80529340	5.08E-05	4.40E-02	RNU5E CKMT2
chr2	25391505	25391911	5.08E-05	4.40E-02	POMC
chr20	43936663	43937467	5.54E-05	4.40E-02	MATN4 RBPJL
chr12	53298383	53299310	5.54E-05	4.40E-02	KRT8
chr20	19866743	19867423	5.54E-05	4.40E-02	RIN2
chr12	16757954	16758465	6.92E-05	4.80E-02	LMO3
chr1	203320190	203320732	6.92E-05	4.80E-02	FMOD

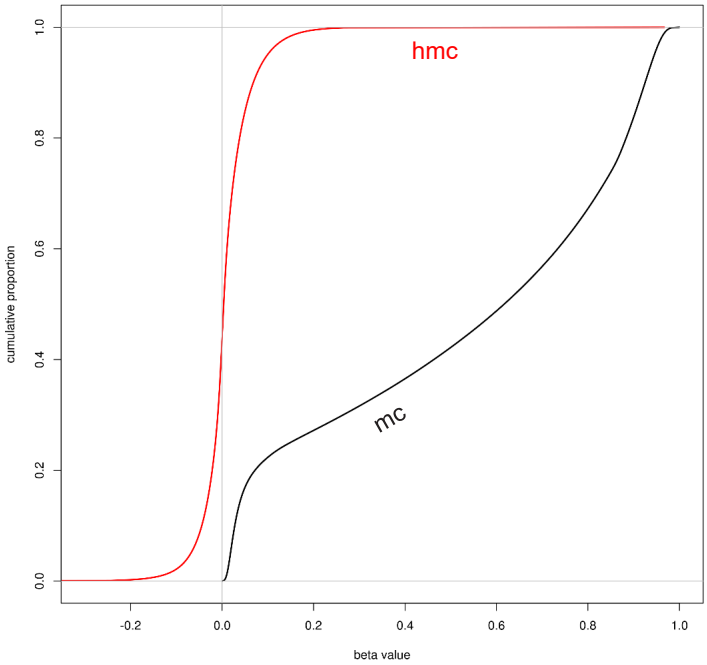
Hydroxymethylation		Methylation			
Naevus	Melanoma	Naevus	Melanoma	In CpG island?	Cancer-related genes?[a]
0.09	-0.02	0.36	0.61	shore island	
0.02	-0.02	0.32	0.34	island shore	
0.04	-0.01	0.38	0.44	island shore	oncogene in pituitary adenoma, pancreatic intraductal papillary mucinous neoplasm, fibrous dysplasia
0.03	-0.01	0.81	0.77	island shore	
0.09	0.01	0.29	0.38	island shore	
0.10	0.00	0.35	0.50	island shore	
0.07	0.00	0.52	0.64	shore shelf	
0.05	0.00	0.25	0.38	island shore	
0.11	0.00	0.26	0.41	shore island	
0.07	-0.01	0.23	0.36	shore island	
0.05	-0.01	0.22	0.35	island shore	
0.13	0.00	0.34	0.54	island shore	
0.07	0.00	0.26	0.35	island shore	
0.03	-0.01	0.27	0.39	island shore	
0.08	0.01	0.30	0.47	island	
0.08	-0.03	0.42	0.59	shore	
0.10	0.02	0.23	0.27	shore	
0.11	0.00	0.31	0.41	island shore	
0.14	0.02	0.18	0.35	island	
0.09	0.00	0.29	0.38	island shore	
0.10	0.00	0.31	0.40	opensea	fusion partner in acute myeloid leukemia
0.04	0.01	0.80	0.86	shore island	
0.19	0.02	0.14	0.26	island shore	
0.13	-0.01	0.33	0.63	shore	
0.18	0.04	0.22	0.30	island shore	
0.04	-0.01	0.16	0.21	island	
0.10	0.00	0.42	0.60	shore island	
0.12	0.00	0.30	0.41	opensea	
0.07	-0.02	0.42	0.61	shore	
0.07	0.00	0.44	0.56	shore island	
0.12	0.01	0.10	0.20	island	
0.12	0.01	0.25	0.24	island	
0.08	0.00	0.17	0.22	island shore	
0.08	0.01	0.25	0.28	island shore	
0.12	0.00	0.21	0.33	island shore	
0.08	0.01	0.08	0.18	island shore	tumor suppressor gene in glioma, prostate, endometrial carcinomas
0.10	-0.01	0.37	0.51	opensea	
0.10	0.00	0.25	0.32	island shore	
0.02	-0.02	0.45	0.51	shore	
0.15	0.01	0.45	0.67	shore	
0.09	0.00	0.41	0.49	opensea	
0.10	0.00	0.20	0.24	opensea	
0.07	0.00	0.35	0.33	opensea	

SUPPLEMENTARY TABLE S2 CONTINUED.

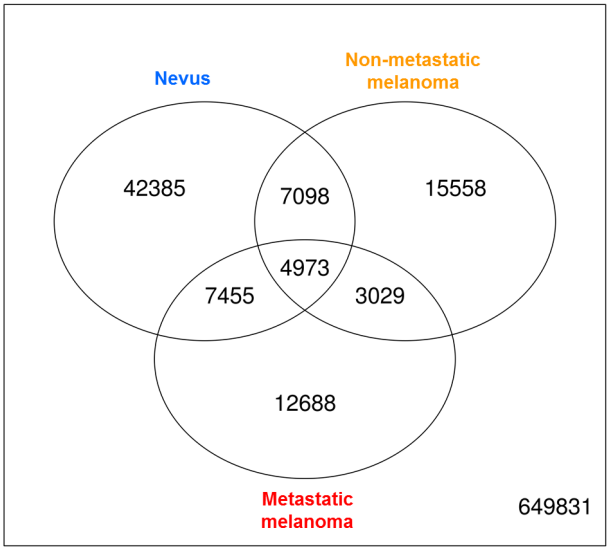
Chr	start	end	p.valueArea	fwerArea	Gene ID
chr16	57831745	57832309	6.92E-05	4.80E-02	KIFC3
chr19	16178030	16178570	6.92E-05	4.80E-02	TPM4
chr16	66400320	66400599	6.92E-05	4.80E-02	CDH5
chr16	54972078	54973128	7.38E-05	4.80E-02	
chr5	140864020	140864834	7.38E-05	4.80E-02	PCDHGA4 PCDHGC4
chr16	31146682	31147199	7.38E-05	4.80E-02	PRSS8
chr22	46481603	46482023	7.38E-05	4.80E-02	LOC400931 MIRLET7BHG
chr17	76128481	76129099	7.38E-05	4.80E-02	TMC8
chr2	85640762	85641438	7.38E-05	4.80E-02	CAPG
chr6	32119616	32120324	7.38E-05	4.80E-02	PRRT1 PPT2
chr11	105479843	105480979	7.38E-05	4.80E-02	GRIA4
chr8	16859451	16860121	7.38E-05	4.80E-02	FGF20
chr7	87935979	87936923	7.38E-05	4.80E-02	STEAP4
chr6	31590513	31590736	7.38E-05	4.80E-02	SNORA38 BAT2
chr5	191127	192103	7.38E-05	4.80E-02	LRRC14B
chr1	234667087	234667549	7.38E-05	4.80E-02	LINC01354
chr6	30698584	30698987	7.38E-05	4.80E-02	FLOT1
chr8	145729106	145729799	7.38E-05	4.80E-02	GPT
chr2	102091048	102091755	7.38E-05	4.80E-02	RFX8
chr2	54785178	54785795	7.38E-05	4.80E-02	SPTBN1
chr6	30850309	30851086	7.85E-05	4.80E-02	DDR1
chr17	6898315	6899888	5.08E-05	4.40E-02	ALOX12
chr6	33263805	33265016	5.54E-05	4.40E-02	RGL2
chr6	33288366	33289280	4.62E-05	4.00E-02	DAXX
chr7	27142100	27143806	5.08E-05	4.40E-02	HOXA2

[a]Data retrieved from Cancer Gene Census - COSMIC (https://cancer.sanger.ac.uk/census)

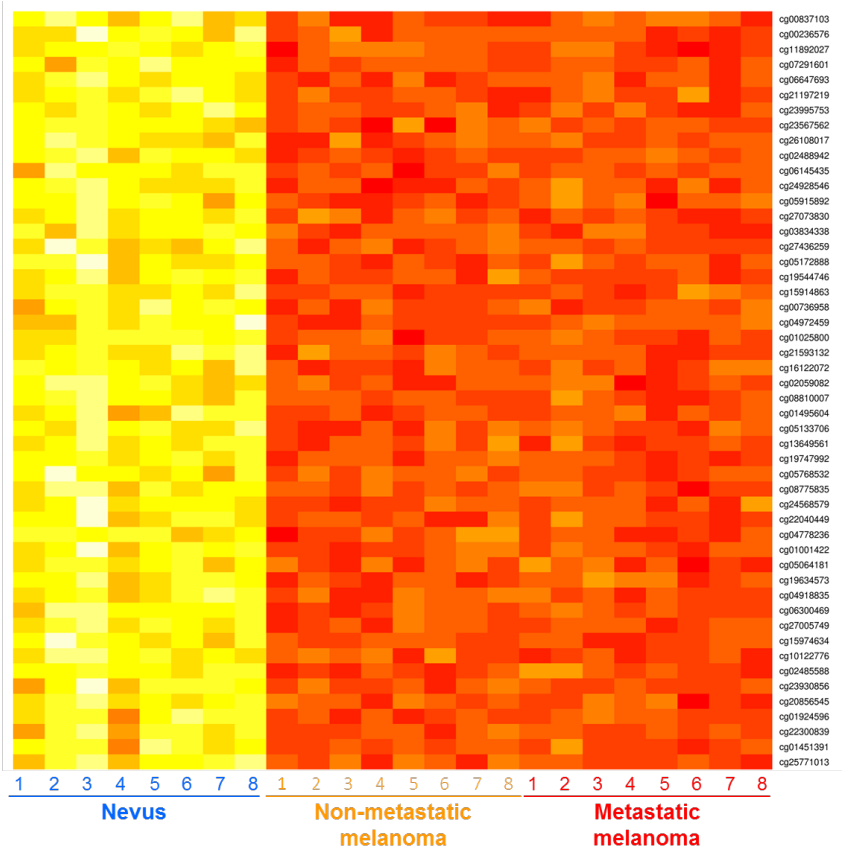
Hydroxymethylation		Methylation			
Naevus	Melanoma	Naevus	Melanoma	In CpG island?	Cancer-related genes?[a]
0.08	0.00	0.43	0.65	shelf opensea	
0.05	-0.01	0.36	0.53	shore island	fusion partner in anaplastic large-cell lymphoma
0.08	-0.01	0.31	0.40	opensea	
0.10	0.00	0.31	0.30	island shore	
0.08	-0.01	0.26	0.29	island shore	
0.11	-0.01	0.36	0.55	opensea	
0.10	0.00	0.28	0.39	island shore	
0.21	0.04	0.28	0.38	shore	
0.04	-0.02	0.31	0.38	island shore	
0.11	0.01	0.35	0.46	shore	
0.07	0.00	0.28	0.32	shore	
0.12	0.01	0.28	0.36	shore island	
0.05	0.00	0.35	0.38	opensea	
0.11	0.02	0.36	0.53	shore	
0.05	0.01	0.23	0.31	island shore	
0.06	-0.04	0.30	0.59	opensea	
0.17	0.04	0.29	0.42	opensea	
0.06	-0.02	0.54	0.73	shore	
0.09	0.01	0.29	0.28	shore island	
0.08	0.00	0.11	0.20	island	
0.16	0.04	0.28	0.51	shore	
0.02	-0.03	0.37	0.57	island shore	
0.02	0.00	0.78	0.81	shelf shore	
0.02	-0.01	0.65	0.71	island shore	oncogene or tumor suppressor gene in pancreatic neuroendocrine tumour, paediatric glioblastoma
0.04	0.00	0.26	0.35	island shore	



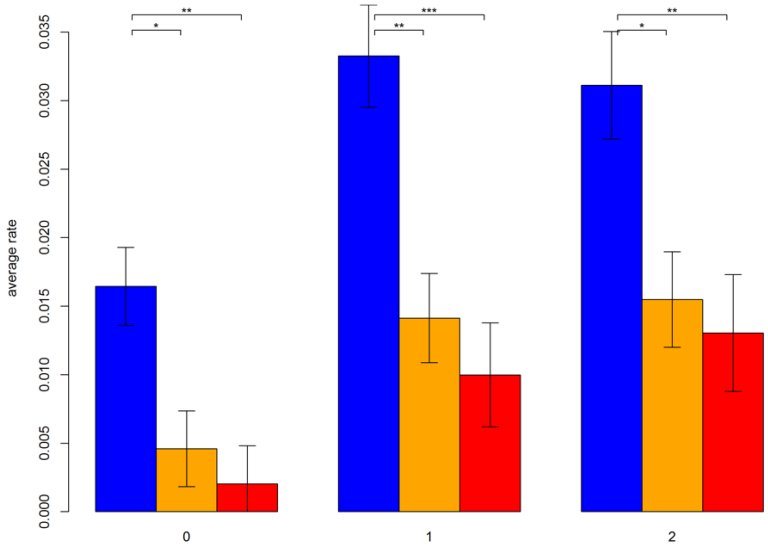
SUPPLEMENTARY FIGURE S1. Cumulative distribution of hmC and mC across all CpG sites analysed. Red line for hmC; black line for mC. All CpG sites show a hmC value between -0.2 and 0.3. The mC distribution is bimodal since there are non-methylated CpGs (0-0.2) or fully methylated CpGs (0.8-1).



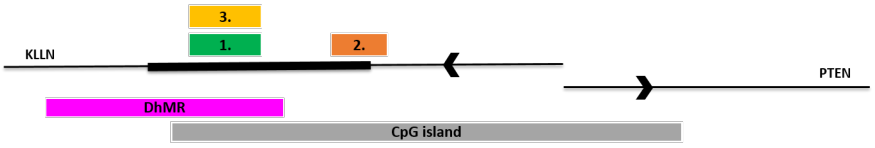
SUPPLEMENTARY FIGURE S2. Venn diagram of the GC-probes for which at least 1 sample within a group showed a Δβ value exceeding the average plus 3 standard deviations (Δβ>0.166).



SUPPLEMENTARY FIGURE S3. Heatmap. The top50 CpG sites statistically significant between nevi and melanomas in order of hmC value.



SUPPLEMENTARY FIGURE S4. Averaged rate of hmC at enhancer regions retrieved from FANTOM5 project (http://FANTOM5.gsc.riken.jp/5/). Blue – nevi; yellow – non-metastatic melanoma; red – metastatic melanoma. Comparison of hmC rate at melanocyte-specific enhancer regions (2) (2593 probes were found in 2136 enhancers) and at general enhancer regions (1) with hmC rate at non-enhancer regions (0).



SUPPLEMENTARY FIGURE S5. Schematic representation of the DhMR in the *PTEN* promoter region (chr10:89621419-89622084). Hypermethylation of the regions 1. (Mirmohammadsadegh et al., 2006) and 2. (Lahtz et al., 2010) have been previously associated with transcriptional repression of the PTEN gene in melanoma. Hypermethylation of region 3. (Roh et al., 2016, same as region 1.) was associated with worse survival in melanoma patients.

5

Interplay between *TERT* promoter mutations and methylation culminates in chromatin accessibility and *TERT* expression

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ABSTRACT

The *telomerase reverse transcriptase* (*TERT*) gene is responsible for telomere maintenance in germline and stem cells, and is re-expressed in 90% of human cancers. CpG methylation in the *TERT* promoter (*TERT*p) was correlated with *TERT* mRNA expression. Furthermore, two hotspot mutations in *TERT*p, dubbed C228T and C250T, have been revealed to facilitate binding of transcription factor ETS/TCF and subsequent *TERT* expression. This study aimed to elucidate the combined contribution of epigenetic (promoter methylation and chromatin accessibility) and genetic (promoter mutations) mechanisms in regulating *TERT* gene expression in healthy skin samples and in melanoma cell lines (n=61). We unexpectedly observed that the methylation of *TERT*p was as high in a subset of healthy skin cells, mainly keratinocytes, as in cutaneous melanoma cell lines. In spite of the high promoter methylation fraction in wild-type (WT) samples, *TERT* mRNA was only expressed in the melanoma cell lines with either high methylation or intermediate methylation in combination with *TERT* mutations. *TERT*p methylation was positively correlated with chromatin accessibility and *TERT* mRNA expression in 8 melanoma cell lines. Cooperation between epigenetic and genetic mechanisms were best observed in heterozygous mutant cell lines as chromosome accessibility preferentially concerned the mutant allele. Combined, these results suggest a complex model in which *TERT* expression requires either a widely open chromatin state in *TERT*p-WT samples due to high methylation throughout the promoter or a combination of moderate methylation fraction/chromatin accessibility in the presence of the C228T or C250T mutations.

KEYWORDS

Regulation, *TERT*, chromatin accessibility, genetics, epigenetics

INTRODUCTION

Approximately 90% of all human cancers share a transcriptional alteration: reactivation of the telomerase reverse transcriptase (*TERT*) gene.[1,2] *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. As the single-stranded 5' ends of chromosomes are shortened with each cellular division, telomeres prevent loss of coding chromosomal DNA.[3-6] Telomerase is only transcribed in a subset of stem cells in growing or renewing tissues, but through reactivation of telomerase expression, cells can extend telomeres or prevent telomeres shrinkage. This is termed telomere maintenance, which is one of the hallmarks of cancer, and allows subsequent indefinite proliferation and immortalization.[3,6-8]

Since the *MYC* oncogene has firstly been identified to activate telomerase, a variety of epigenetic or genetic mechanisms in the gene body or *TERT* promoter (*TERT*p) have followed, such as CpG methylation, histone modifications, mutations, germline genetic variations, structural variations, DNA amplification or chromosomal rearrangements.[3,5,7]

A widely investigated mechanism that could induce *TERT* reactivation is the presence of mutations in the gene promoter.[7,9] Horn and Huang *et al.* identified two mutually exclusive *TERT*p point mutations that are correlated to *TERT* mRNA expression by creating binding motifs for the transcription factor E26 transformation-specific/ternary complex factor (ETS/TCF).[7,9] These mutations, chr5:1,295,228 C>T and chr5:1,295,250 C>T in hg19 (−124 bp and −146 bp from the translation start site), henceforth respectively dubbed C228T and C250T, were first identified in melanoma. Furthermore, these mutations showed high prevalence in and were correlated with poor prognosis of cutaneous melanomas.[4,5,10-12]

An additional mechanism by which a gene can be made accessible to transcription factors, facilitating gene expression, is hypomethylation of promoter CpG islands, a hallmark of euchromatin.[13,14] Methylation located in the gene body, however, shows a positive correlation with active gene expression.[15] In stark contrast to most genes, *TERT*p hypermethylation may also allow gene expression since transcriptional repressors rely on unmethylated promoter CpGs, such as CCCTC-binding factor (CTCF)/cohesin complex or MAZ.[16-18] As such, in combination with transcription factor binding, dissociation of the repressor may result in *TERT* expression.[3,16,19,20] Castelo-Branco *et al.* proposed that methylation of a specific CpG site in *TERT*p, cg11625005 (position 1,295,737 in hg19) was associated with paediatric brain tumours progression and poor prognosis.[20] This finding was later supported by the study from Barthel *et al.*, in which the CpG methylation was found to be correlated with *TERT*

expression in samples lacking somatic *TERT* alterations and to be generally absent in normal samples adjacent to tumour tissue.[3]

Chromatin organisation, its plasticity and dynamics at *TERT*p region have been reported as relevant players in regulation of gene expression by influencing the binding of transcription factors.[21,22] Cancer cells are positively selected to escape the native repressive chromatin environment in order to allow *TERT* transcription.[23]

In the present study, we aim to elucidate the interaction of genetic and epigenetic mechanisms in regulation of *TERT*p. We approach this by using novel droplet digital PCR (ddPCR)-based assays.[24] Human-derived benign skin cells (keratinocytes, dermal fibroblasts, melanocytes, skin biopsy samples and naevi) and melanoma cell lines were analysed. The *TERT*p mutational status was assessed along with the absolute presence of methylation in the *TERT*p at a CpG-specific resolution. The effect of chromatin accessibility in *TERT* expression was evaluated in a subset of cultured melanoma cell lines.

RESULTS

NGS-BASED DEEP BISULFITE SEQUENCING AND DEVELOPMENT OF A DDPCR ASSAY TO ASSESS *TERTp* METHYLATION FRACTION

We first aimed to quantitatively measure the *TERT*p methylation at a CpG-specific resolution in primary skin samples and melanoma cell lines. DNA of 44 primary skin biopsy samples and melanoma cell lines was bisulfite-converted (BC) and analysed using NGS-based deep bisulfite sequencing to assess the methylation fraction (MF) in a region of *TERT*p encompassing 31 CpG sites. The *TERT*p MF was high in some healthy skin samples, such as normal skin (~30%), naevi (~30%) and cultured keratinocytes (~50%). In the latter group, in fact, the MF was as high as in cutaneous melanoma cell lines (Figure 1 and Figure 7a). In contrast, the fibroblasts and low-passage cultured melanocytes show the lowest MF observed in this cohort. Since the cutaneous melanoma originates from melanocytes of the skin, we found the difference in MF between normal melanocytes and cutaneous melanoma cells quite remarkable.

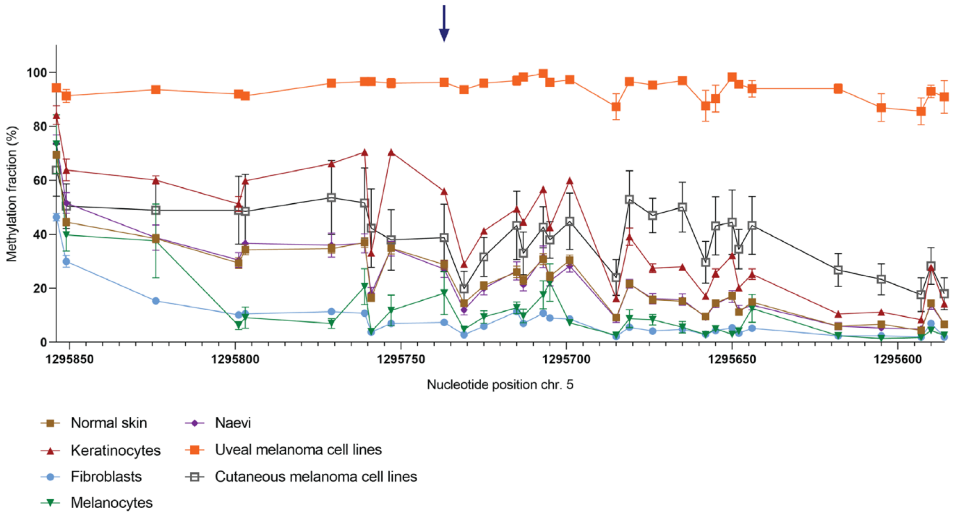

FIGURE 1. Methylation fraction (MF) of 31 CpG sites around cg11625005 in 35 primary skin samples and 9 melanoma cell lines. DNA samples were bisulfite-converted (BC) and analysed through NGS-based deep sequencing. Connected scatter plot representing the MF per cell type group in absolute distance between measured CpG sites. Blue arrow: cg11625005 (position 1,295,737). Samples included: fibroblasts (n=5), melanocytes (n=5), naevi (n=6), normal skin samples (n=11), keratinocytes (n=8), cutaneous melanoma cell lines (n=6) and uveal melanoma cell lines (n=3).

In order to validate the *TERT*p MF obtained through NGS in a quantitative manner, we have developed a ddPCR assay (Figure 2a) using methylation-sensitive restriction enzymes (MSREs) HgaI and AvaI, which recognise the CpG on position 1,295,737 (cg11625005) and 1,295,731 in hg19, respectively. Castelo-Branco *et al.* showed that methylation of the cg11625005 in *TERT*p, was associated with tumour progression and poor prognosis of childhood brain tumours.[20] Barthel *et al.* affirmed a correlation between methylation and *TERT* expression in samples lacking somatic *TERT* alterations and a lower methylation level in normal samples.[3] Indeed, in our study, the MF of fibroblasts was as low as that of the unmethylated control DNA, whereas that of the keratinocytes was higher than most of the cutaneous melanoma cell lines (Figure 2b). The MF of cg11625005 (position 1,295,737) obtained through NGS and by ddPCR were highly correlated (R^2=0.82, p<0.001) (Figure 2c). The MF of 1,295,731 assessed through ddPCR even yielded a stronger correlation (R^2=0.96, p<0.001) (Figure 2d).

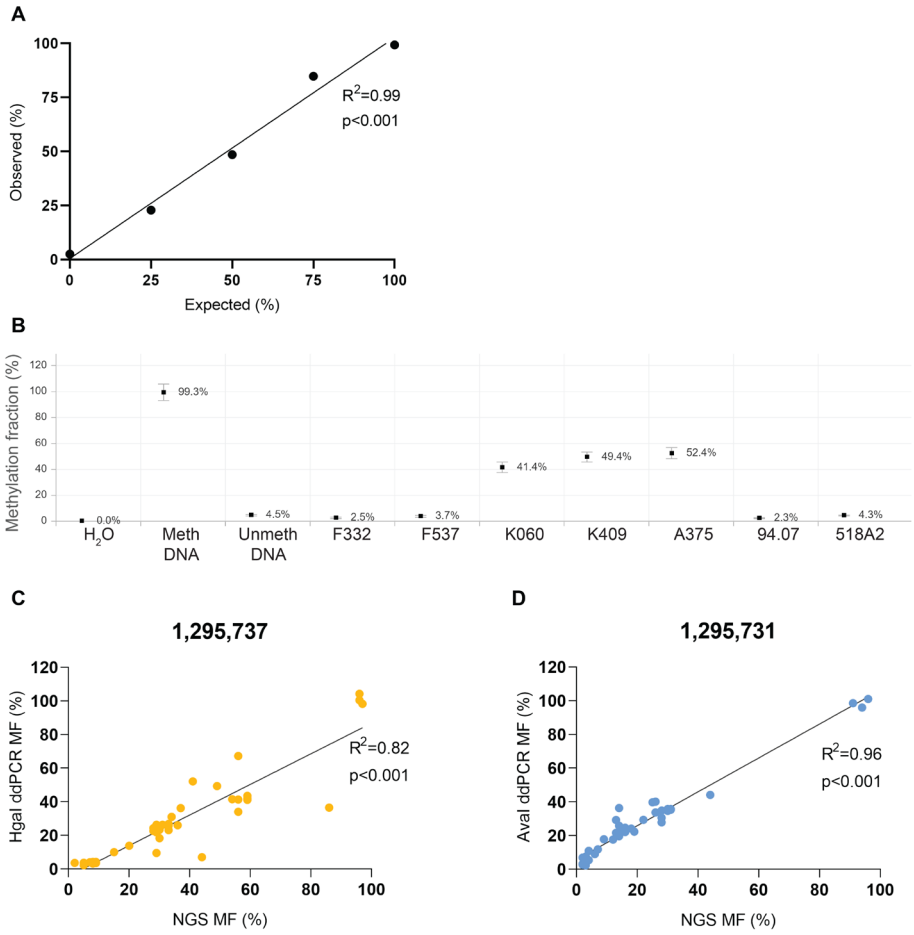

FIGURE 2. Methylation fraction (MF) analysed through ddPCR. MDNA and UDNA are commercially available methylated and unmethylated DNA, respectively. (a) Calibration curve using different expected ratios (25%, 50% and 75%) of methylated DNA and F332 to demonstrate the quantitative capacity of ddPCR. Linear regression and correlation analysis were performed to compare the expected to observed ratios ($F_{(1,3)}$=209.2, r=0.99, p<0.001). (b) MF of cg11625005 in a subset of healthy primary skin samples – fibroblasts (F332 and F537) and keratinocytes (K060 and K409) and cutaneous melanoma cell lines (A375, 94.07 and 518A2) incubated with MSRE Hgal. MF was plotted with 95% CI through RoodCom WebAnalysis (version 1.9.4).(c & d) Correlation plots between MF obtained through golden standard NGS-based deep bisulfite sequencing versus ddPCR using either the MSRE Hgal (c) or Aval (d), which digest unmethylated CpG in position 1,295,737 and 1,295,731, respectively, in a batch of 44 samples: fibroblasts (n=5), melanocytes (n=5), naevi (n=6), normal skin samples (n=11), keratinocytes (n=8), cutaneous melanoma cell lines (n=6) and uveal melanoma cell lines (n=3). Linear regression and correlation analysis were performed ($F_{(1,40)}$=178.1, r=0.90 and $F_{(1,41)}$=934.4, r=98, respectively, p<0.001).

ABSENCE OF CORRELATION BETWEEN METHYLATION FRACTION AND *TERT* EXPRESSION

Cancer cells are commonly characterised by hypermethylation of promoter CpG islands resulting in repression of tumour suppressor genes. However, in *TERT*, promoter hypermethylation was found to be associated with higher expression, since CTCF repressors of *TERT* transcription do not bind methylated sequences.[3,16,17,19] In our sample cohort, there was no correlation between *TERT* methylation of cg11625005 and mRNA expression (n=31, Figure 3 and Figure 7b).

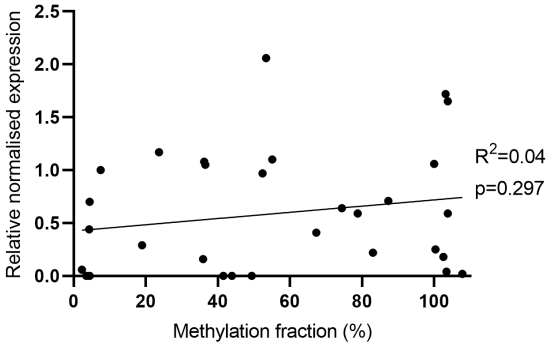

FIGURE 3. Correlation between methylation fraction (%) and *TERT* mRNA expression in total of 31 samples: fibroblasts (n=3), melanocytes (n=1), keratinocytes (n=2), cutaneous melanoma cell lines (n=19) and uveal melanoma cell lines (n=6). Linear regression and correlation analysis were performed to compare ($F_{(1,29)}$=1.13, r=0.19, ns p=0.297).

EVALUATION OF *TERT*p MUTATIONS IN A COLLECTION OF SKIN SAMPLES AND MELANOMA CELL LINES

Besides promoter methylation, somatic mutations are also known to be correlated with *TERT*p reactivation. Therefore, we characterised the *TERT*p mutational status of the sample cohort. Sanger sequencing on one naevus, fresh skin and cutaneous melanoma cell lines 518A2, 607B, A375, 94.07 and 93.08 revealed melanoma-associated *TERT* C250T and C228T mutations (Figure 4a). Aiming to use the ddPCR method to evaluate the mutational load of the samples, the *TERT* C250T and C228T mutation assays were validated in three samples of which the mutation was identified in sequencing analysis, 518A2, 607B and A375 (Figure 4b). Following the test runs, the C228T and C250T assays were used on the extended sample cohort (n=61) (Supplementary Table S5 and Figure 7c). All *TERT*p-mutated samples were cutaneous melanoma cell lines, however OCM8 and 94.13 cutaneous cell lines tested wild-type. The C250T mutation was not present in combination with the C228T mutation in any sample, confirming that the mutations are mutually exclusive.

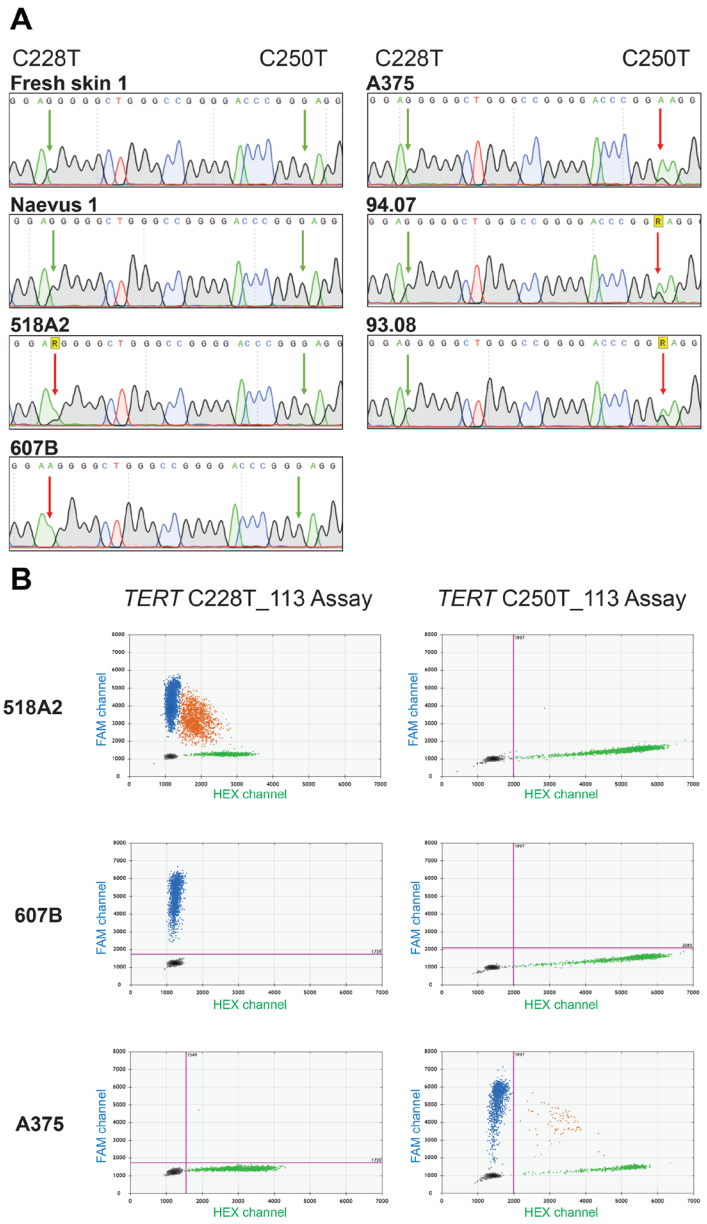

FIGURE 4. *TERT*p mutational status of primary skin samples and cutaneous melanoma cell lines. (a) The *TERT*p region encompassing the C228T and C250T mutations was sequenced through Sanger sequencing using McEvoy's[25] *TERT*p forward primer. The *TERT*p region of fresh skin 1, Naevus 1, 518A2, 607B, A375, 94.07, 93.08 is shown. The left and right arrows respectively indicate the positions 1,295,228 and 1,295,250. R: one-letter code for bases G or A; Green arrow: wild-type; red arrow: C>T mutation on the complementary strand. (b) Evaluation of *TERT*p mutations through commercial Bio-

Rad TERT assays in 518A2, 607B and A375 melanoma cell lines. 2D ddPCR plots of the results from the C228T mutation assay (left) and C250T mutation assay (right). The blue cloud represents mutant copies; the green cloud represents WT copies.

ABSENCE OF CORRELATION BETWEEN MUTATIONAL STATUS AND *TERT* EXPRESSION

As the presence of mutations in the gene promoter induces *TERT* reactivation, we assessed the correlation between mutational status with *TERT* mRNA expression (n=31). When WT and mutated samples (either C228T or C250T) were compared, regardless of origin of the tissue, no significant differences for *TERT* mRNA expression were found (Figure 5). Moreover, *TERT* expression was exclusive to the melanoma cell lines, either with or without *TERT*p mutations (Figure 7b).

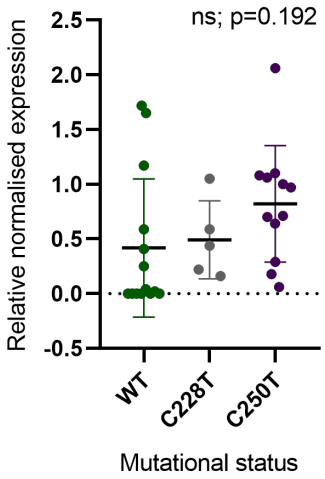

FIGURE 5. Correlation between *TERT*p mutational status and *TERT* mRNA expression in total of 31 samples: fibroblasts (n=3), melanocytes (n=1), keratinocytes (n=2), cutaneous melanoma cell lines (n=19) and uveal melanoma cell lines (n=6) (One-way ANOVA, $F_{(2,28)}$=1.75, ns p=0.192).

TERT EXPRESSION IS CORRELATED TO CHROMATIN ACCESSIBILITY

In contrast to most genes, methylation of the *TERT*p positively correlates with its mRNA expression.[3,16,17,19] Although we were not able to confirm this finding, we investigated whether besides promoter methylation, other mechanisms could contribute to chromatin accessibility to transcription factors affecting *TERT*p regulation. Therefore, we analysed chromatin state in a subset of melanoma cell lines (cutaneous, 518A2, 607B, 94.07, A375,

93.08 and OCM8; and uveal, OMM2.5 and Mel270) by ddPCR methodology instead of qPCR for an accurate quantification. The positive control gene *GAPDH*, a housekeeping gene that is generally expressed in all conditions, and thus 100% accessible, was used. The accessibility in the region around cg11625005 shows a high variability, being over 90% in uveal cell lines while being intermediate to low in cutaneous melanoma cell lines (Figure 6a, Figure 7d and Supplementary Table S6). When comparing the accessibility around cg11625005 to the methylation fraction of this CpG, a significant positive correlation was observed (R^2= 0.89, $p<0.001$) (Figure 6b). Another positive correlation (R^2=0.59, $p<0.05$) was found when comparing the accessibility of the same region to the normalised *TERT* mRNA expression levels in these samples (Figure 6c). In actuality, in this subset of 8 cell lines, the *TERT*p methylation and gene expression show a statistically significant ($p<0.05$) positive correlation (Figure 6d). The 3 cell lines with higher MF are those with the highest chromatin accessibility (OMM2.5, Mel270 and OCM8). Remarkably, these are also the cell lines with WT-*TERT*p, in which the chromatin accessibility was significantly higher than in the mutated subgroup (Figure 6e).

In addition, we investigated whether the *TERT* accessibility originated from the mutant or the wild-type allele. For this purpose, we assessed the fractional abundance of mutated allele, in the subgroup of 4 *TERT*p-mutated cutaneous cell lines before and after nuclease digestion. 607B cell line was not included since it is homozygous for the mutation and not informative. In 3 out of 4 cell lines preferential digestion of the mutant allele showed that mutated alleles were more accessible than WT alleles (Figure 6f).

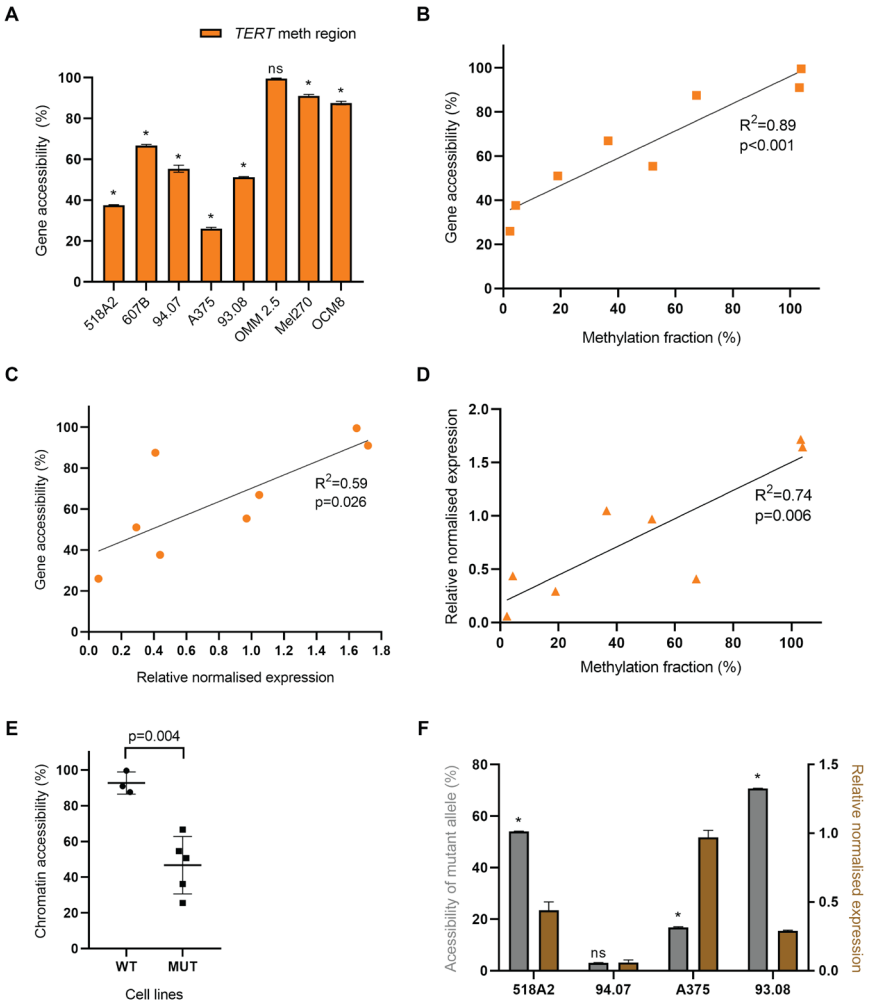

FIGURE 6. Accessibility of *TERT*p around cg11625005 in 8 melanoma cell lines. Cell lines (518A2, 607B, 94.07, A375, 93.08, OMM2.5, Mel270 and OCM8) were analysed with the EpiQ chromatin kit, and ddPCR was performed using primers and probes for positive control gene *GAPDH* and for the *TERT* methylation region, a 231-bp amplicon around cg11625005. Accessibility (%) was calculated by the ratio of the digested sample to its matched undigested sample, subtracted from 1, and subsequently normalised against the positive control *GAPDH*. (a) Accessibility of the *TERT* methylation region relative to *GAPDH* (mean ± SD, multiple t-tests, one t-test per cell line, *p<0.001, ns p=0.149). (b and c) Correlation plots of gene accessibility around cg11625005 with the MF (%) of cg11625005 obtained through ddPCR (b), or with normalised expression levels via qPCR (c). Linear regression and correlation analysis were performed ($F_{(1,6)}$=49.9, r=0.95, p<0.001 and $F_{(1,6)}$=8.6, r=0.77, p<0.05, respectively). (d) Correlation plot between MF (%) of cg11625005 obtained through ddPCR and normalised expression levels via qPCR. Linear regression and correlation analysis were performed ($F_{(1,6)}$=16.92, r=0.86, p<0.05). (e) Comparison of WT (OMM2.5,

Mel270 and OCM8) and mutated (518A2, 607B, 94.07, A375, 93.08) *TERT*-expressing cell lines subsets regarding chromatin accessibility (two-tailed unpaired t-test; t=4.63, df=6; p<0.005). (f) Accessibility of mutant allele (%) in a subset of 4 *TERT*p-mutated cutaneous cell lines (518A2, 94.07, A375 and 93.08) calculated as described in Material and Methods (mean ± SD, multiple t-tests, one t-test per cell line, ns p=0.171; *p<0.001) and the *TERT* mRNA expression in the respective cell lines (mean ± SEM).

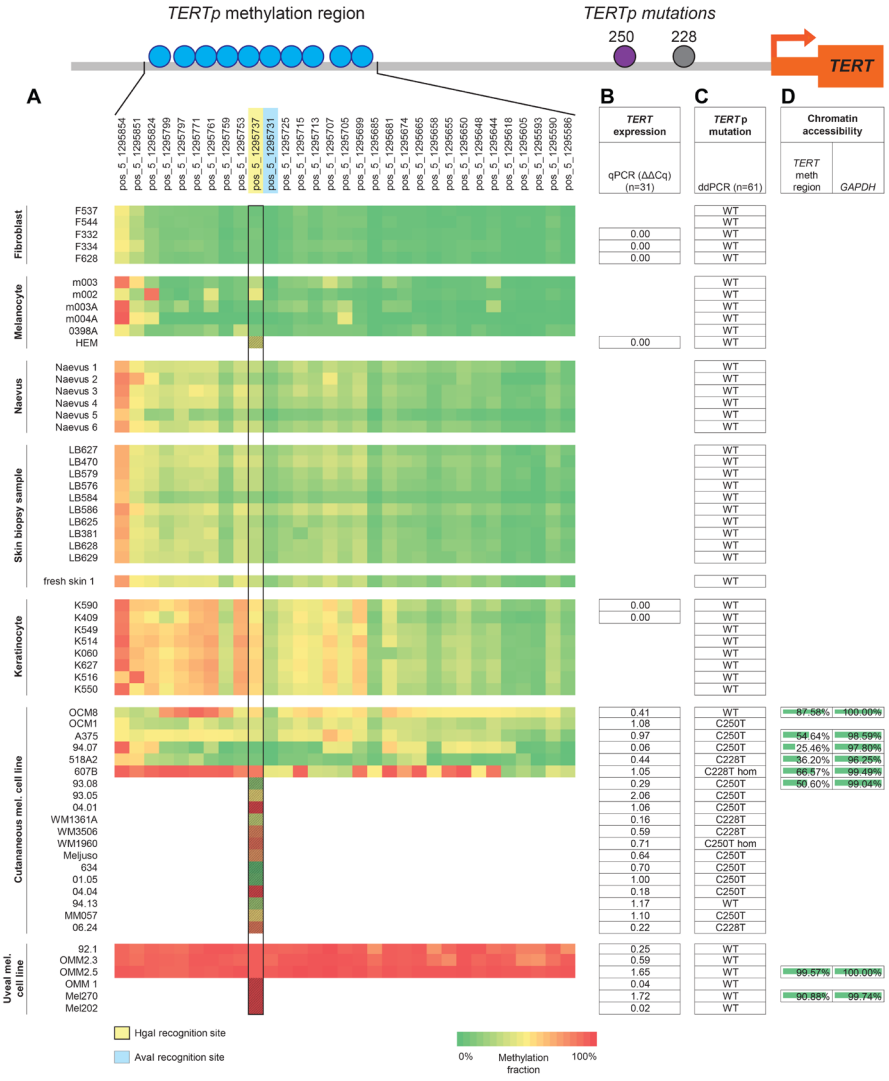

FIGURE 7. Results overview. Schematic representation of *TERT*p with the relative positions of cg11625005 (position 1,295,737 in hg19) to the *TERT*p mutations (position 1,295,228 and 1,295,250) and the transcription start site (TSS). (a) Heat-map of methylation fraction (MF) in 31 CpG sites (top) in 44 samples (left). Yellow-marked CpG cg11625005 (position 1,295,737) is recognised by MSRE HgaI.

Blue-marked CpG in 1,295,731 is recognised by MSRE AvaI. Black rectangle: MF at the cg11625005 measured either by NGS (clear squares, n=44) and by ddPCR (patterned squares, n=17; these samples were not included in the 44-sample batch subjected to NGS). (b) *TERT* mRNA expression in 31 samples by qPCR analysed through the ΔΔCT method in Bio-Rad CFX manager software (version 3.1, Bio-Rad). (c) *TERT*p mutations evaluated through ddPCR with commercial *TERT* C250T and C228T Mutation Assays in total 61 samples. (d) Analysis of the chromatin accessibility in 8 cultured cell lines for *TERT* methylation region using *GAPDH* as a positive control.

DISCUSSION

By using advanced quantitative methods, we investigated the epigenetic and genetic regulation of *TERT*p in benign and malignant skin cells. Innovative ddPCR-based assays were developed and validated to assess *TERT* promoter methylation and chromatin accessibility. These methods avoid semi-quantitative qPCR and provide absolute quantification even in samples that are challenged by CG-rich DNA sequences, low concentration and integrity.

In the present study the methylation fraction was assessed by NGS interrogating 31 CpGs in the *TERT*p region across 44 healthy, benign and malignant tumour samples. Remarkably, high methylation levels were observed in a variety of normal samples. Mainly in keratinocytes methylation levels exceeded those of cutaneous melanoma cell lines. Previous studies on brain tumours and skin melanoma, observed a general absence of methylation in a specific CpG in *TERT*p, cg11625005, in healthy control samples.[3,20] Of note, although the authors state absence of methylation we can observe a β-value of ~0.4 (fluorescence ratio provided by Illumina 450K array, ranging from 0 to 1) in their normal samples.[3] In our cohort, the methylation fraction at this CpG was quantified by ddPCR, which validated our results obtained through NGS. Moreover, in our study, methylation of cg11625005 did not stand out across the CpGs in *TERT*p but seemed to be affected along with adjacent CpGs in this genomic region in all samples (Figure 7a). This result suggests that context-related methylation around cg11625005 is biologically relevant as opposed to methylation of one specific CpG.

*TERT*p mutations has been described as a genetic mechanism responsible for induction of *TERT* reactivation.[7,9] Over the years that followed, a variety of epigenetic or genetic alterations in the gene body or *TERT*p have been identified, such as promoter methylation, mutations, structural variations, DNA amplification, or promoter rearrangements.[3,5,19]

In accordance with previous studies, regardless of the methylation status, human benign

cells neither harbour *TERT*p mutation nor express *TERT,* thereby supporting the principal oncological concept that a benign cell does not undergo undefined proliferation (Figure 8). Although we have not found a positive correlation between presence of *TERTp* mutations or *TERTp* methylation levels and mRNA expression values, all tumour cell lines showed *TERT* expression, supporting that these mechanisms contribute to telomerase-activation in cancer, separate or in combination.[7,9,19]

A plethora of histone modifications result in chromatin remodelling that may change accessibility of the *TERT*p to transcription factors, such as ETS/TCF.[7] Schwartz *et al.* state that the degree of chromatin folding is correlated with gene transcription and is thought to impact the regulation of DNA-dependent processes.[26] Therefore, we explored the level of chromatin accessibility and its interaction with methylation levels and mRNA expression in 6 cutaneous and 2 uveal melanoma cell lines. In fact, we found a positive correlation between chromatin accessibility and methylation levels as well as mRNA expression that ultimately explains the correlation between methylation fraction and *TERT* expression. Then, we investigated whether both wild-type and mutant alleles were equally affected by similar patterns of chromatin organization and assessed the mutational fraction upon digestion with nuclease in heterozygous cell lines, assuming that the nuclease only digests DNA open chromatin regions. We could infer that, mutated alleles are more accessible, possibly favouring the binding of transcription factors and consequently *TERT* mono-allelic expression. Our findings in the 518A2 cell line, harbouring the C228T *TERT*p mutation, are similar to the results from a study by Stern *et al.*, in which it was found that the active mutant allele is hypomethylated.[27] These observations are consistent with the canonical influence of methylation on transcriptional regulation. In contrast, 94.07 cell line also presents a very small methylation fraction. However both alleles were equally resistant to nuclease digestion, which might explain the lowest *TERT* expression levels among all cell lines. Therefore, it still supports the link between local chromatin accessibility and gene regulation.[26] To fully disclose the molecular mechanisms behind *TERT* expression the heterozygous mutant cell lines A375 and 93.08 provide good models as they allow to study a repressed and expressed allele within the same cell.

Another remarkable observation in our study is that in WT *TERT*-expressing uveal melanoma cell lines, the methylation of the whole *TERT*p region is close to 100% with a significantly higher chromatin accessibility compared to *TERT*p-mutated cell lines. Accordingly, Stern *et al.* also demonstrate that cell lines with WT *TERT*p display much higher levels of methylation.[27] These characteristics of WT *TERT*p cell lines may lead to biallelically *TERT* activation under distinct epigenetic conditions from those in mutated *TERT*p.

Interestingly, these results suggest a complex model in which *TERT* expression requires either a widely open chromatin state in *TERT*p-WT samples due to hypermethylation throughout the promoter or mono-allelic expression of the accessible mutated allele in combination with moderate (probably allele-specific) methylation fraction (Figure 8).

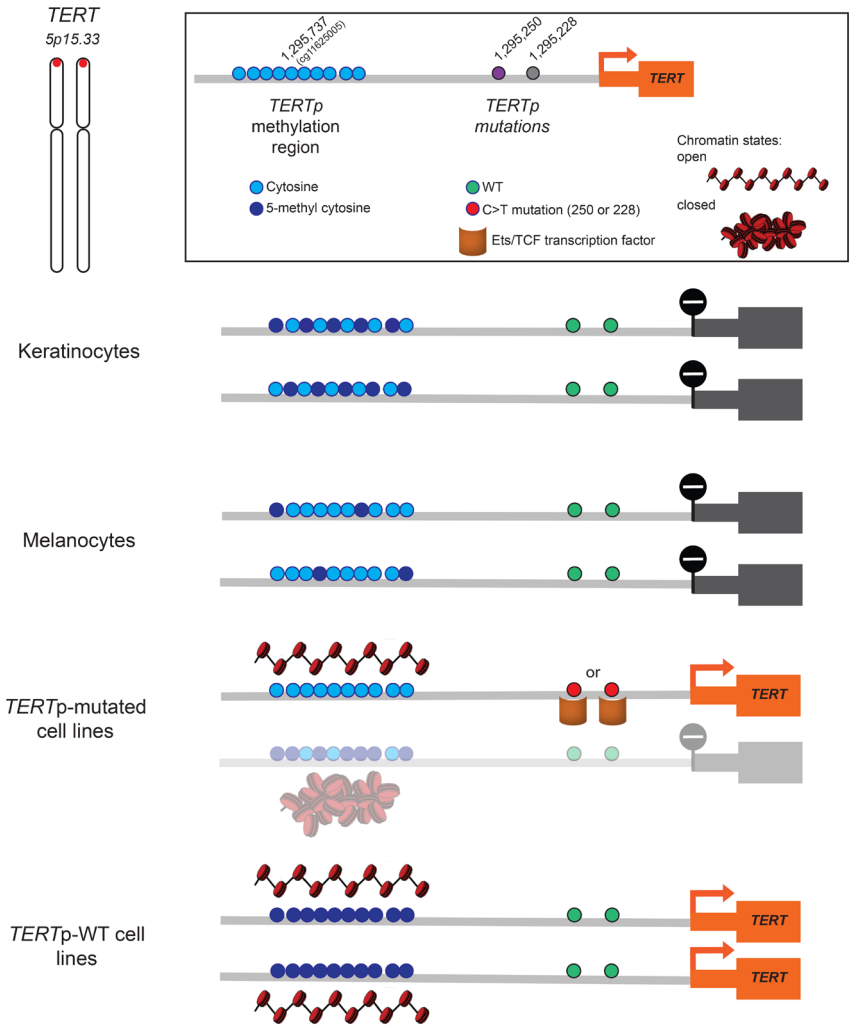

FIGURE 8. Proposed model of *TERT* transcriptional regulation. Regardless of MF at the *TERT*p methylation region, both keratinocytes and melanocytes do not show TERT expression. In *TERT*p-mutated cell lines, an intermediate MF positively correlated with chromatin accessibility, in combination with C228T/C250T *TERT* mutations allows monoallelic *TERT* expression. In *TERT*p-WT cell lines, the MF is close to 100% with a significantly higher chromatin accessibility leading to high expression levels. Chromatin schemes adapted from Schwartz *et al.*[26]

Furthermore, Huang and colleagues reported that some cancer cell lines show mono-allelic expression of *TERT* even in the absence of *TERT*p mutations.[28]

Previous studies have reported the association between *TERT*p hypermethylation and poor patient survival in melanoma and other cancers, indicating that it might be a relevant prognostic marker.[20,27,29-31] In primary melanoma it needs to be assessed if *TERT*p methylation is predictive of worse prognosis. Thus, the quantification of *TERT* methylation through ddPCR might be relevant in the clinic to assess patient prognosis.

The dynamics of epigenetic mechanisms in *TERT* genetic regulation is complex. Further investigations are needed to address the correlation of allele-specific differences in chromatin accessibility and promoter methylation with allele-specific mRNA expression.

MATERIAL AND METHODS

SAMPLES, DNA EXTRACTION AND PCR

Surplus female breast skin and nevi tissues were obtained from 11 and 6 anonymous patients that underwent cosmetic surgery, respectively. Surgeries for mama reduction (performed between 2010 and 2018) and naevi (performed between 2008 and 2009), were conducted according to declaration of Helsinki principles. Epidermis and dermis were separated after removal of adipose tissue followed by enzymatic digestion and primary fibroblast (n=5) and keratinocyte (n=8) cell suspensions were obtained and cultured as described before.[32] Keratinocytes were used at passage 2, while fibroblasts were used at passage 3-5.

Low-passage cultured melanocytes (n=5) – m003, m003A, m002, m004A and 0398A – were cultured as previously described.[33] HEMs were cultured more recently in the medium 254 supplemented with HMGS-2 (Gibco/ThermoFisher) and Penicillin (100 U/ml), and Streptomycin (100 µg/ml; both from Lonza, Verviers, Belgium).

We also included 19 early-passage cutaneous melanoma cell lines derived from metastatic lesions cultured for research purposes and adoptive T-cell transfer.[34] Cell lines were cultured and DNA and RNA extracted between 2017 and 2019. The 518A2, 607B, 04.01, 04.04, 94.13, 93.05, 94.07, 93.08, 634, 01.05, and 06.24 cell lines were a kind gift from Dr. Els Verdegaal (Department of Medical Oncology, LUMC). Meljuso was obtained from Prof. Neefjes (Department of Cell and Chemical Biology, LUMC). WM1361A, WM3506, WM1960 cell lines were a kind gift from Dr. KL Scott (Baylor College of Medicine, Houston, USA). MM057 and A375 were kindly provided by Prof. JC Marine (VIB, Leuven, Belgium). OCM8

and OCM1 were provided by Dr. Mieke Versluis (Department of Ophthalmology, LUMC).[35] All cell lines were cultured with Dulbecco's modified eagle medium (DMEM, low glucose, pyruvate; Gibco/ThermoFisher) supplemented with 10% FCS, Penicillin (100 U/ml), and Streptomycin (100 µg/ml; both from Lonza, Verviers, Belgium) and glutamax (100X, Gibco). For the 6 uveal cell lines provided by Dr. Mieke Versluis (Department of Ophthalmology, LUMC), the establishment and culturing conditions have been described before: OMM 1[36], OMM 2.3, OMM 2.5 and Mel270[37], Mel202[38], 92.1[39]. All cell lines used in our study were tested negative for mycoplasm and recently subjected to STR profiling.

The batch thus consisted of 36 primary skin type samples and 25 melanoma cell lines, totalling 61 samples (Table 1).

TABLE 1. Samples overview

Control samples					Melanoma cell lines	
Skin biopsy samples	Fibroblasts	Melanocytes	Keratinocytes	Naevi	Cutaneous	Uveal
LB627	F537	m003	K590	Naevus 1	04.01	OMM 2.3
LB470	F544	m002	K409	Naevus 2	WM1361A	OMM 1
LB579	F332	m003A	K549	Naevus 3	93.05	OMM 2.5
LB576	F334	m004A	K514	Naevus 4	WM3506	Mel270
LB584	F628	0398A	K060	Naevus 5	WM1960	Mel202
LB586		HEM	K627	Naevus 6	Meljuso	92.1
LB625			K516		634	
LB381			K550		OCM8	
LB628					OCM1	
LB629					518A2	
Fresh skin 1					607B	
					94.07	
					A375	
					93.08	
					94.13	
					01.05	
					04.04	
					MM057	
					06.24	

DNA was isolated using the QIAamp DNA Blood Mini Kit and the DNeasy Blood & Tissue Kit (both from Qiagen, Hilden, Germany).

Conventional PCR was performed using the PCR-sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA), containing 10X reaction buffer, MgCl2 (50mM), dNTP mix (10nM, Fermentas/Thermo Fisher Scientific), primer mix (900nM each), PlatinumX Taq enzyme (2.5U), 50ng DNA and Aqua B. Braun RNase-free water. A PCR for CG-rich sequences was performed on 50ng DNA using the PCRX Enhancer System (Thermo Fisher

Scientific), containing 10X PCRX amplification buffer, MgSO4 (50mM), dNTP mix (10nM), primer mix (900nM each), PlatinumX Taq enzyme (2.5U) and Aqua B. Braun RNase-free water. The samples were amplified in C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

PROMOTER METHYLATION DETERMINATION

Bisulfite conversion and next-generation sequencing (NGS)-based deep bisulfite sequencing

In this experiment 44 samples were included: fibroblasts (n=5), melanocytes (n=5), naevi (n=6), normal skin samples (n=11), keratinocytes (n=8). cutaneous melanoma cell lines (n=6) an uveal melanoma cell lines (n=3). DNA was bisulfite-converted (BC) using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer protocol (version 1.2.2). BC samples were amplified using the PCRX Enhancer System in the program: 1 cycle of 95°C for 3 minutes, 8 cycles of 95°C for 30 seconds, 58°C for 30 seconds, reducing 1°C/cycle, and 68°C for 1 minute, then 36 cycles of 95°C and 53°C for 30 seconds each, and 68°C for 1 minute, followed by 1 cycle of 68°C for 3 minutes. Tailed primers were used for amplification (900nM each; S1 Table). Samples were sequenced through next-generation sequencing (NGS), MiSeq, 2x300bp paired-end, at Leiden Genome Technology Centre (LGTC). Bisulfite sequencing reads were quality trimmed using PRINSEQ (v0.20.4 lite) and aligned to GRCh37 using Bismark (v0.20.0) and Bowtie 2 (v2.3.4.3).[40-42]

Novel design of a ddPCR assay using methylation-sensitive restriction enzymes (MSREs) to determine *TERT*p methylation fraction

The methylation fraction (MF) of the CpG (cg11625005) in position 1,295,737 was determined by an in-house designed ddPCR assay in combination with HgaI methylation-sensitive restriction enzyme (MSRE) that cleaves this CpG when unmethylated, as described by Nell *et al.*[24] 100ng DNA sample was incubated with HgaI (2U/µl) and appurtenant 10X NEBuffer 1.1 (both from New England Biolabs, Bioké, Leiden, The Netherlands) for 60 minutes at 37°C and 65°C for 20 minutes. To assess the MF of a CpG adjacent to cg11625005, located in 1,295,731, the MSRE AvaI (10U/µl; New England Biolabs) was employed, which recognises this CpG and cleaves it when unmethylated. Incubation of the DNA samples with AvaI was performed with 10X CutSmart buffer for 15 minutes at 37°C and subsequently 65°C for 20 minutes. For ddPCR reaction, 60ng DNA digested or undigested by HgaI, 2x ddPCR SuperMix for Probes (no dUTP), primers (900nM each), a FAM-labelled in-house-designed probe for the CpG site of interest (250nM, Sigma, St. Louis, MO, USA), and 20X HEX-labelled CNV *TERT* reference primer/probe (Bio-Rad) for total *TERT* amplicon count. The primers and probe sequences are presented in S2 Table. The amplification protocol used: 1 cycle of 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 60°C for 1 minutes, and 1 cycle of

98°C for 10 minutes, all at ramp rate 2°C/s. Droplets were analysed through a QX200 droplet reader (Bio-Rad) using QuantaSoft software version 1.7.4 (Bio-Rad). Raw data was uploaded in online digital PCR management and analysis application Roodcom WebAnalysis (version 1.9.4, https://www.roodcom.nl/webanalysis/)[24], in which the MF was calculated by dividing the CNV of the digested sample with that of the paired undigested sample.

ASSESSMENT OF MUTATIONAL STATUS

Sanger sequencing

The presence of the C228T and C250T *TERT*p mutations in some samples was evaluated by conventional Sanger sequencing. DNA samples were amplified through the PCRX Enhancer System (Thermo Fisher Scientific) using primers (Sigma-Aldrich) and amplification program described by McEvoy *et al.*[25]

Mutation analysis using commercial TERT C250T and C228T mutation assays

For most of the samples, the *TERT*p mutations were detected by the ddPCR technique according to protocol described by Corless *et al.*[43], using the *TERT* C250T_113 Assay and C228T_113 Assay (unique assay ID dHsaEXD46675715 and dHsaEXD72405942, respectively; Bio-Rad). Both assays include FAM-labelled probes for the C250T and C228T mutations respectively, HEX-labelled wild-type (WT) probes, and primers for a 113-bp amplicon that encompasses the mutational sites. The ddPCR reaction mix comprised 1X ddPCR Supermix for Probes (No dUTP), Betaine (0.5M; 5M stock), EDTA (80mM; 0.5M stock, pH 8.0, Thermo Fisher Scientific), CviQI restriction enzyme (RE; 2.5U; 10U/µl stock, New England BioLabs), the *TERT* assay, and 50ng DNA. Droplets were generated in QX200 AutoDG system (Bio-Rad) and amplified in T100 Thermal Cycler (Bio-Rad) according to the recommended cycling conditions and analysed through a QX200 droplet reader (Bio-Rad) using QuantaSoft software version 1.7.4 (Bio-Rad).

CHROMATIN ACCESSIBILITY

Cell culture and treatment to assess chromatin states

Cutaneous melanoma cell lines A375, 518A2, 607B, 94.07, 93.08, OMM2.5, Mel270 and OCM8 were cultured for 22 days in 9-cm Cellstar® cell culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany) with Dulbecco's modified eagle medium (DMEM; Sigma-Aldrich) supplemented with 10% FCS, Penicillin (100U/ml), and Streptomycin (100µg/ml; both from Lonza, Verviers, Belgium) until roughly 95% confluent. Then, different densities (10,000, 20,000, 40,000 and 80,000 cells) of the above-mentioned cell lines were seeded in duplicate into a 48-well plate (Corning Costar, Sigma-Aldrich) required for the EpiQ chromatin assay. The EpiQ™ Chromatin Analysis Kit (Bio-Rad) was performed according to manufacturer's instructions. Briefly, after 2 days each cell line was 85%-95% confluent. The

cells were permeabilised and treated with EpiQ chromatin digestion buffer with or without nuclease for 1 hour at 37°C. Following incubation with EpiQ stop buffer for 10 minutes at 37°C, the DNA samples were purified using alcohol and DNA low- and high-stringency wash solutions. The genomic DNA was eluted in DNA elution solution.

Novel design of a ddPCR assay to assess chromatin opening state

The analysis was performed using ddPCR rather than qPCR, to achieve quantifiable results using *GAPDH* expression as positive control. The reaction mix consisted of 2x ddPCR Supermix for Probes (No dUTP, Bio-Rad), 20x HEX-labelled CNV *TERT* reference primer/probe (Bio-Rad), 50ng DNA, and primers (900nM each) and FAM-labelled probes (250nM) for *GAPDH*, or the methylation region around cg11625005 (S3 Table). Samples were amplified according to the program of the CNV *TERT* reference primer/probe as described. Gene accessibility was quantified by the digestion fraction between the digested and undigested samples, subtracted from 1, multiplied by 100.

Allele-specific chromatin accessibility

The mutational fraction upon digestion with nuclease (EpiQ™ Chromatin Analysis Kit aforementioned) was assessed in cutaneous melanoma cell lines with heterozygous *TERT*p mutations, 518A2, 94.07, A375 and 93.08. The analysis was performed by ddPCR using the *TERT* C250T_113 Assay and C228T_113 Assay (unique assay ID dHsaEXD46675715 and dHsaEXD72405942, respectively; Bio-Rad) as described above. The mutation fraction from undigested and digested samples were compared and the accessibility of mutant allele was calculated as follows:

$$Accessibility\ of\ mutant\ allele\ = \frac{mutational\ fraction\ undigested - mutational\ fraction\ digested}{mutational\ fraction\ undigested} \times 100$$

RNA ISOLATION, CDNA SYNTHESIS AND QUANTITATIVE REAL-TIME PCR

RNA was obtained using the FavorPrep Tissue Total RNA Extraction Mini Kit (Favorgen Biotech, Vienna, Austria) according to manufacturer's instructions for animal cells. cDNA was synthesised through the iScript™ cDNA Synthesis Kit (Bio-Rad) according to recommended protocol. *TERT* mRNA expression was assessed by qPCR performed with 3.5ng DNA, IQ SYBR Green Supermix (2x; Bio-Rad), and 0.5µM PCR primers (Sigma-Aldrich; Supplementary Table S4) in a Real-Time PCR Detection System CFX96 (Bio-Rad) and normalised to reference gene expression (*RPS11*, *TBP* and *CPSF6*, Supplementary Table S4). Data was analysed through the ΔΔCT method in Bio-Rad CFX manager software (version 3.1, Bio-Rad).

STATISTICAL ANALYSIS

In this study we used the GraphPad Prism software (version 8.0.1 for Windows, GraphPad Software, CA, USA) to perform all the statistical tests. Prism 8 has a wide library of analysis and in our paper we have used the linear regressions and correlations (in Figure 2a,c and d; Figure 3; Figure 6b,c and d), one-way ANOVA (Figure 5) and multiple t-tests without correction for multiple comparisons, one t-test per cell line, *p<0.001 (Figure 6a and f) and two-tailed unpaired t-test (Figure 6e). A p-value<0.05 was considered statistically significant. The methylation fraction obtained using ddPCR was calculated with 95% confidence interval by dividing the CNV of the digested sample with that of the paired undigested sample. Raw data was uploaded in online digital PCR management and analysis application Roodcom WebAnalysis (version 1.9.4, https://www.roodcom.nl/webanalysis/)[24] (in Figure 2b).

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SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLE S1. Tailed primers used for amplification of 325-bp region in bisulfite-converted samples.

Forward primer (5'-3')	[GATGTGTATAAGAGACAG]AGGGGTTATGATGTGGAGGT
Reverse primer (5'-3')	[CGTGTGCTCTTCCGATCT]TTACTCATAATAAAAACCCCTC

Note: Primer tail between square brackets

SUPPLEMENTARY TABLE S2. Primers and probe sequences to amplify the 106-bp amplicon in a novel design of a ddPCR assay to determine the methylation fraction.

Forward primer (5'-3')	GTGAAGGGGAGGACGGAGG
Reverse primer (5'-3')	GTGTTGCAGGGAGGCACT
Probe (5'-3')	TAGACGCGGCTGGGGACGAA

SUPPLEMENTARY TABLE S3. Primers and probe sequences to amplify the 231-bp region encompassing 31 CpG sites around the cg11625005 in a novel ddPCR assay to assess the chromatin state.

	Forward primer (5'-3')	Reverse primer (5'-3')	Probe (5'-3')
GAPDH	CCTTGACTCCCTAGTGTCCT	ATTTATAGAAACCGGGGGCG	CGGGGCCCACACGCTCGGT
TERT methylation region	GCCTAGGCTGTGGGGTAAC	CCCGTCCAGGGAGCAA	GCGGCGACCCTTTGGCCGC

SUPPLEMENTARY TABLE S4. Primer and probe sequences for *TERT* expression in qPCR.

		Forward primer (5'-3')	Reverse primer (5'-3')
TERT	ex9-10	ATCCTCTCCACGCTGCTCT	CCAACAAGAAATCATCCACCA
Reference genes	*RPS11*	AACATCGGTCTGGGCTTC	AGTGAAGGGGCATTTCTTGT
	TBP	CACGAACCACGGCACTGATT	TTTTCTTGCTGCCAGTCTGGAC
	CPSF6	AAGATTGCCTTCATGGAATTGAG	TCGTGATCTACTATGGTCCCTCTCT

SUPPLEMENTARY TABLE S5. Overview of the methylation fraction (measured by ddPCR and NGS), mutational status and *TERT* mRNA expression of our sample cohort (n=61).

	Type of samples	Sample ID	Methylation fraction (cg11625005)		*TERT*p mutation	*TERT* expression
			ddPCR (n=59)	NGS (n=44)	ddPCR (n=61)	qPCR (ΔΔCq) (n=31)
1	Uveal cell lines	OMM 2.3	103.8%	96%	WT	0.59
2		OMM 1	103.4%		WT	0.04
3		OMM 2.5	103.8%	97%	WT	1.65
4		Mel270	103.2%		WT	1.72
5		Mel202	107.8%		WT	0.02
6		92.1	100.4%	96%	WT	0.25
7	Cutaneous cell lines	04.01	100.0%		C250T	1.06
8		WM1361A	35.9%		C228T	0.16
9		93.05	53.4%		C250T	2.06
10		WM3506	78.8%		C228T	0.59
11		WM1960	87.3%		C250T hom	0.71
12		Meljuso	74.4%		C250T	0.64
13		634	4.4%		C250T	0.70
14		OCM8	67.3%	56%	WT	0.41
15		OCM1	36.2%	37%	C250T	1.08
16		518A2	4.3%	8%	C228T	0.44
17		607B	36.5%	86%	C228T hom	1.05
18		94.07	2.3%	5%	C250T	0.06
19		A375	52.4%	41%	C250T	0.97
20		93.08	19.0%		C250T	0.29
21		01.05	7.5%		C250T	1.00
22		04.04	102.6%		C250T	0.18
23		94.13	23.7%		WT	1.17
24		MM057	55.1%		C250T	1.10
25		06.24	83.0%		C228T	0.22
26	Skin biopsy samples	LB627	21.9%	29%	WT	
27		LB470	26.0%	36%	WT	
28		LB579	23.4%	30%	WT	
29		LB576	18.3%	30%	WT	
30		LB584	10.1%	15%	WT	
31		LB586	31.0%	34%	WT	
32		LB625		25%	WT	
33		LB381	26.3%	29%	WT	
34		LB628	22.7%	28%	WT	
35		LB629	24.3%	28%	WT	
36		fresh skin 1	25.8%	33%	WT	
37	Keratinocytes	K590	41.5%	54%	WT	0.00
38		K409	49.4%	49%	WT	0.00
39		K549	34.0%	56%	WT	
40		K514	43.5%	59%	WT	
41		K060	41.4%	56%	WT	
42		K627	42.0%	59%	WT	
43		K516	41.1%	59%	WT	
44		K550	34.1%	56%	WT	

SUPPLEMENTARY TABLE S5 CONTINUED.

	Type of samples	Sample ID	Methylation fraction (cg11625005)		*TERT*p mutation	*TERT* expression
			ddPCR (n=59)	NGS (n=44)	ddPCR (n=61)	qPCR (ΔΔCq) (n=31)
45	Melanocytes	m003	9.5%	29%	WT	
46		m002	7.1%	44%	WT	
47		m003A		7%	WT	
48		m004A	3.6%	2%	WT	
49		0398A	4.3%	9%	WT	
50		HEM	43.9%		WT	0.00
51	Fibroblasts	F537	3.7%	5%	WT	
52		F544	4.0%	7%	WT	
53		F332	3.5%	9%	WT	0.00
54		F334	4.4%	8%	WT	0.00
55		F628	3.3%	8%	WT	0.00
56	Naevi	Naevus 1	26.4%	31%	WT	
57		Naevus 2	13.8%	20%	WT	
58		Naevus 3	26.9%	33%	WT	
59		Naevus 4	25.8%	31%	WT	
60		Naevus 5	10.2%	15%	WT	
61		Naevus 6	23.1%	33%	WT	

SUPPLEMENTARY TABLE S6. Overview of the methylation fraction (measured by ddPCR and NGS), mutational status and *TERT* mRNA expression and chromatin accessibility in the subset of melanoma cell lines present of our cohort (n=25).

	Type of samples	Sample ID	Methylation fraction (cg11625005)		*TERT*p mutation	*TERT* expression	Gene accessibility	
			ddPCR	NGS	ddPCR	qPCR (ΔΔCq)	*TERT* meth site	*GAPDH*
1	Uveal cell lines	OMM 2.3	103.8%	96%	WT	0.59		
2		OMM 1	103.4%		WT	0.04		
3		OMM 2.5	103.8%	97%	WT	1.65	99.6%	100.0%
4		Mel270	103.2%		WT	1.72	90.9%	99.7%
5		Mel202	107.8%		WT	0.02		
6		92.1	100.4%	96%	WT	0.25		
7	Cutaneous cell lines	04.01	100.0%		C250T	1.06		
8		WM1361A	35.9%		C228T	0.16		
9		93.05	53.4%		C250T	2.06		
10		WM3506	78.8%		C228T	0.59		
11		WM1960	87.3%		C250T hom	0.71		
12		Meljuso	74.4%		C250T	0.64		
13		634	4.4%		C250T	0.70		
14		OCM8	67.3%	56%	WT	0.41	87.6%	100.0%
15		OCM1	36.2%	37%	C250T	1.08		
16		518A2	4.3%	8%	C228T	0.44	36.2%	96.3%
17		607B	36.5%	86%	C228T hom	1.05	66.6%	99.5%
18		94.07	2.3%	5%	C250T	0.06	25.5%	97.8%
19		A375	52.4%	41%	C250T	0.97	54.6%	98.6%
20		93.08	19.0%		C250T	0.29	50.6%	99.0%
21		01.05	7.5%		C250T	1.00		
22		04.04	102.6%		C250T	0.18		
23		94.13	23.7%		WT	1.17		
24		MM057	55.1%		C250T	1.10		
25		06.24	83.0%		C228T	0.22		

6

Summary and Discussion

Melanoma is an aggressive form of skin cancer, causing significant mortality, due to its capacity for metastatic dissemination. It develops from the pigment-producing cells, the melanocytes. In order to become malignant, these cells accumulate multiple genetic and epigenetic alterations.

During the last decade the advent of next generation sequencing methodology has allowed the identification of novel inherited pathogenic gene variants in patients with familial melanoma and patterns of somatic alterations in sporadic cases.

In the present thesis, we explored inherited and acquired epigenetic alterations in melanoma susceptibility and development. Epigenetics literally means 'on top of genetics' and refers to mechanisms that regulate gene expression not involving alterations in the DNA.[1] There are 3 main epigenetics mechanisms: DNA (hydroxy)methylation (covalent modifications of DNA bases), histone modifications (post-translational modifications on the amino-terminal tail of histones) and chromatin remodelling (repositioning of nucleosomes by protein complexes).[2] The epigenetic enzymes can be classified as writers, readers and erasers depending on their function and have different implication in cancer. The *writers* add various chemical modifications on DNA and histone tails; the *readers* are the proteins that bind the modifications and interpret them; and the *erasers* are responsible for removing these chemical tags.[3]

We aimed to address epigenetic alterations in melanoma susceptibility by studying a novel putative melanoma-susceptibility gene encoding a histone 3 lysine 79 (H3K79) methyltransferase, a *writer* according to the above-mentioned classification, was functionally assessed. In addition, epimutations as a putative cause of familial melanoma using genome-wide analyses. We also aimed to explore the epigenetic regulation of an essential gene in melanoma pathogenesis, *TERT*. Finally, we analysed the genomic distribution and functional significance of DNA hydroxymethylation in melanoma.

CAN A MUTATED EPIGENETIC ENZYME BE RESPONSIBLE FOR MELANOMA PREDISPOSITION?

To reveal new candidate susceptibility genes in melanoma we performed whole exome sequencing (WES) on DNA isolated from blood cells from two members of a family with four melanoma cases, not explained by established high penetrance melanoma-susceptibility genes. WES identified 10 rare, co-segregating, predicted deleterious missense gene variants. Subsequent co-segregation analysis revealed that only variants in the *DOT1L* (R409H) and

the *SLCO4C1* (P597A) genes were present in the other two affected members of this family. The lack of expression of *SLCO4C1* in melanocytes combined with gene function did not encourage us to explore this gene variant further. In Chapter 2, we describe a new germline missense variant in the *DOT1L* gene co-segregating with melanoma in all affected members of a Dutch melanoma family. DOT1L is the unique histone methyltransferase responsible for methylating the nucleosome core on lysine 79 of histone H3 (H3K79).[4-6] In addition, DOT1L regulates transcription elongation, establishes cell cycle checkpoints, and maintains genomic stability.[7,8] Dysregulation of DOT1L has been associated with a number of cancers either as an oncogene or tumour suppressor gene.[4] In ovarian cancer cells, when *DOT1L* expression was knocked-out by CRISPR/Cas9 technology, cell invasion and cancer stem-like cell properties were significantly promoted.[9] Studies in gastric cancer showed that *DOT1L* expression is significantly higher in gastric malignant tumours correlating to the degree of differentiation, lymph node metastasis and TNM staging.[10] Recently, in colorectal cancer DOT1L has been described as an important player in DNA double-stand break repair via homologous recombination.[11]

Three new somatic mutations (M55L, P271L, and P505L) in the *DOT1L* gene that negatively affect the catalytic activity of the methyltransferase were identified in melanoma cells.[12] Loss of *DOT1L* (by silencing or mutation) impaired the DNA damage repair induced by UV-B radiation, thereby promoting melanoma development *in vivo*. Accordingly, we found that mouse ES cells express a catalytically inactive *DOT1L* mutant (G165R) were more sensitive to UV radiation, consistent with a protective role of DOT1L. However, when we functionally explored the germline R409H variant, we could neither detect histone methyltransferase activity reduction in melanoma and mESCs nor an effect on UV-induced survival in mESCs. Different explanations for this lack of effects can be pointed out: the dynamic changes in and alternative functions of H3K79me were missed in the assays used; the R409H mutant is not by itself pathogenic; the role of the R409H variant in melanocytes is not recapitulated in the cell model used in our study; the R409H variant affects a methyltransferase-independent function of DOT1L. However, in mammalian cells this activity of DOT1L has been shown to be required for several critical functions, such as reactivation of repressed genes, cell cycle progression in lung cancer cell lines, and leukemic transformation in CALM-AF10 MLL-rearranged leukemia.[13-15]

Subsequently, several variants in *DOT1L* gene have been observed in independent familial and sporadic melanoma cases from the UK (personal communication, Dr. Harland, Leeds University). The variants G1320R and Y115F, both assessed as damaging/deleterious by Polyphen and SIFT prediction tools, were described in a family with 2 cases of melanoma. The variant A591V co-segregated with melanoma in a family with 3 affected members.

Furthermore, two somatic variants in the *DOT1L* gene (S1440T and L871P) were found in independent sporadic patients with early-onset of melanoma and no family history (see Table 2, Chapter 2[16]).

Although our studies on the R409H variant do not conclusively prove pathogenicity, we believe that a variant co-segregating with melanoma in a family with four affected members reinforce the findings by Zhu *et al.* of *DOT1L* as a melanoma susceptibility gene. We consider it worthwhile to examine whether this variant has methyltransferase-independent functional consequence and to investigate *DOT1L* variants in future studies involving large familial melanoma cohorts.

MAY HERITABLE EPIGENETIC EVENTS EXPLAIN MELANOMA SUSCEPTIBILITY?

Whole exome and whole genome sequencing analyses of familial melanoma have identified a few rare pathogenic gene variants, but failed to explain the cause of melanoma predisposition in more than half of the families.[17] This might be due to the fact that most of the identified variants are extremely rare and segregate in only a few families, along with polygenic inheritance mechanisms as well as the effects of UV radiation. In addition hereditary epigenetic alterations have been proposed to explain the 'missing heritability' of melanoma susceptibility.

Over the years, the focus has been redirected to epigenetic mechanisms that may explain the melanoma predisposition in these families. Holliday defined epimutations as heritable promoter hypermethylation leading to gene silencing.[18] In hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) not caused by mutations in DNA mismatch repair genes, epimutation of the *MLH1* gene has been identified as causative mechanism in multiple families.[19] Subsequently, epimutation of the *MSH2* mismatch repair gene was demonstrated in another family with HNPCC.[20] In several other cancer types with familial occurrence, including ovarian cancer and retinoblastoma, candidate epimutations have been found.[21,22]

Whether a heritable epimutation can be the result of transgenerational epigenetic inheritance in the absence of an underlying genetic cause is controversial.[23,24] In fact, epimutations were described as primary, those occurring in the absence of an underlying DNA sequence alteration, or secondary, where genetic variation drives the propensity for hypermethylation at a specific locus.[25] The extent to which heritable epigenetic alterations might confer predisposition to cancer and melanoma in particular remains to be established.

Therefore, besides the putative genetic cause, we have aimed to assess whether heritable epigenetic events could account for melanoma predisposition in families where no genetic cause could be found. Possible epimutation of the *CDKN2A* gene has been explored as a mechanism to explain the melanoma susceptibility in melanoma families. For this gene, our group and others concluded that in 64 Swedish, 22 Dutch and 114 American patients with hereditary melanoma, *CDKN2A* epimutation was not the causative mechanism for melanoma susceptibility.[26-28]

In Chapter 3, we evaluated 5 families with a history of melanoma in multiple generations where no pathogenic gene variants in any of the currently established or candidate high penetrance melanoma susceptibility genes had been found. DNA from peripheral blood of 2 affected members of each family was assessed by 450K Illumina arrays.[29] In this study we made use of DNA methylation data from a valuable reference group of 1000 healthy individuals from the Biobank-based Integrative Omics Studies (BIOS) consortium. The occurrence of epimutation of the recently established melanoma susceptibility genes (*CDKN2A*, *CDK4*, *BAP1*, *TERT*, *POT1*, *TERF2IP*, *ACD, MBD4, POLH, POLE, EBF3, GOLM1, MC1R* and *MITF)* was examined in these familial melanoma patients. Secondly, loss-of-imprinting, a distinct epigenetic mechanism of inheritance by which certain genes are silenced based on its parental origin epigenetic mark, was examined.[30] Genomic imprinting is involved in fetal growth and plays a role in carcinogenesis[31], being the association between the *IGF2–H19* locus and Wilms tumour the best-described in cancer.[32] Thirdly, a genome-wide analysis of approximately 450,000 CpGs located in all human gene promoters was performed. This unbiased genomic analysis of single CpG sites was complemented by investigation of differentially methylated regions (dmrs).

In summary, there was no significant difference from the reference group in methylation level at any of the melanoma susceptibility genes. Nor did we find any indication of loss-of-imprinting at the CpG sites located at the imprinted loci. The exploratory analysis found 6 hypermethylated CpGs in both affected members of a melanoma family compared to a healthy cohort as well as 35 hypomethylated CpGs. Although we did find the 6 hypermethylated CpGs located in the promoter regions of *RABGGTB, SND1, SCAF11, ZNF638, THAP1* and *SFSWAP* genes, none of them have been reported as a cancer predisposition gene or functions related to melanoma.[33] Besides these also the dmrs in the promoters of *CCNI, CD47* and *USP46* genes showed minor differences compared to healthy controls. Two of 35 hypomethylated CpGs were located in cancer-related genes, but were not found to be plausible candidates either. This was the first time that heritable epigenetic events in melanoma have been analysed in a genome-wide fashion interrogating 99% of human genes. We conclude that none of the hyper/hypomethylated CpG sites and dmrs in these

families provide a relevant pathogenic explanation for melanoma inheritance. However, it is still possible that pathogenic heritable DNA methylation alterations might occur in a minor proportion of melanoma families or that epimutations may affect other potentially regulatory regions. Recently, constitutional mosaic epimutations in *MGMT, MLH1* and *BRCA1* genes have been reported as the epigenetic cause of a significant number of glioblastoma, colorectal and ovarian/breast cancers, respectively.[34] Therefore, constitutional methylation of key tumour suppressor genes may represent an initiating event of carcinogenesis that might be present in a mosaic fashion due to a post-zygotic epigenetic event in melanocyte precursor cells and not be retrievable in DNA from blood.

Uncovering the causes of predisposition to melanoma in families is of major clinical relevance since it could improve the risk estimation, genetic counselling and testing and enable targeted clinical surveillance of patients at high risk of melanoma. Accordingly, further studies for such constitutional epigenetic events could aid cancer prevention programs.

CAN AN ALTERED DNA HYDROXYMETHYLATION PATTERN BE A BIOMARKER OF MELANOMA?

The role of DNA methylation in cancer has been widely explored. Cutaneous melanoma demonstrates altered patterns of DNA methylation that are associated with genetic instability and transcriptional repression of tumour suppressor genes. A more recently discovered epigenetic modification, that has since received increasing attention of the cancer research field, is DNA hydroxymethylation. Active DNA demethylation entails a hydroxymethylation step mediated by TET enzymes, which catalyze conversion of 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC). Hydroxymethylcytosine can thus be considered as an intermediate step in the active demethylation of DNA.

In Chapter 4, we aimed to identify CpG sites and regions with differential hydroxymethylation and methylation levels when comparing melanoma (metastatic and non-metastatic) and melanocytic nevus samples that might be used as diagnostic and prognostic markers. In addition we intended to gain insights into the functional significance of this DNA modifications by studying its distribution. A study from Lian *et al.* had previously reported the loss of hydroxymethylation as an epigenetic hallmark of melanoma.[35] Oxidative bisulfite chemistry combined with arrays that simultaneously interrogate hmC and mC at 850,000 CpG sites throughout the genome were used in our study. This methodology followed by bioinformatic analysis revealed significant differences in the global hmC patterns of melanoma and nevus samples, being more pronounced than in mC patterns. Although the

levels of hmC were uniformly lower in metastatic than in non-metastatic melanoma, the patterns of distribution were not significantly different. DNA hmC loss may be explained by passive dilution of the hmC mark due to DNA replication in proliferating melanoma cells and by insufficient active demethylation.[36,37] Within the pattern of global hmC depletion, specific CpG sites and regions could be identified with significantly lower hydroxymethylation in melanoma than in nevus, pointing to epigenetic deregulation at specific loci. We identified 22,164 single differentially hydroxymethylated CpG sites and 68 regions that are strong candidate biomarkers for diagnosis.[38-40] Among the 68 DhMRs, 5 localized to cancer-related genes: *PTEN, DAXX*, *GAS7*, *GNAS* and *TPM4*. *PTEN* is an essential tumour suppressor gene in melanoma. Many previous studies report downregulation of PTEN in melanoma compared to nevus, *PTEN* inactivation through promoter hypermethylation, and enhanced melanoma formation upon PTEN downregulation.[35,41-45] These findings strengthen our hypothesis that hmC depletion at the *PTEN* regulatory region in melanoma has functional significance by affecting expression of this tumour suppressor gene. Briefly, in normal skin, the development of melanocytic nevi is caused by activating mutations of the *BRAF* or *NRAS* oncogenes, which provides a proliferative stimulus to the cells at first but eventuates in a state of stable cell cycle arrest (oncogene-induced senescence). We found that at this stage the *PTEN* promoter region shows high hmC and low mC levels. The accumulation of mC due to reduced active demethylation (loss of hmC) at this region regulating *PTEN* expression may lead to transcriptional silencing of this essential tumour suppressor gene facilitating escape from oncogene-induced senescence and contribute to malignant transformation.

Recently, Bonvin *et al.* made use of a well-established genetically engineered *Nras* mutation–driven mouse model of spontaneous melanoma to disclose the roles of specific epigenetic alterations in melanomagenesis. They showed that in mice, genetic ablation of *Tet2* in combination with *Nras*^{Q61K}-driven melanoma mouse model promotes melanoma initiation by an overall decrease in hmC and specific hmC gains in selected gene bodies, accelerates melanoma progression and decreases melanoma-free survival. They also suggest that the presence/absence of hmC modulates the binding of epigenetic factors resulting in changes in chromatin structure rather than directly affecting gene transcription.[46] Similar to our results a striking hmC depletion in melanoma compared to nevus samples was observed exceeding those of mC patterns. Altogether these studies suggest that active demethylation may protect promoter and enhancer regions from methylation-associated silencing and therefore loss of hmC might contribute to malignant progression. Our findings call for further functional studies of the role of DNA hydroxymethylation at the *PTEN* locus and other tumour suppressor gene loci.

The reasons behind hmC reduction have been widely explored and some studies report that mutational inactivation and/or downregulation of *TET2* might explain the loss of hmC as well as mutant IDH proteins.[35,47] While wild-type IDH protein produce αKG, the co-substrate of TET enzymes, the mutant IDH transform it into 2-HG, an oncometabolite that is a competitive inhibitor of TET. Resetting the differential mC and hmC levels towards a functional demethylation pathway might be an interesting target to cancer therapy.[48] Recently, a new combinatorial treatment between vitamin C and ML309, *IDH1*-mutant inhibitor, showed a synergistic effect in colon cancer cells, significantly decreasing the 2-HG levels to levels comparable with WT cells, leading to increased levels of global hydroxymethylation and even increased expression of tumour suppressors.[49]

HOW IS *TERT* GENE EPIGENETICALLY REGULATED?

Located on chromosome locus 5p15.33, telomerase reverse transcriptase (*TERT*) encodes the catalytic subunit of the ribonucleoprotein telomerase and is capable of extending the repetitive, non-coding DNA sequence of telomeres following mitosis.[50] Telomeres become shorter at each cell division, until reaching the Hayflick limit, which induces replicative senescence and growth arrest.[51] Through *TERT* reactivation, the cells keep the ability of extending their telomeres or prevent their shortening allowing immortalization of malignant cells and tumour progression as occurs in 90% of human cancers. This limitless replicative potential by active telomere elongation is one of the hallmarks of cancer.[52-54]

Expression of TERT is a key determinant of telomerase activity in human cells. Activating *TERT* promoter (*TERT*p) mutations are reported in 30–80% of familial and sporadic melanomas.[55,56] The well-known influence of epigenetic mechanisms in the regulation of gene expression in cancer led us to explore how the *TERT* gene can be epigenetically reactivated. As mentioned above in this thesis the canonical effect of hypermethylation in the promoter regions of cancer cells is to repress genetic transcription.[57,58] Contrary to common concepts, CpG methylation in the *TERT*p was previously correlated with higher *TERT* mRNA expression.

In Chapter 5, we aimed to characterize the genetic-epigenetic interaction in the regulation of the *TERT* gene in healthy skin samples and melanoma cell lines. The two hotspot mutations in *TERT*p, dubbed C228T and C250T, together with *TERT*p methylation, chromatin accessibility and *TERT* expression were assessed. The methylation fraction was evaluated by NGS in a set of 31 CpGs in the *TERT* promoter region and validated by custom ddPCR-based assays, namely for the CpG cg11625005 that deserved special attention. Previously

in brain tumours and melanoma this CpG showed absence of methylation in normal cells.[52,59] However, in our study, the methylation fraction was quite high in healthy skin samples, naevi and cultured keratinocytes. Furthermore, our results suggest that context-related methylation of a genomic region might be biologically more relevant as opposed to methylation of one specific CpG. The two point mutations in the *TERT*p described in melanoma create new transcription factor binding motifs and correlate with upregulation of the *TERT* gene. In our cohort, we checked the presence of these mutations through ddPCR-based assays. Altogether, *TERT* expression was only found in tumour cell lines, with or without *TERT*p mutation and a broad range of promoter methylation levels (5%-100% methylation fraction). Thus, we hypothesise that another level of regulation was responsible for mRNA expression. Since distinct histone modifications influences chromatin remodelling and ultimately gene accessibility to transcription factors, such as ETS/TCF, we investigated to which extent the chromatin was accessible and whether this had occurred in a mono- or biallelic fashion. We observed a positive correlation between chromatin accessibility and methylation levels as well as mRNA expression leading us to conclude that mutated alleles were more accessible, possibly favouring the binding of transcription factors and consequently *TERT* mono-allelic expression of the mutant allele. Our results are in line with the study from Stern *et al.* and Huang *et al.*, where the authors found that the mutant allele was hypomethylated and allowed monoallelic *TERT* expression.[60,61] Thus, these observations are consistent with the canonical influence of methylation on transcriptional regulation. On the contrary, the WT *TERT*-expressing uveal melanoma cell lines show a methylation fraction close to 100% and significantly higher chromatin accessibility. These characteristics of WT *TERT*p cell lines may lead to biallelic *TERT* activation. In fact, in this case *TERT* seems to be an exception, since possible biallelic hypermethylation leads to upregulation.

Taking everything into account we might conclude that *TERT* activation is under distinct epigenetic regulation in mutated and WT *TERT*p cell lines. While in mutant *TERT*p, methylation executes its canonical function, repressing transcriptional regulation, in WT *TERT*p, methylation carries out a non-canonical role, leading to transcriptional activation in these cell lines. The dynamics of epigenetic mechanisms in *TERT* genetic regulation is complex, however we believe that our results contribute to the full understanding of all (epi) genetic mechanisms that collectively reactivate *TERT*.

TERT was first recognized for the telomere maintenance and over the years many other non-canonical roles have been reported, such as involvement in regulation of non-telomeric DNA damage responses, promotion of cell growth and proliferation and control of mitochondrial integrity following oxidative stress. Moreover, TERT behaves as a regulator of

genetic transcription through chromatin modulation.[62,63] Beyond the telomere maintenance, *TERT* is crucial to other aspects of the tumour microenvironment, such as angiogenesis, inflammation and cancer cell stemness.[64] Although TERT repression may sensitize cells to conventional chemotherapy in a telomere independent manner[65-67], due to the above mentioned telomere-independent functions this approach can lead to severe side effects.[63] Therefore, further study is required to elucidate the conventional and alternative molecular mechanisms of telomerase beyond telomere maintenance, in order to develop new anticancer strategies targeting telomerase or telomeres.[63,68]

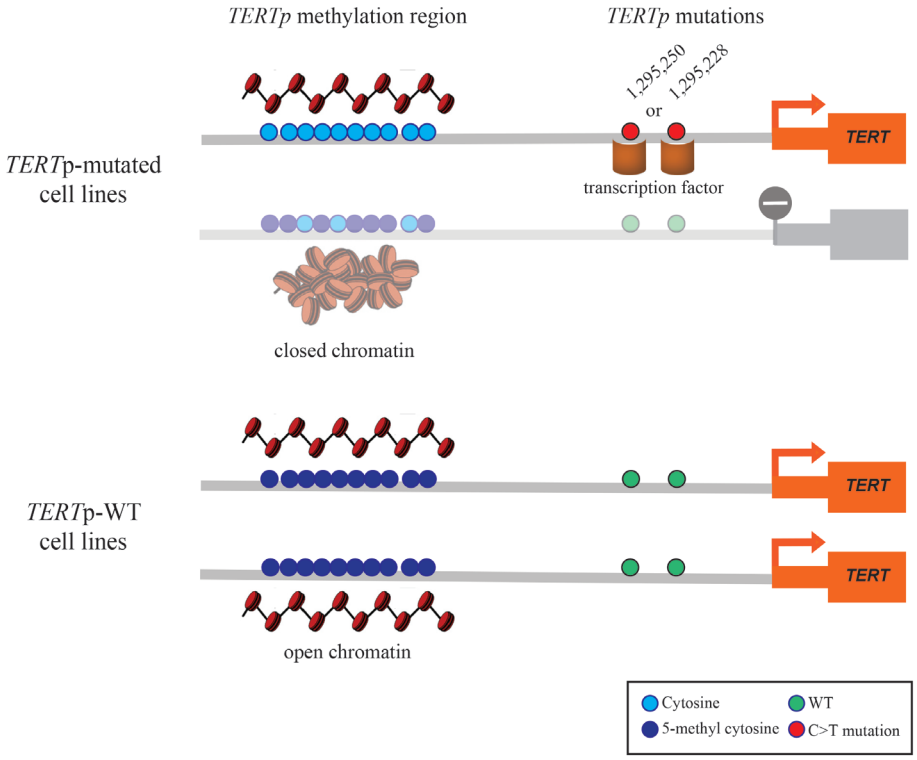

FIGURE 1. Proposed model of *TERT* transcriptional regulation. In *TERT*p-mutated cell lines, active mutant allele is hypomethylated, highly accessible and allows monoallelic *TERT* expression. In *TERT*p-WT cell lines, the MF is close to 100% with a significantly higher chromatin accessibility leading to high expression levels due to biallelic *TERT* activation.

HOW DO OUR FINDINGS CONTRIBUTE TO THE UNDERSTANDING OF EPIGENETIC ALTERATIONS IN THE SUSCEPTIBILITY TO AND DEVELOPMENT OF MELANOMA?

In our study, we discovered R409H variant in the *DOT1L* gene using WES in a family. Although we were not able to definitely prove the functional effect of the R409H variant in the *DOT1L* gene, encoding a H3K79 methyltransferase, our findings reinforce the ones by previous studies, which pointed to *DOT1L* as a melanoma susceptibility gene with a role in DNA damage repair.[11,12] *DOT1L* is not involved in transcriptional regulation of the DNA repair genes, but rather promotes the assembly of the nucleotide excision repair complex on chromatin by interacting with XPC and stimulating its recruitment to DNA lesions.[12] Loss of *DOT1L* impaired DNA damage repair induced by UV-B radiation, thereby promoting melanoma development *in vivo*. To fully disclose the functional consequences in regard to melanoma risk of the variant identified in our study, an engineered mouse model to harbour the *DOT1L* R409H variant might be valuable tool.

As previously observed in patients with hereditary nonpolyposis colorectal cancer and familial chronic lymphocytic leukemia, heritable epigenetic events might be a plausible explanation for melanoma predisposition.[19,20,69-72] For the first time we performed genome-wide analysis assessing all CpGs located in gene promoters of candidate and established susceptibility genes, imprinted genes and gene promoters throughout the genome. We conclude that none of the hypermethylated CpGs and differentially methylated regions identified do constitute a pathogenic epimutation in predisposition to melanoma in these families. Nevertheless, it is still possible that pathogenic heritable DNA methylation alterations might either exist in a mosaic pattern through a post-zygotic epigenetic event, potentially only affecting melanocytes or occur in other potentially regulatory regions rather than in promoters or in a small number of melanoma families.

Beyond the epigenetic silencing of tumour suppressor genes or inherited epigenetic alterations involved in the predisposition to cancer, we also explored how epigenetic alterations may contribute to progression towards malignancy. Specific CpG sites and regions identified with differential distribution and significantly lower hydroxymethylation levels in melanoma than in nevus can be used to aid in distinguishing malignant from benign melanocytic lesions.[38-40] Moreover, the presence of hmC at the *PTEN* regulatory region in nevus, and its loss in melanoma, may signify that active demethylation can protect promoter and enhancer regions from methylation-associated silencing of tumour suppressor genes. Loss of hmC might therefore contribute to malignant progression, in accordance with hmC

depletion observed at the *PTEN* promoter region in melanoma.

Genetic regulation comprises different processes and pathways. We have shown that a holistic model encompassing *TERT*p mutation, methylation and other epigenetic mechanisms as chromatin accessibility are crucial to dictate *TERT* gene expression. Either a widely open chromatin state in *TERT*p-WT samples due to hypermethylation throughout the promoter or mono-allelic expression of the accessible mutated (C228T or C250T) allele in combination with moderate (probably allele-specific) methylation fraction are required.
In general, the studies presented in this thesis show associations between epigenetic states and cellular gene expression patterns or phenotypes. However demonstration of causality would require methods to specifically introduce such epigenetic alterations and investigate their consequences. Whereas CRISPR/Cas9 technology has become a valuable tool to engineer genetic alterations, similar methods to introduce epigenetic changes still need further development.

SUMMARY AND FUTURE PERSPECTIVES

Over recent years, our understanding of the genetic variation underlying cutaneous melanoma susceptibility has increased, with the discovery of pathogenic gene variants that explain half of melanoma susceptibility in families. The focus has been redirected to the epigenetic mechanisms that may either explain the melanoma predisposition in the remaining families and to reveal how certain genes are repressed or activated in melanoma. Nowadays, the integration of genomic/epigenomic/transcriptomic/metabolomic data seems to be of major importance. We generated a comprehensive view of interaction between genome and epigenome of cancer cells by addressing hydroxymethylation as a possible protection against hypermethylation-associated silencing at promoter regions of relevant genes as well as by revealing how mutations, methylation and chromatin organization may interact to regulate the expression of a gene involved in the immortalization of cancer cells. Many questions still remain for instance on hydroxymethylation distribution at the promoter region that may interfere with allele-specific chromatin accessibility and expression. We were also able to identify potential epigenetic diagnostic markers for melanoma and (epi) genetic alterations that must be taken into consideration in future studies of melanoma predisposition.

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A

NEDERLANDSE SAMENVATTING

Melanoom is het meest agressieve en dodelijke type huidkanker, omdat het kan uitzaaien naar interne organen, waarna de ziekte nauwelijks meer genezen kan worden.

Het is gebruikelijk om te horen over cellen, chromosomen, DNA, genen en veranderingen hieraan wanneer men spreekt over kanker. De chromosomen bevinden zich in de celkern en bestaan uit lange DNA-strengen die veel genen bevatten. De meest voorkomende veranderingen, ofwel mutaties, die bij kanker in de DNA strengen voorkomen verstoren de structuur of de hoeveelheid en daarmee de functie van specifieke eiwitten in kankercellen. DNA mutaties kunnen verschillende gevolgen hebben voor de cel en erfelijke mutaties kunnen ook van invloed zijn op de vatbaarheid voor kanker.

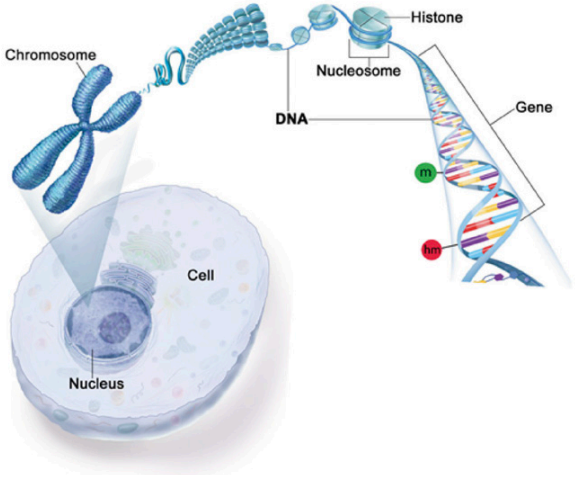
WAAR STAAT EPIGENETICA VOOR?

Mutaties zijn veranderingen in volgorde van de nucleotiden waaruit de DNA-streng is opgebouwd. De structuur en functie van het DNA kan echter ook veranderd worden zonder dat deze nucleotiden veranderen. Het DNA kan gemodificeerd worden en daardoor op een andere wijze in de celkern opgevouwen worden. Deze wijzigingen die het functioneren van het DNA beïnvloeden worden tezamen ‘epigenetica’ genoemd. Het Griekse voorvoegsel “epi” betekent “bovenop” of “naast” de traditionele genetica. De term ‘epigenetica’ werd oorspronkelijk door Conrad Waddington in 1942 voorgesteld om de moleculaire mechanismen te beschrijven die onafhankelijk zijn van veranderingen in de DNA sequentie. Er zijn drie belangrijke epigenetische mechanismen: DNA (hydroxy)methylatie (covalente modificaties van DNA nucleotiden), histonmodificaties (posttranslationele modificaties aan het amino-uiteinde van histonen en chromatine-hermodellering (repositionering van nucleosomen door eiwitcomplexen).

In de studies opgenomen is dit proefschrift is onderzocht hoe verworven en geërfde epigenetische veranderingen een rol spelen bij de vatbaarheid voor en ontwikkeling van melanoom.

Ons doel is om nieuwe melanoom susceptibiliteitsgenen en erfelijke epigenetische alteraties te identificeren die een verklaring kunnen zijn voor melanoom aanleg in een significante proportie van de met melanoom aangedane families (familiair melanoom) waarbij de oorzaak nog onbekend is. Hiervoor hebben we de rol bestudeerd van een nieuwe vermoedelijke melanoom susceptibiliteitsgen die codeert voor histon 3 lysine 79 (H3K79) methyltransferase. De mutatie in het DOT1L gen werd gevonden in een familie waarvan verschillende leden melanoom hadden ontwikkeld. We hebben experimenten met gekweekte cellen uitgevoerd om te achterhalen welke biologische processen werden beïnvloed door deze genmutatie. We konden op basis van onze en eerdere resultaten

concluderen dat erfelijke mutaties in het DOT1L gen invloed heeft op de gevoeligheid voor UV-blootstelling en zo een verhoogd risico op melanoom met zich mee kunnen brengen. Een ander onderzoeksproject had tot doel om na te gaan of erfelijke epigenetische veranderingen de oorzaak kunnen zijn van familiair melanoom in die gevallen waarvoor geen genetische oorzaak kan worden geïdentificeerd. Verschillende epigenetische mechanismen, zoals de aanwezigheid van epimutaties aan alle bekende melanoom susceptibiliteitsgenen, verlies van genomische imprinting, gevolgd door genoombrede promoter hypermethylatie, -werden onderzocht in het DNA van steeds 2 leden met melanoom uit 5 families. Onze resultaten tonen aan dat erfelijke DNA methylatie van genpromoters geen plausibele oorzaak zijn van familiair melanoom.



FIGUUR. Structuur van DNA. De chromosomen zijn gelokaliseerd in de celkern en bestaan uit lange DNA-strengen die vele genen bevatten. Chromosomen bevatten eiwitten die histonen worden genoemd, waar het DNA omheen is gebonden. DNA bestaat uit vier componenten die nucleotiden worden genoemd (hier weergegeven in geel, rood, blauw en paars). DNA-methylatie (m) en DNA-hydroxymethylatie (hm) zijn de weergegeven epigenetische veranderingen [Aangepast overgenomen van TereseWinslow LLC]

Verder onderzochten we ook het voorkomen van bovengenoemde DNA methylatie (m in Figuur) en DNA hydroxymethylatie (hm in Figuur) betrokken bij de de-methylatie van DNA in het DNA van melanomen en goedaardige gepigmenteerde huidafwijkingen (nevus, moedervlek). Het doel was om meer inzicht te krijgen in de distributie van DNA hydroxymethylatie en daarmee in de rol van deze epigenetische veranderingen in de

kwaadaardige melanoomcellen. We waren in staat om specifieke nucleotiden en regio's met hydroxymethylatie specifiek in het DNA van melanoomcellen te identificeren die zouden kunnen worden gebruikt bij de diagnostiek van dit tumortype. Daarnaast werden in het DNA van melanoomcellen regio's met hydroxymethylatie gelokaliseerd die kunnen bijdragen aan de ontwikkeling van melanoom.

Aangezien zowel genetische als epigenetische modificaties vaak naast elkaar 'werken' om maligne transformatie te veroorzaken, hebben we ook de gecombineerde bijdrage onderzocht van epigenetische (DNA methylatie en chromatine hermodellering) en genetische (genpromotor mutaties) mechanismen bij het reguleren van een belangrijk gen dat betrokken is bij de vorming van melanoom, het TERT gen. Door activatie van het TERT gen kan celdood voorkomen worden en kunnen cellen ongelimiteerde capaciteit om te delen verkrijgen, twee belangrijke kenmerken van kankercellen. We konden een complex samenspel tussen mutaties, methylatie en veranderingen van de chromatinestructuur ter plaatse van het TERT gen waarnemen die correleren met activatie van dit gen in goedaardige en kwaadaardige weefsels en cellen.

RESUMO EM PORTUGUÊS

O melanoma é o tipo de cancro de pele mais agressivo e mortal devido à sua capacidade de metastização, o que o torna difícil de controlar.

Quando se fala de cancro, é muito comum ouvir falar de células, cromossomas, DNA, genes e alterações. Os cromossomas estão confinados aos núcleos das nossas células e consistem em longas cadeias de DNA contendo inúmeros genes. As alterações mais comumente descritas em cancro estão relacionadas com alterações na sequência de DNA, mutações, que dificultam a correta produção de proteínas. Estas apresentam diferentes implicações para a célula e podem estar envolvidas na predisposição para cancro.

O QUE SIGNIFICA EPIGENÉTICA?

Além das mutações, os componentes da cadeia de DNA podem ser afetados por diversas modificações com um impacto negativo na geração de uma nova proteína. Ao conjunto destas modificações que afetam o "comportamento" dos componentes do DNA dá-se o nome de "epigenética". O prefixo grego "epi" significa "em cima da" ou "além da" tradicional genética. O termo epigenética foi originalmente proposto por Conrad Waddington em 1942 para descrever os mecanismos moleculares independentes das alterações na sequência de DNA. Existem 3 mecanismos epigenéticos principais: (hidroxi)metilação do DNA (modificações covalentes das bases de DNA); modificações das histonas (modificação pós-traducional de histonas) e remodelação de proteínas (reposicionamento de nucleossomas por complexos proteicos).

Nesta tese de doutoramento, pretendemos investigar o papel das alterações epigenéticas herdadas e adquiridas na suscetibilidade para melanoma e seu desenvolvimento.

Alterações epigenéticas hereditárias podem explicar a predisposição para melanoma numa proporção significativa de famílias com história familiar desta doença, para as quais a causa é ainda desconhecida. Assim, estudámos o papel de uma mutação num potencial gene de suscetibilidade que codifica a metiltransferase do amino-ácido lisina 79 na histona 3. Interessantemente, observámos que esta alteração no gene *DOT1L* co-segrega com melanoma em vários casos da mesma família, enfatizando o seu papel na transmissão da doença. Através de técnicas de biologia celular avaliámos que processos biológicos eram afetados ao incorporar esta alteração em determinadas células. Concluímos, baseados em estudos anteriores e nos nossos próprios resultados, que o estudo desta alteração merece ser mais aprofundado, uma vez que as mutações no gene *DOT1L* parecem afetar a sensibilidade à radiação UV, potenciando a transformação neoplásica. Além disso, investigámos se alterações epigenéticas podem ser a causa de melanoma numa grande proporção de casos familiares para os quais nenhuma causa genética foi apontada.

Diferentes mecanismos epigenéticos foram investigados no DNA de 10 indivíduos de 5 famílias: em particular estudámos 2 indivíduos diagnosticados com melanoma por família, procurando alterações de metilação hereditárias (epimutações) em todos os genes de suscetibilidade para melanoma, investigando perda de imprinting, e ainda avaliando a metilação dos respetivos promotores. Os nossos resultados mostram que epimutações não parecem constituir uma causa plausível de melanoma familiar nestas famílias.

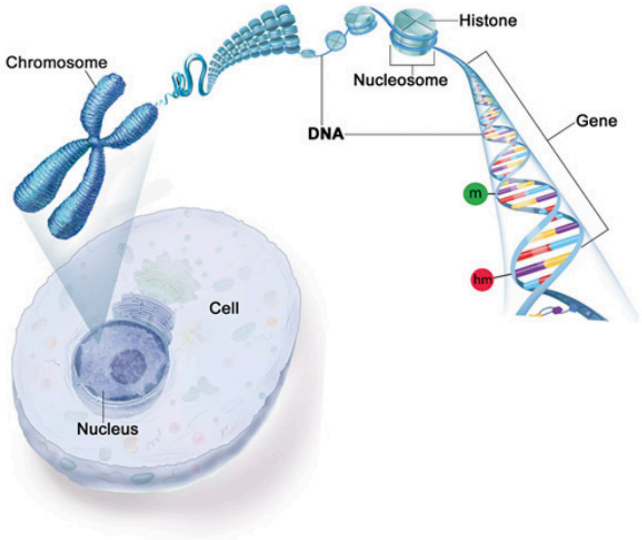


FIGURA. Estrutura de DNA. Os cromossomas estão confinados ao núcleo das células e são longas cadeias de DNA contendo diversos genes. Contém ainda proteínas chamadas histonas que se ligam ao DNA. O DNA é constituído por 4 nucleótidos diferentes (amarelo, vermelho, azul e roxo). A metilação (m) e hidroximetilação (hm) são as alterações epigenéticas representadas [adaptado de © 2015 Terese Winslow LLC, U.S. Govt. has certain rights and Dictionary of Cancer Terms PDQ Cancer Information Summaries, National Cancer Institute (US)].

Analisámos a distribuição genómica da metilação de DNA (m na Figura) e o processo oposto, desmetilação de DNA, também conhecido como hidroximetilação (hm na Figura), em amostras benignas (nevos normais, os comuns sinais na pele) e amostras cancerígenas (melanoma) de diferentes pacientes. O objetivo foi esclarecer como é que a hidroximetilação está envolvida na progressão maligna das células e que processos são, portanto, potencialmente modificáveis. Fomos capazes de identificar regiões genómicas específicas com um significado funcional que podem constituir novos e valiosos biomarcadores de

diagnóstico que parecem contribuir para a evolução de melanoma.

Uma vez que as modificações genéticas e epigenéticas cooperam na promoção da transformação neoplásica, também examinámos de que forma a metilação de DNA e remodelação da cromatina (mecanismos epigenéticos) e mutações do promotor (mecanismos genéticos) contribuem para regulação de um gene essencial na patogénese de melanoma, *TERT*. O gene *TERT* participa no processo de proliferação descontrolada e na prevenção de morte celular, duas características importantes das células neoplásicas. Os nossos resultados sugerem um modelo complexo no qual a expressão do gene *TERT* requer um estado de cromatina descondensada devido à elevada taxa de metilação ao longo do promotor em amostras sem mutações do promotor, ou a combinação de uma fração moderada de metilação/acessibilidade da cromatina em amostras com mutações no promotor (C228T ou C250T) do gene.

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ABOUT THE AUTHOR

Catarina Salgado was born in Porto, Portugal, on the 22nd of August 1989. In 2007 started her bachelor degree in Biology at University of Porto. In 2009-2010 her first internship was performed at Institute of Pathology and Immunology (Portugal) within a project related to familial amyloidotic polyneuropathy, a type of amyloidosis endemic of the north of Portugal. Afterwards, between 2010 and 2012 she followed a Master Degree in Molecular Oncology granted by University of Porto and Portuguese Institute of Oncology and developed the master's project on familial thyroid carcinoma entitled "Exome-Sequencing of a familial thyroid cancer: genetic and functional characterization" under supervision of Dr. Hugo Prazeres and Prof. Paula Soares. Alongside she also contributed to other oncobiology research projects in Soares lab, which led to her first scientific publications (Atlas Genet Cytogenet Oncol Haematol, 2014; J Clin Endocrinol Metab, 2014; Turk Patoloji Derg, 2015; Surgery, 2016) and the Everis Award 2015. In August 2015, she was accepted as a PhD fellow, supported by the prestigious Innovative Training Network grant program by the European Union and integrated in the MELGEN network, at the Leiden University Medical Center (The Netherlands) to where she moved in January 2016. Since then, under supervision of Dr. Remco van Doorn and Dr. Nelleke Gruis, she established several collaborations with specialists from different fields (genomics, cell biology, bioinformatics and medical oncologists), supervised a master student (Celine Roelse) and co-supervised a bachelor student (Casper Wenzel) and orally presented her research at several national and international scientific conferences. All different aims of the PhD project resulted in first-author publications in peer-review journals (Melanoma Res, 2019; Genes Chromosomes Cancer, 2020; Clin Epigenetics, 2020; PLoS One, 2020). She participated as a Board Member in the LUMC Association for PhD candidates (2017-2019) as well as a scientist member of two international organizations developing outreach activities for children (Native Scientist and Letters to a Pre-Scientist).

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