Epigenetic alterations in the predisposition to and progression of melanoma

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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).

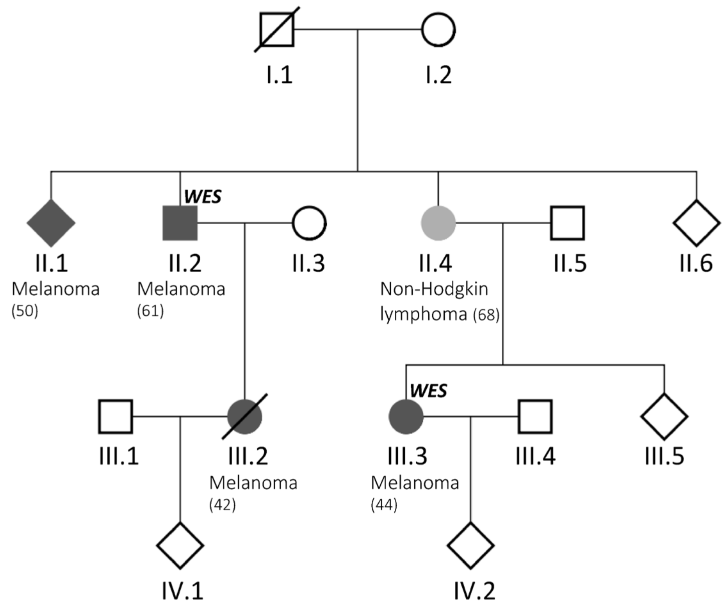

FIGURE 1. Family pedigree. The melanoma affected members are in dark grey color (II.1, II.2, III.2, III.3). The age of diagnosis is indicated in brackets. The melanoma cases subjected to whole-exome sequencing are indicated by an 'WES'.

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)

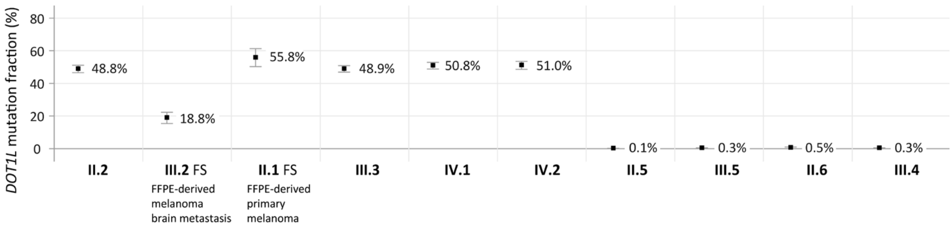

FIGURE 2. Droplet digital PCR results showing the *DOT1L* mutation fraction in DNA samples extracted from whole blood samples [II.2, III.3 (both subjected to WES) and IV.1 and IV.2], a primary melanoma (II.1 FS) and a brain metastasis (III.2 FS). The mutation fraction is around 50% in mutation-carriers 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).

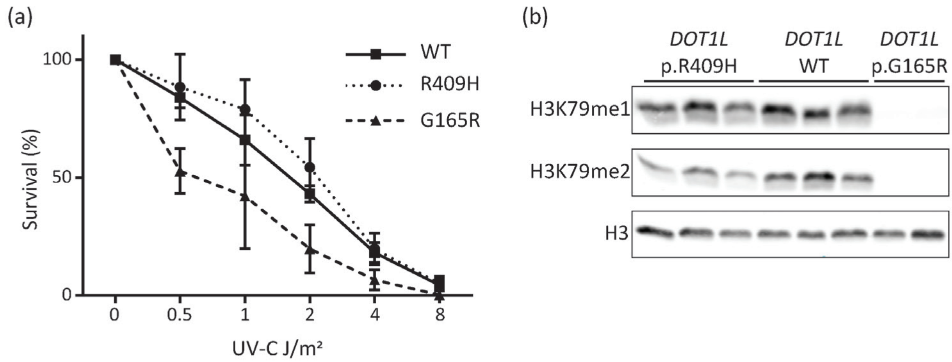

FIGURE 3. UV-survival assay of WT and *DOT1L*-mutant mouse embryonic stem cells (mESCs). (a) Colony formation capacity upon UV-C irradiation (dose range: 0.5, 1, 2, 4, 8 J/m^2) in wild-type, *DOT1L*-R409H and *DOT1L*-G165R mutated mESCs (n = 3 independent replicates, error bars represent s.d.) (b) Immunoblot 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.

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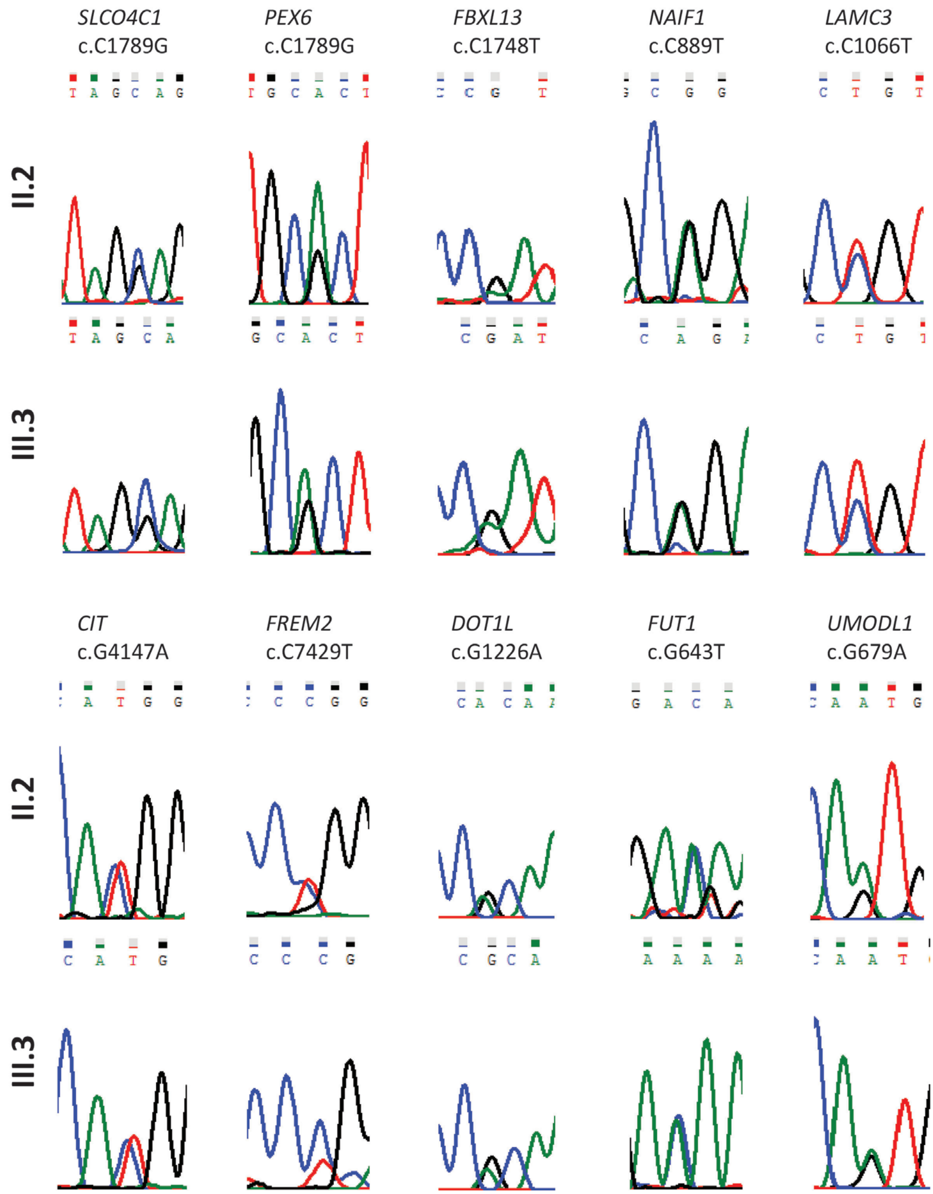
SUPPLEMENTARY MATERIAL

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

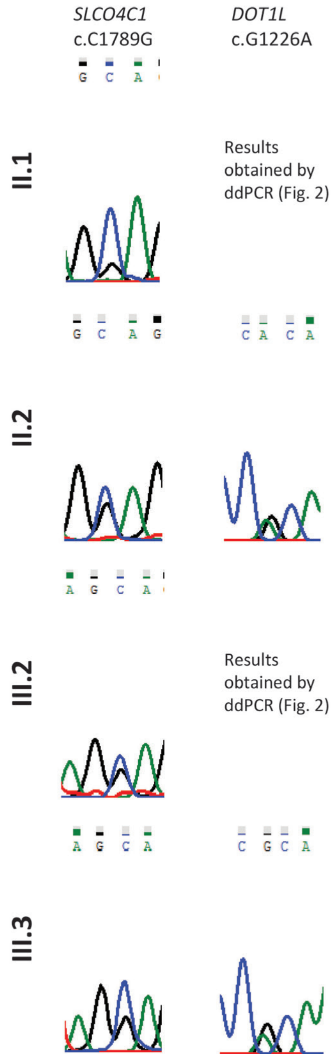
Oligonucleotide	Sense	Antisense
R409H guide 1	CACCGCTTGGGCCGCCCACGTTTC	AAACGAAACGTGGGCGGCCCAAGC
R409H guide 2	CACCGGATGGCTGGCCGGAAACGT	AAACACGTTTCCGGCCAGCCATCC
R409H guide 3	CACCGAGATGGCTGGCCGGAAACG	AAACCGTTTCCGGCCAGCCATCTC
R409H guide 4	CACCGGGCTGGCCGGAAACGTGGG	AAACCCCACGTTTCCGGCCAGCCC
G165R guide 1	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.

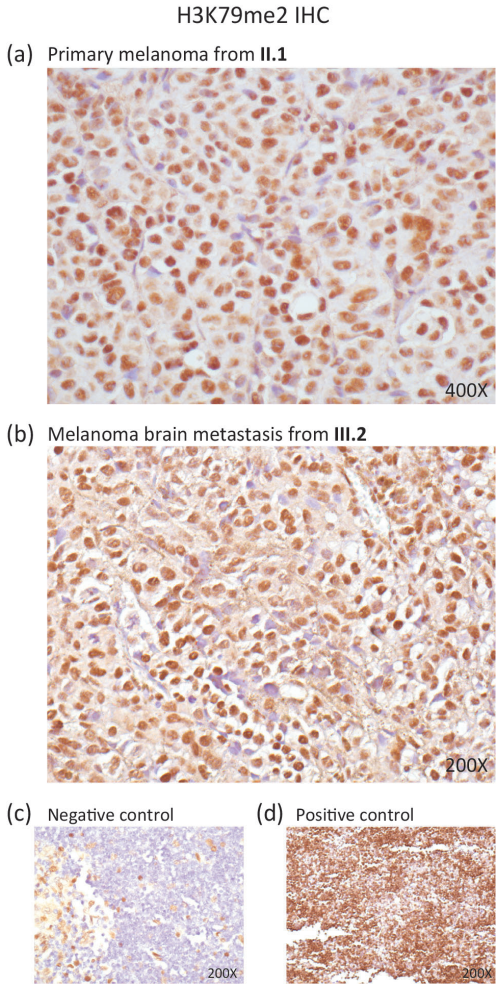
HDR template	
R409H	TCCCTCCAAAGCCCGGAAGAAGAAACTGAACAAGAAAGGGAGAAAGATGGCTGGCCACAAGAGGG GGCGGCCCAAGAAAATGAGTGCTGCCAGTGCCGAACGCAAGTCTAAGAAGAGCCA
G165R	AGCCTTGACTTTTTTTGGGACTCAAGGTCGGCTCAAAGACCATGCTGCAAGACACTCACGAGAGCCC AGGTCGACAAACAGGTCATCCTCTGTCATCTTGATCTCATCAATCATCTGGGC



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.