Loss of Wild-Type CDKN2A Is an Early Event in the Development of Melanoma in FAMMM Syndrome

TO THE EDITOR

The development of melanoma involves a sequence of genetic and epigenetic alterations. Somatic mutations typically sequentially induce MAPK pathway activation (BRAF and NRAS), upregulation of telomerase (TERT), and disruption of the G1/S cell cycle checkpoint (CDKN2A), in addition to other pathogenic alterations (Shain et al., 2018). Biallelic CDKN2A loss is the most common genetic alteration distinguishing melanocytic nevi from invasive melanomas (Shain et al., 2015). Homozygous deletion of CDKN2A has been reported in a small proportion of dysplastic nevi but never in common nevi. Studies in primary human melanocytes have revealed that CDKN2A deletions confer migratory and invasive behavior (Tran et al., 2002; Zeng et al., 2018).

At least one third of hereditary melanoma cases are caused by heterozygous germline mutations of CDKN2A, designated as familial atypical multiple mole melanoma (FAMMM) syndrome (Bergman et al., 1990). This dominant high penetration melanoma susceptibility gene encodes two tumor suppressor proteins that are translated in alternate reading frames. The \( \alpha \) transcript encodes p16\(^{\text{INK4A}} \), a protein that mediates G1 arrest by inhibiting the phosphorylation of the cyclin D1-CDK4/6 complex. The \( \beta \) transcript encodes p14\(^{\text{ARF}} \), which inhibits MDM2, thereby promoting p53 activity (Sharpless and DePinho, 1999). In carriers of germline CDKN2A mutations, the wild-type allele is functionally inactivated in melanoma by a second somatic event, commonly through deletion (Curtin et al., 2005). In the Netherlands, a specific founder mutation, a 19-base pair deletion in exon 2 of CDKN2A (c.225_243del, p.(A76Cfs*64)) known as the p16-Leiden mutation, is the most frequent cause of hereditary melanoma (Gruis et al., 1995b). CDKN2A loss-of-heterozygosity (LOH) has been demonstrated previously in primary and metastatic melanomas of patients with FAMMM syndrome (Gruis et al., 1995a; Hashemi et al., 1999). The timing of wild-type CDKN2A inactivation in hereditary melanoma development because of germline CDKN2A mutation is unknown.

The objective of this study was to investigate CDKN2A LOH in melanocytic neoplasms of patients with FAMMM syndrome. To analyze allelic imbalances at the CDKN2A locus, we developed a custom single nucleotide polymorphism (SNP)-based digital PCR method enabling absolute quantification of both alleles (Nell et al., 2019). The rs2811708 SNP, located in intron 1 of CDKN2A 2 kilobases upstream of the p16-Leiden mutation, has a minor allele frequency of 26% in the Dutch population. A second SNP, rs3731237, located 4 kilobases downstream of the p16-Leiden mutation with a minor allele frequency of 26%, was included to validate the presence of allelic imbalances at 9p21 (Boomsma et al., 2014). Capillary sequencing analysis was performed on blood DNA of homozygous and heterozygous p16-Leiden mutation carriers to show linkage of rs2811708-T and rs3731237-C with the p16-Leiden mutation (Figure 1b and c). Combined, this SNP-based digital PCR approach allows for quantification of CDKN2A allelic imbalances and loss in melanocytic neoplasms of patients with FAMMM syndrome.

The pathological diagnosis of all lesions was made by two melanoma pathologists independently. Patient consent was not necessary as the genetic analysis of nevus and melanoma biopsy material was in compliance with the Dutch code of conduct for responsible use of human tissue in the...
context of health research. Evaluation of common melanocytic nevi confirmed the absence of cytonuclear or tissue architectural atypia (Figure 1 d). We chose to analyze common benign nevi because dysplastic nevi as intermediate lesions can be difficult to distinguish from early-stage melanoma. In blood DNA of two \textit{CDKN2A} mutation carriers, there was 50% fractional abundance of the intronic \textit{CDKN2A} SNP (rs2811708), consistent with heterozygosity (Figure 2). Remarkably, allelic imbalances of rs2811708 at the \textit{CDKN2A} locus, indicative of LOH, were detected in 7 of 13 (54%) common melanocytic nevi from patients with FAMMM syndrome (Figure 2a). In informative cases heterozygous for rs3731257, allelic imbalances located downstream of \textit{CDKN2A} confirmed loss of the entire \textit{CDKN2A} locus (Supplementary Figure S1a). To investigate the presence of deletions across chromosome 9, indicative of genomic instability, a SNP at chromosome 9q (rs4745670) with a minor allele frequency of 70% was analyzed. Allelic imbalances at 9q were not detected in any of the tested nevi, and eight were \textit{BRAF}^{V600E} positive (Figure 2b). In nevi with \textit{CDKN2A} LOH, the \textit{BRAF}^{V600E} mutation occurred before \textit{CDKN2A} loss (Supplementary Table S1, Supplementary Figure S2a). The clinical and pathological characteristics do not distinguish nevi with \textit{CDKN2A} LOH and nevi without LOH (Supplementary Table S2). Collectively, these data demonstrate subclonal biallelic loss of \textit{CDKN2A} already in a subset of common melanocytic nevi of these patients with FAMMM syndrome.

In primary melanomas, allelic imbalances consistent with \textit{CDKN2A} LOH were observed in 9 of 14 (64%) cases by the intronic SNP (rs2811708) (Figure 2c). We found that 5 of 14 (36%) melanomas had allelic imbalances of chromosome 9q, suggesting additional deletions across chromosome 9, previously reported in sporadic and familial melanomas (Figure 2d, Supplementary Figure S1b) (Isshiki et al., 1994). In one melanoma case (1023), fractional abundance of 9q loss was significantly lower than \textit{CDKN2A} LOH (Supplementary Figure S2b), signifying that chromosome 9q loss occurs in a subclone of cells that already were affected by \textit{CDKN2A} LOH. Additional mutation analysis showed that 7 of 13 (54%) analyzed melanomas tested positive for the \textit{BRAF}^{V600E} mutation. Most analyzed melanoma samples were positive for a \textit{TERT} promoter mutation (Figure 2d, Supplementary Table S1). These data show \textit{CDKN2A} LOH as a common event in patients with familial melanoma, leading to biallelic inactivation of \textit{CDKN2A}. Moreover, in melanoma, \textit{CDKN2A} LOH may be followed by loss at 9q, possibly indicating additional targets on chromosome 9 in melanoma development.

Biallelic \textit{CDKN2A} inactivation may be more prevalent through intragenic

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{SNP-specific dPCR analysis of \textit{CDKN2A}. (a) Diagram of the \textit{CDKN2A} locus and two common SNPs analyzed, one located in intron 1 of \textit{CDKN2A}, 2 kb upstream the \textit{p16-Leiden} mutation (rs2811708) and a second SNP (rs3731257) at 4 kb downstream the \textit{p16-Leiden} mutation. (b) Example of genotyping from heterozygous \textit{p16-Leiden} carriers for rs2811708 [G/T] and rs3731257 [C/T] and a homozygous \textit{p16-Leiden} carrier who was also homozygous for rs2811708-[T] and rs3731257-[C]. (c) Schematic of linkage between rs3731257-[C] and rs2811708-[T] allele with the \textit{p16-Leiden} mutation. (d) Histopathology of common melanocytic nevus 1071 from a \textit{p16-Leiden} mutation carrier. Bar = 200 \textmu m. bp, base pair; Chr, chromosome; del, deletion; dPCR, digital PCR; kb, kilobase; MT, mutant; SNP, single nucleotide polymorphism; WT, wild-type.}
\end{figure}
mutation and promoter hypermethylation that was not investigated here. The digital PCR method optimized for this study is specifically able to detect gene variants at a single locus, allowing quantification of allelic imbalances and hotspot mutations. It is not suited to simultaneously quantify various mutations distributed over a gene such as \textit{PTEN}. Moreover, our analyses are restricted by small amounts of available DNA from the dissected melanocytic lesions. For many samples, we have insufficient DNA to perform new analysis on a gene, in addition to \textit{CDKN2A}, \textit{BRAF}, and \textit{TERT}. Because of the fact that p16\textsuperscript{INK4A} is not uniformly expressed in nevi and truncated p16\textsuperscript{INK4A} protein encoded by mutant \textit{CDKN2A} is recognized by most antibodies at the same level as p16\textsuperscript{INK4A} wild-type protein, confirmation of p16\textsuperscript{INK4A} loss was not possible at the protein level.

In patients with FAMMM syndrome, \textit{CDKN2A} inactivation can occur at an earlier stage of genomic evolution of melanocytic neoplasia that diverges from sporadic melanoma. The subclonal loss of \textit{CDKN2A} in benign nevi of patients with FAMMM syndrome has similarity to the development of \textit{BAP1}-inactivated melanocytic tumors in patients with \textit{BAP1}-tumor predisposition syndrome (Zhang et al., 2019). Our results suggest a model of genetic events, with the \textit{BRAF}\textsuperscript{V600E} mutation occurring before \textit{CDKN2A} LOH in subclones of cells in nevi. In melanomas, there are additional \textit{TERT} promoter mutations and loss at chromosome 9q indicative of genomic instability. It is plausible that melanocytic nevi harboring subclones of cells with \textit{CDKN2A} LOH might be at a higher risk to develop into melanoma.

Data availability statement
All data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCIDs
Eirini Christodoulou: http://orcid.org/0000-0003-4153-7974
Rogier J. Nell: http://orcid.org/0000-0003-3530-3725
Rob M. Verdijk: http://orcid.org/0000-0003-1437-214X
Nelleke A. Gruis: http://orcid.org/0000-0002-5210-9150
Pieter A. van der Velden: http://orcid.org/0000-0002-5710-9256

Figure 2. Absolute quantification of genetic events in melanocytic neoplasms from patients with FAMMM syndrome. (a) Fractional abundance of rs2811708-T in blood DNA, a negative control FFPE scar sample, and common melanocytic nevi from \textit{p16-Leiden} mutation carriers. (b) Summary of \textit{CDKN2A} LOH (rs2811708), \textit{BRAF}\textsuperscript{V600E} mutation, and 9q LOH (rs4745670) in common melanocytic nevi from patients with FAMMM syndrome (n = 13). (c) Fractional abundance of rs2811708-T in blood DNA, FFPE scar, and melanomas from \textit{p16-Leiden} mutation carriers. (d) Summary of \textit{CDKN2A} LOH (rs2811708), \textit{BRAF}\textsuperscript{V600E} mutation, 9q LOH (rs4745670), and \textit{pTERT} mutation (c.1-146C>T or c.1-124C>T) in melanomas of patients with FAMMM syndrome (n = 14). Error bars represent 95% CIs. Color legend: black = positive (mutant/LOH confirmed), white = negative (wild-type/no LOH), gray = not available (low DNA, low number of droplets). CI, confidence interval; FAMMM, familial atypical multiple mole melanoma; FFPE, formalin-fixed paraffin-embedded; LOH, loss-of-heterozygosity.
TO THE EDITOR

Current risk stratification methods for primary cutaneous melanoma are based on clinicopathologic variables and are limited in their ability to identify patients at risk of metastasis (Morton et al., 2014). It has previously been shown that other ways of characterizing melanoma, such as using the tumor cell adhesion marker integrin β3 through immunohistochemistry (Hieken et al., 1996) or RT-qPCR (Meves et al., 2015), are useful in discriminating between primary cutaneous melanomas that had or had not metastasized, for example, the sentinel lymph nodes (SLNs). We sought to understand whether other molecular markers like the programmed cell death protein-1 (PD-1) and/or programmed cell death-ligand 1 (PD-L1) immune checkpoint are useful in the risk stratification of melanoma.

PD-L1 expression on tumor cells inhibits tumor rejection by engaging the PD-1 receptor on cytotoxic T cells, which inhibits T-cell receptor function.

PD-1, PD-L1, and BIM as Predictors of Sentinel Lymph Node Metastasis in Primary Cutaneous Melanoma

Journal of Investigative Dermatology (2020) 140, 2301–2304; doi:10.1016/j.jid.2020.03.948

TO THE EDITOR

Current risk stratification methods for primary cutaneous melanoma are based on clinicopathologic variables and are limited in their ability to identify patients at risk of metastasis (Morton et al., 2014). It has previously been shown that other ways of characterizing melanoma, such as using the tumor cell adhesion marker integrin β3 through immunohistochemistry (Hieken et al., 1996) or RT-qPCR (Meves et al., 2015), are useful in discriminating between primary cutaneous melanomas that had or had not metastasized, for example, the sentinel lymph nodes (SLNs). We sought to understand whether other molecular markers like the programmed cell death protein-1 (PD-1) and/or programmed cell death-ligand 1 (PD-L1) immune checkpoint are useful in the risk stratification of melanoma.

PD-L1 expression on tumor cells inhibits tumor rejection by engaging the PD-1 receptor on cytotoxic T cells, which inhibits T-cell receptor function.

PD-1, PD-L1, and BIM as Predictors of Sentinel Lymph Node Metastasis in Primary Cutaneous Melanoma

Journal of Investigative Dermatology (2020) 140, 2301–2304; doi:10.1016/j.jid.2020.03.948

TO THE EDITOR

Current risk stratification methods for primary cutaneous melanoma are based on clinicopathologic variables and are limited in their ability to identify patients at risk of metastasis (Morton et al., 2014). It has previously been shown that other ways of characterizing melanoma, such as using the tumor cell adhesion marker integrin β3 through immunohistochemistry (Hieken et al., 1996) or RT-qPCR (Meves et al., 2015), are useful in discriminating between primary cutaneous melanomas that had or had not metastasized, for example, the sentinel lymph nodes (SLNs). We sought to understand whether other molecular markers like the programmed cell death protein-1 (PD-1) and/or programmed cell death-ligand 1 (PD-L1) immune checkpoint are useful in the risk stratification of melanoma.

PD-L1 expression on tumor cells inhibits tumor rejection by engaging the PD-1 receptor on cytotoxic T cells, which inhibits T-cell receptor function.

PD-1, PD-L1, and BIM as Predictors of Sentinel Lymph Node Metastasis in Primary Cutaneous Melanoma

Journal of Investigative Dermatology (2020) 140, 2301–2304; doi:10.1016/j.jid.2020.03.948

TO THE EDITOR

Current risk stratification methods for primary cutaneous melanoma are based on clinicopathologic variables and are limited in their ability to identify patients at risk of metastasis (Morton et al., 2014). It has previously been shown that other ways of characterizing melanoma, such as using the tumor cell adhesion marker integrin β3 through immunohistochemistry (Hieken et al., 1996) or RT-qPCR (Meves et al., 2015), are useful in discriminating between primary cutaneous melanomas that had or had not metastasized, for example, the sentinel lymph nodes (SLNs). We sought to understand whether other molecular markers like the programmed cell death protein-1 (PD-1) and/or programmed cell death-ligand 1 (PD-L1) immune checkpoint are useful in the risk stratification of melanoma.

PD-L1 expression on tumor cells inhibits tumor rejection by engaging the PD-1 receptor on cytotoxic T cells, which inhibits T-cell receptor function.

PD-1, PD-L1, and BIM as Predictors of Sentinel Lymph Node Metastasis in Primary Cutaneous Melanoma

Journal of Investigative Dermatology (2020) 140, 2301–2304; doi:10.1016/j.jid.2020.03.948
SUPPLEMENTARY MATERIALS AND METHODS

Study population
This study was performed on a cohort of 20 heterozygous and one homozygous carrier of a germline-inactivating CDKN2A mutation (*p16-Leiden*). Blood DNA from CDKN2A mutation carriers was available for analysis. Tumor DNA was derived from 18 melanoma and 17 common melanocytic nevi (not matched from the same lesion) formalin-fixed paraffin-embedded tissue samples from CDKN2A mutation carriers and extracted by microdissection with the QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) or with the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega, Madison, WI). Because of quality control measurements, the analysis was restricted to 14 melanoma and 13 common melanocytic nevi. The pathological diagnosis of all lesions was made by two melanoma pathologists independently. The common melanocytic nevi did not show morphologically distinct nevus cell subsets.

Digital PCR analysis
A digital PCR assay was designed targeting a common trinucleotide single nucleotide polymorphism (SNP) within the intronic region of CDKN2A, rs2811708, located 2 kilobases upstream from the 19–base pair deletion (*p16-Leiden* mutation) site (G/AT, chr9:21973422, rs2811708-[T] allele frequency in the Dutch population is 26%). To validate the assay performance and confirm the copy number amplification within the CDKN2A region, a different intronic SNP, rs3731257, located 4 kilobases downstream of the *p16-Leiden* mutation site was amplified by digital PCR (C/T, chr9:21966221, rs3731257-[T], allele frequency in the Dutch population is 26%). To control for allelic imbalances and losses in 9p21, the 9q region was targeted by amplifying a SNP, rs4745670, located within the GNAQ intronic region (T/A, chr9:80423139, rs4745670-[A] allele frequency in the Dutch population is 70%) (Boomsma et al., 2014) (Supplementary Table S3). This SNP-based digital PCR approach follows the design guidelines of a mutation-specific digital PCR reaction as described previously (Versluis et al., 2015).

Sanger Sequencing analysis long run (BaseClear, Leiden, The Netherlands) was performed to validate primer combinations of the different SNP assays, and chromatograms were analyzed using Chromas software (Technelysium, South Brisbane, Australia). The two most frequent TERT promoter mutations (c.1-146C>T and c.1-124C>T) and the *BRAF* V600E mutation were examined using mutation detection digital PCR assays predesigned by Bio-Rad ( Hercules, CA). Raw digital PCR results were acquired using QuantaSoft (version 1.7.4, Bio-Rad) and imported in online digital PCR management and analysis application Roodcom WebAnalysis (version 1.9.4, available via https://webanalysis.roodcom.nl). The fractional abundance (%) of the alteration of interest (CDKN2A loss-of-heterozygosity [LOH], TERT promoter, 9q LOH, and *BRAF* V600E) was calculated by dividing the mutant allele counts over the total allele counts. The mutant allele fraction was determined and multiplied by two to obtain the mutant cell fraction:

$$\text{% cells with mutation} = \frac{2 \cdot \text{[mutation]}}{\text{[mutation] + [wildtype]}}$$

Allelic SNP imbalances were analyzed using in-house developed digital PCR assays (Supplementary Table S3). Assuming the allele linked to the *p16-Leiden* deletion remains stable, the copy number value (average number of total CDKN2A alleles per cell) was calculated as follows:

$$\text{CNV} = 1 + \frac{\text{Var}_{\text{unlinked}}}{\text{Var}_{\text{linked}}}$$

A copy number significantly lower than 2 was interpreted as being the sum of normal cells (copy number value = 2) and cells with LOH (copy number value = 1). The presence of LOH was therefore determined as follows:

$$\text{% cells with LOH} = (2 - \text{CNV}) \cdot 100\%$$

For the 9q SNP (rs4745670), the variant with the highest concentration was assumed to be stable and the fraction of cells with LOH was determined as described.
Supplementary Figure S1. Validation of allelic imbalances at the CDKN2A locus in melanocytic neoplasms of patients with FAMMM syndrome. (a) Absolute quantification of rs2811708-[T] and rs3731257-[C] fractional abundance in blood DNA, common melanocytic nevi, and melanomas. (b) Absolute quantification of a copy number control SNP, rs4745670, located within the intronic region of 9q and fractional abundance of [A] allele in informative (heterozygous for SNP) blood DNA, common melanocytic nevi, and melanomas. FAMMM, familial atypical multiple mole melanoma; SNP, single nucleotide polymorphism.
Supplementary Figure S2. Summary of genetic events in common melanocytic nevi and primary melanomas. All values represent estimates of the fraction of cells having a certain alteration, relative to the complete sample (all cells). This is either a mutation of BRAFV600E and pTEFT (c.1-146C>T or c.1-124C>T) or LOH for CDKN2A (rs2811708) and 9q loss (rs4745670). (a) Common melanocytic nevus samples 1065 and 1071 (b) Primary melanoma 1023. Error bars represent 95% CIs based on Poisson statistics. CI, confidence interval; LOH, loss-of-heterozygosity.