Genetic dependencies in hereditary and sporadic melanoma

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Chapter 4

Loss of wild-type *CDKN2A* is an early event in the development of melanoma in FAMMM syndrome

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ABSTRACT

The development of melanoma involves a sequence of genetic and epigenetic alterations. *CDKN2A* is a key tumor suppressor gene that is commonly inactivated in invasive melanoma, but not in benign precursor lesions. Heterozygous germline mutations in *CDKN2A* cause hereditary melanoma, also termed Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome. The objective of this study was to investigate *CDKN2A* loss-of-heterozygosity (LOH) in melanocytic neoplasms of FAMMM syndrome patients. Here we applied digital PCR methodology for absolute quantification of allelic imbalance of SNPs at the *CDKN2A* locus. Allelic imbalance consistent with *CDKN2A* LOH was observed in 9/14 (64%) primary melanomas. Remarkably, *CDKN2A* LOH was present in 7/13 (54%) common melanocytic nevi with no histopathological atypia. Digital PCR provided insight into tumor heterogeneity and the order of genetic events by quantification of *CDKN2A* LOH relative to $BRAF^{V600E}$, *TERT* promoter mutation and chromosome 9q loss. In nevi, a subclonal fraction of cells demonstrated *CDKN2A* LOH and this genetic event occurred subsequent to *BRAF* mutation. *TERT* promoter mutation and loss at chromosome 9q were observed later in melanoma development of FAMMM syndrome patients. In FAMMM syndrome patients *CDKN2A* inactivation can occur at an earlier stage of genomic evolution of melanocytic neoplasia.

INTRODUCTION

Approximately 10% of patients diagnosed with melanoma report a positive family history of this aggressive cutaneous malignancy. Familial or hereditary melanoma is arbitrarily defined as the occurrence of three or more cases of melanoma within a family [1]. At least a third of melanoma families are caused by germline heterozygous mutations of the *CDKN2A* gene [2]. Hereditary melanoma due to germline *CDKN2A* mutation is designated familial atypical multiple mole melanoma syndrome (FAMMM syndrome) [3]. *CDKN2A*-mutation carriers have an estimated 70% lifetime risk of developing melanoma and are at increased risk for pancreatic cancer, head and neck cancer as well as other tumor types [4, 5]. This dominant high penetrance melanoma susceptibility gene, encodes for two tumor suppressor proteins that are translated in alternate reading frames from the alpha and beta transcript [6]. The larger α transcript encodes for $p16^{INK4A}$, a protein that mediates G1 arrest by inhibiting the phosphorylation of Cyclin-D1-CDK4/6 complex [7, 8]. The smaller β transcript encodes for $p14^{ARF}$ which inhibits MDM2, thereby promoting p53 activity [9, 10].

In carriers of germline *CDKN2A* mutations, the wild-type allele is functionally inactivated in melanoma by a second somatic event, commonly through deletion [11]. The importance of *CDKN2A* as a tumor suppressor gene is underscored by the high frequency of somatic mutation, chromosomal deletion and promoter hypermethylation, estimated at 40%, in sporadic melanoma [12, 13]. In the Netherlands, a specific founder mutation, a 19-bp deletion in exon 2 of the *CDKN2A* gene (c.225_243del, p.(A76Cfs*64)) known as the *p16-Leiden* mutation, is the most frequent cause of hereditary melanoma [14]. Loss of $p16^{INK4A}$ function, that occurs in hereditary as well as sporadic melanoma, not only disrupts the G1 cell cycle checkpoint, but also promotes invasive behavior of melanoma cells due to deregulation of the BRN2 transcription factor [15].

The most frequent de-regulated pathway in melanocytic transformation is the mitogen-activated protein kinase (MAPK) pathway [16]. The two most common mutated genes are *BRAF* and *NRAS* occurring in a mutually exclusive pattern. In melanomas of germline *CDKN2A* mutation carriers *BRAF* and *NRAS* mutations were reported in 43% and 11% respectively [17]. In addition, the genetic landscape of sporadic melanomas frequently involves upregulation of telomerase reverse transcriptase (*TERT*) expression through promoter mutation in primary melanoma [16]. *TERT* promoter mutations create ETS/TCF transcription factor binding motifs that increase *TERT* gene expression. These mutations can be found in both *BRAF* and *NRAS* mutant cases with the majority occurring at two hotspots [18, 19]. In sporadic melanoma, somatic mutations typically sequentially induce MAPK pathway activation (*BRAF, NRAS*), upregulation of telomerase

(*TERT*) and disruption of the G1/S cell cycle checkpoint (*CDKN2A*) in addition to many other pathogenic alterations [16]. Bi-allelic loss of the *CDKN2A* locus is the most common genetic alteration distinguishing melanocytic nevi from invasive melanomas [19]. *CDKN2A* LOH has been previously demonstrated in primary and metastatic melanomas of FAMMM syndrome patients [20, 21]. The timing of wild-type *CDKN2A* inactivation in the development and progression of hereditary melanoma due to germline *CDKN2A* mutation remains to be resolved.

To evaluate the occurrence and timing of somatic events in melanoma-genesis for FAMMM syndrome patients, we developed an innovative digital PCR (dPCR) method allowing accurate quantification of somatic wild-type *CDKN2A* allele loss in patients' tumors including melanomas and common melanocytic nevi. Moreover, dPCR was used to quantify presence of $BRAF^{V600E}$ mutation and the two most frequent *TERT* promoter mutations.

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 (FFPE) tissue samples from *CDKN2A* mutation carriers and extracted by macrodissection with the QIAamp DNA Micro Kit (Qiagen) or with the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega, Leiden, The Netherlands). Due to 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 (dPCR) analysis

A digital PCR assay was designed targeting a common tri-nucleotide single nucleotide polymorphism (SNP) within the intronic region of *CDKN2A*, rs2811708, located 2kb upstream the 19-bp deletion (*p16-Leiden* mutation) site (G/A/T, chr9:21973422, rs2811708-[T] AF in the Dutch population is 26%, [19]) (Table 1). To validate the assay performance and confirm the copy number amplification within *CDKN2A* region, a different intronic SNP, rs3731257, located 4kb downstream the *p16-Leiden* mutation site was amplified by dPCR (G/A, chr9:21966221, rs3731257-A, AF in the Dutch population is 26% [19]) (Table 1, Figure 1). To control for allelic imbalance and loss in 9p21, the 9q region was targeted by amplifying a SNP, rs4745670, located within *GNAQ* intronic region (T/A, chr9:80423139, rs4745670-[A] AF in the Dutch population is 70% [19]) (Table 1). This SNP-based digital PCR approach follows the design guidelines of a mutation specific digital PCR reaction as described previously [52].

Sanger Sequencing analysis long-run (BaseClear, Leiden, The Netherlands) (Table 1) 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 dPCR assays predesigned by Bio-Rad (Hercules, California, United States). Raw digital PCR results were acquired using *QuantaSoft* (version 1.7.4, Bio-Rad Laboratories) and imported in an 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* 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:

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

Allelic SNP imbalance was analyzed using in-house developed digital PCR assays (Table 1). 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:

$$CNV = 1 + \frac{[var_{unlinked}]}{[var_{linked}]}$$

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

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

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 above.

Table 1 Primer/Probe combinations used for digital PCR and sanger sequencing analyses

Primer/Probe name	Sequence 5'to 3'
CDKN2A_rs2811708_F	ACCAATGTAGTTAGGATTCTAAGCCA
CDKN2A_rs2811708_R	AGGAAAAAGAAAAGTGGATAGTTTTGA
variant_rs2811_probe	TGTTCTTCTCCCTTCTCCATTA-FAM
normal_rs2811_probe	TGTTCTTCTCCCGTCTCCATTA-HEX
CDKN2A_rs3731257_F	TTGGTTCAGCACTCACTTGG
CDKN2A_rs3731257_R	AGCAAAAGCTGCCAGAATTG
variant_rs3731257_probe	AAACTTTCTTATTGTTTCCCAGAG-FAM
normal_rs3731257_probe	AAACTTTCTTACTGTTTCCCAGAG-HEX
9q_rs4745670_F	AAGAGCTTTCTGAAAGGGGAA
9q_rs4745670_R	AGCCCTGCATTTGTCTTCCT
variant_rs4745670_probe	CATCTTAGAATCATCAGCATTCACC-FAM
normal_rs4745670_probe	CATCTTAGAAACATCAGCATTCACC-HEX

RESULTS

A Single Nucleotide Polymorphism (SNP)-based digital PCR approach to detect allelic imbalance in FAMMM syndrome patients

Detection of a 19 bp-deletion (*p16-Leiden* mutation) in degraded DNA derived from formalin-fixed paraffin embedded (FFPE) tissue samples turned out to be troublesome, also due to the size difference between wild-type and mutant *CDKN2A* target that caused an amplification bias (Supplemental Figure 1). Therefore, an approach was developed for accurate copy number determination through SNP-specific dPCR technology [22]. The SNP rs2811708 located in intron 1 of the *CDKN2A* gene and 2kb upstream of the *p16-Leiden* mutation had a MAF of 26% in the Dutch population (Figure 1A) [23]. A SNP located 4kb downstream the *p16-Leiden* mutation, rs3731257, was included to confirm the presence of allelic imbalance within 9p21 and had a MAF in the Dutch population of 26%, (Figure 1A) [23]. To investigate the presence of deletions across chromosome 9 or gross genomic instability in melanomas, a SNP at chromosome 9q (rs4745670) located in an intron of the *GNAQ* gene was included with MAF in the Dutch population of 70% (Figure 1A) [23].

To distinguish the different genotypes of common SNPs located within the 9p21 locus (rs2811708 and rs3731257) and rs4745670 located at 9q, capillary sequencing analysis was performed on blood DNA of *p16-Leiden* mutation carriers (Figure 1B). The minor variant allele rs2811708-[T], was found to be linked to this pathogenic germline variant (Figure 1C). This was concluded from analyzing all available *p16-Leiden* carriers and specifically from homozygous carriers of the *p16-Leiden* allele who were also homozygous for rs2811708-[T] (Figure 1B and C). For the confirmative SNP, rs3731257-[C] was present in all *p16-Leiden* carriers and homozygous carriers of the *p16-Leiden* allele were also homozygous for rs3731257-[C] (Figure 1B). This shows that the normal allele [C] was linked to the *p16-Leiden* mutation (Figure 1C).

Since we confirmed linkage of the different SNPs to the *p16-Leiden* mutation and included a common SNP on the 9q arm as a control, this SNP-based digital PCR approach allowed for quantification of *CDKN2A* allelic imbalance and loss in tissues of FAMMM syndrome patients.

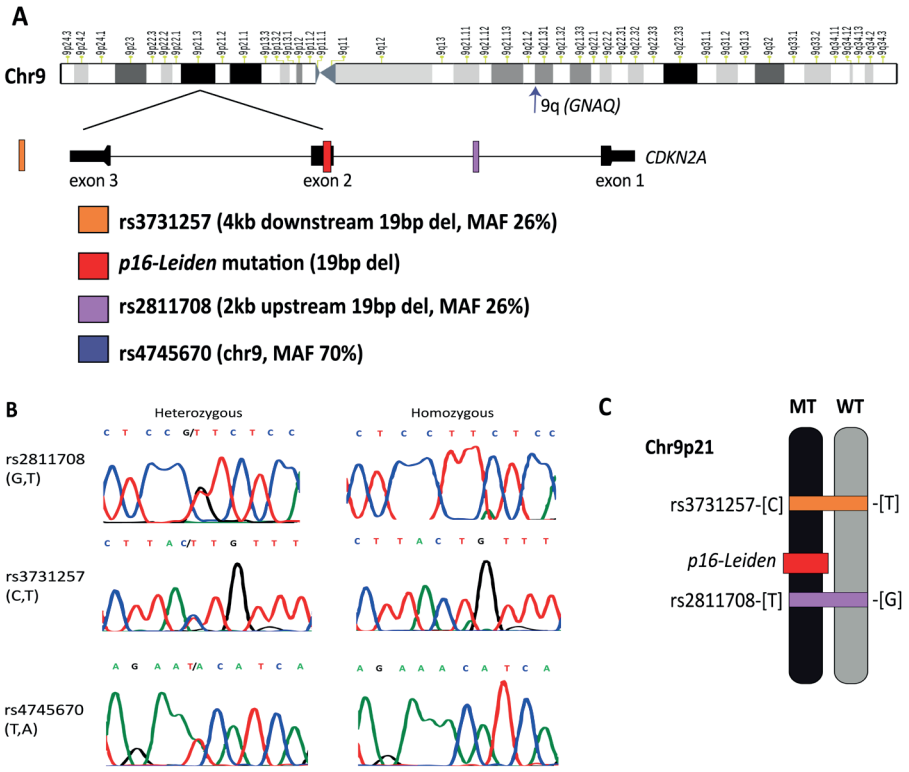

Figure 1 SNP-specific dPCR analysis schematic A) Schematic of the genomic location used to design the digital PCR (dPCR) assays amplifying three common SNPs within chromosome 9: one located in intron 1 of *CDKN2A*, 2kb upstream the *p16-Leiden* mutation (rs2811708) and a second SNP located within the intronic region of 9p21 (rs3731257) at 4kb downstream the *p16-Leiden* mutation. A third SNP was located within the intronic region of 9q (rs4745670) and was used as a control. **B)** Example of genotype from a heterozygous individual for rs2811708 [G/T], rs3731257 [C/T] and rs4745670 [T/A] and a homozygous *p16-Leiden* carrier who was also homozygous for rs2811708-[T], rs3731257-[C] and rs4745670-[A]. **C)** Schematic of linkage between rs3731257-[C] and rs2811708-[T] allele to the *p16-Leiden* mutation (WT- wild-type, MT-mutant).

Loss-of-heterozygosity (LOH) of the CDKN2A locus in melanomas from FAMMM syndrome patients

After having confirmed the specificity of our digital PCR assays, we analyzed melanoma lesions from *CDKN2A* mutation carriers to study allelic imbalance (Figure 2A, Supplemental table 1). The pathological diagnosis of all lesions was made by two melanoma pathologists independently. Absolute quantification showed that in blood DNA of a *CDKN2A* mutation carrier there was 50% fractional abundance of an upstream SNP adjacent to the *CDKN2A* locus (rs2811708) consistent with heterozygosity (Figure 2A). Loss of wild-type *CDKN2A* allele was detected in 9/14 (64%) melanomas of FAMMM syndrome patients by the upstream SNP (rs2811708) (Figure 2). Additional mutation analysis in melanomas showed that 7/14 (50%) tested positive for a $BRAF^{V600E}$

mutation and 6/14 (43%) tested positive for a *TERT* promoter mutation (Figure 2B). Allelic imbalance within 9p21 locus was validated by the fractional abundance of the downstream SNP (rs3731257) in informative (heterozygous for SNP) cases (Figure 3A). These data show wild-type *CDKN2A* LOH as a common event in patients with familial melanoma, leading to the bi-allelic inactivation of *CDKN2A*.

In order to investigate loss at chromosome 9, we quantified a common SNP on the 9q arm (rs4745670). We found that 5/14 (36%) of melanoma had a significant imbalance in amplification (Supplemental figure 2A, Figure 2B) indicating additional deletions across chromosome 9, an event that has been reported previously in sporadic and familial melanomas [24]. Since we found wild-type *CDKN2A* loss to be an early event, in a similar proportion of cells as the *BRAF*^{V600E} mutation, we investigated the frequency of *CDKN2A* allelic imbalance in common melanocytic nevi.

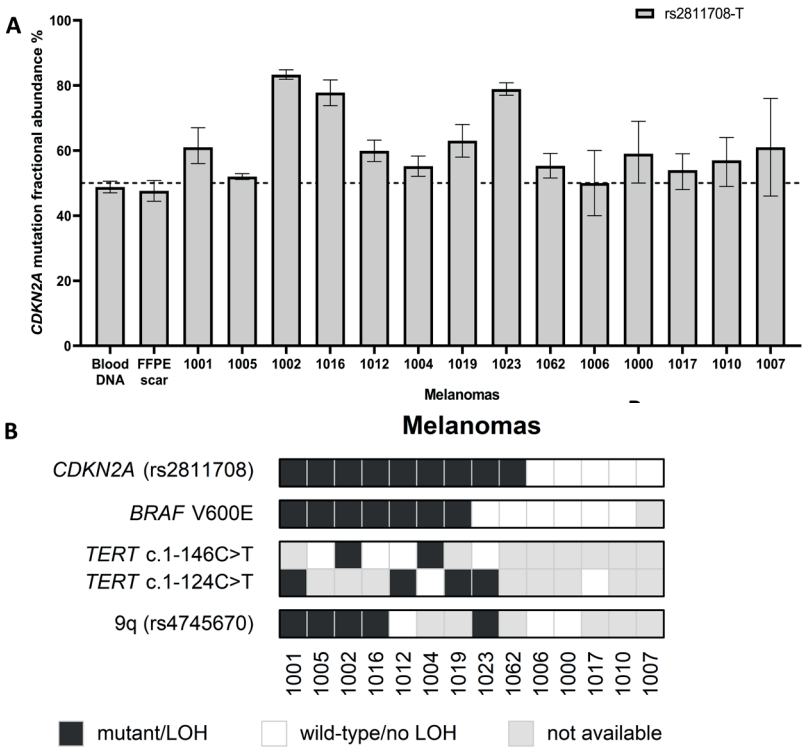

Figure 2 Absolute quantification of genetic events in melanoma lesions from FAMMM syndrome patients. A) Absolute quantification of rs2811708-[T] fractional abundance in blood DNA and primary melanoma lesions from *p16-Leiden* mutation carriers. Error bars represent the 95% confidence intervals (CIs) based on Poisson statistics **B)** Summary of *CDKN2A* loss-of-heterozygosity (LOH) (rs2811708), *BRAF*^{V600E} mutation, 9q LOH (rs4745670) and p*TERT* mutation (c.1-146C>T or c.1-124C>T) in melanomas of FAMMM syndrome patients (n=14). Color legend = positive (mutant or LOH confirmed), white = negative (wild-type or no LOH), grey = not available (not informative, low DNA, low number of droplets or not analyzed).

CDKN2A LOH in common melanocytic nevi from FAMMM syndrome patients

Given the high absolute abundance of loss of wild-type *CDKN2A* in melanomas, we analyzed allelic imbalance in common melanocytic nevi from FAMMM syndrome patients (Supplemental table 1). It is well established that the *CDKN2A* locus remains intact in nevi, whereas homozygous deletion is a common event in sporadic melanomas and is the main cause of inactivation of this tumor suppressor gene [19]. We chose to analyze common, benign nevi without clinical or histopathological atypia, because dysplastic nevi as intermediate lesions can be difficult to distinguish from early stage melanoma.

CDKN2A LOH has been previously shown at primary melanoma and metastasis stage of FAMMM syndrome patients [20, 21]. Remarkably, in this study loss of the wild-type *CDKN2A* allele was detected in 7/13 (54%) common melanocytic nevi from FAMMM

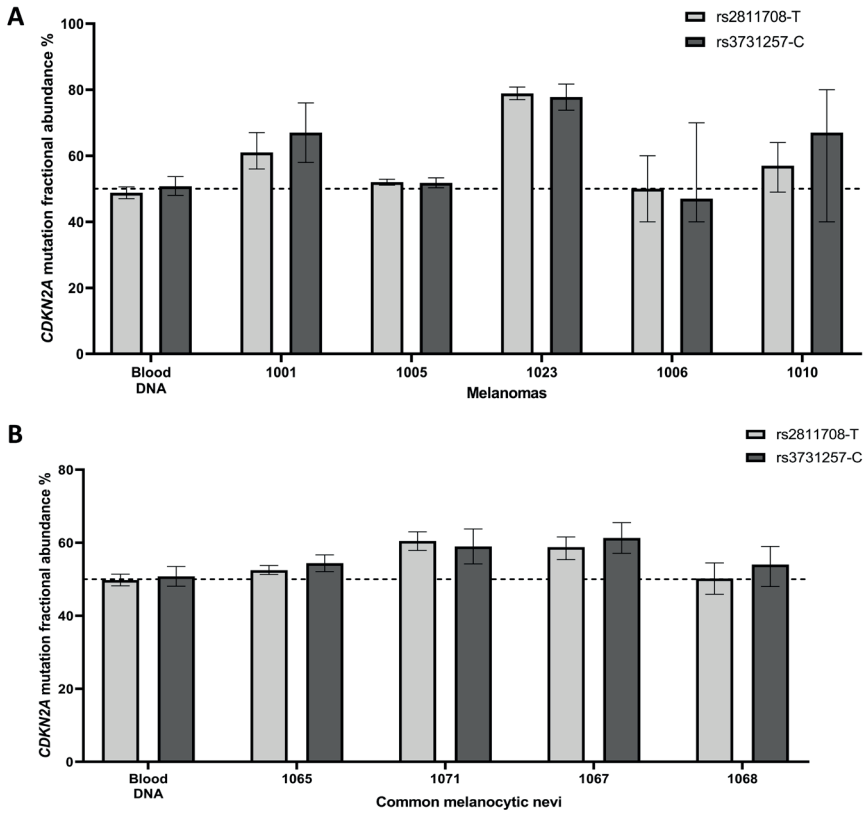

Figure 3 Validation of allelic imbalance within 9p21 locus in melanomas and common melanocytic nevi. Absolute quantification of rs2811708-[T] and rs3731257-[C] fractional abundance in blood DNA and **A)** Primary melanoma lesions **B)** Common melanocytic nevi

syndrome patients, based on absolute quantification of rs2811708 allelic imbalance (Figure 4A). In addition, 8/13 (62%) common nevi tested positive for *BRAF*^{V600E} mutation and five *BRAF* mutant nevi showed significant *CDKN2A* LOH (Figure 4B). As for the melanoma samples, in all informative nevus samples, allelic imbalance of rs3731257 located downstream of *CDKN2A* confirmed loss of the entire *CDKN2A* locus (Figure 3B). We did not detect allelic imbalance at 9q (*GNAQ* locus) in any of the nine tested nevi (Supplemental figure 2B, Figure 4B). The identification of subclonal *CDKN2A* allelic imbalance in common melanocytic nevi instigated histopathological re-evaluation of the lesions which confirmed absence of cytonuclear or tissue architectural atypia (Supplemental figure 3). The clinical and pathological characteristics do not distinguish nevi with *CDKN2A* LOH and nevi without LOH (Supplemental Table 2). Collectively, these data demonstrate bi-allelic loss of *CDKN2A* already at the common melanocytic nevus stage in *CDKN2A* mutation carriers.

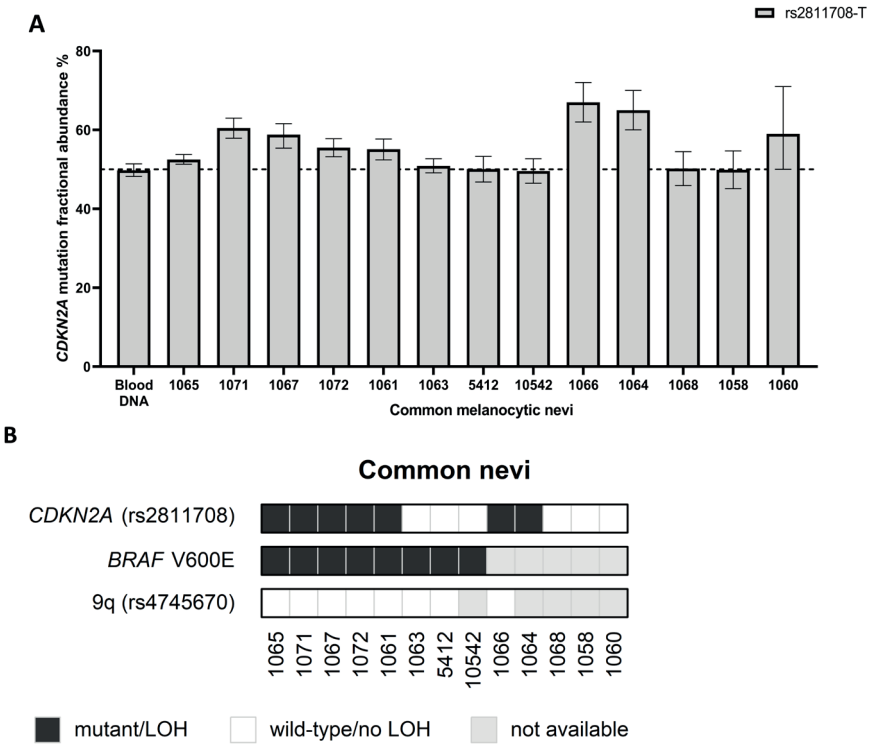

Figure 4 Absolute quantification of genetic events in common melanocytic nevi from FAMMM syndrome patients. A) Absolute quantification of rs2811708-[T] fractional abundance in blood DNA and common melanocytic nevi from *p16-Leiden* mutation carriers. Error bars represent the 95% CIs based on Poisson statistics **B)** Summary of *CDKN2A* LOH (rs2811708), *BRAF*^{V600E} mutation and 9q LOH (rs4745670) in common melanocytic nevi from FAMMM syndrome patients (n=13). Color legend = positive (mutant or LOH confirmed), white = negative (wild-type or no LOH), grey = not available (low DNA, low number of droplets, not analyzed).

Genetic events in nevi and melanomas of FAMMM syndrome patients

Based on combined absolute quantification of mutations and losses, we attempted to deduce the order of genetic events in melanoma and common melanocytic nevi from patients with FAMMM syndrome. In *BRAF* mutant melanocytic nevi, *CDKN2A* LOH was detected in a significantly smaller proportion of the cells than the *BRAF*^{V600E} mutation (Figure 5A, Supplemental Table 1). This suggests that *BRAF* mutation occurs prior to *CDKN2A* LOH. We found no deletions at chromosome 9q in the nevi.

The *BRAF* mutant melanoma biopsy samples contained between 21% and 87% of cells harboring a *BRAF*^{V600E} mutation, due to different proportions of admixed resident and infiltrating cells. Copy number alterations for *BRAF* and *TERT* were observed in a single melanoma lesion that was positive for *CDKN2A* LOH (Supplementary Table 1). The cell fraction harboring *BRAF*^{V600E} mutation, *CDKN2A* LOH and *TERT* mutation was similar in all investigated samples, precluding determination of the order of these ubiquitous genetic events (Figure 5B, Supplemental Table 1). In five melanoma samples, allelic imbalance at 9q (rs4745670) was found; in one case (1023), the fractional abundance of this SNP was significantly lower than that of the *CDKN2A* SNPs (Figure 5B), showing that loss of chromosome 9q occurs in a subclone of cells that already were affected by loss of wild-type *CDKN2A*.

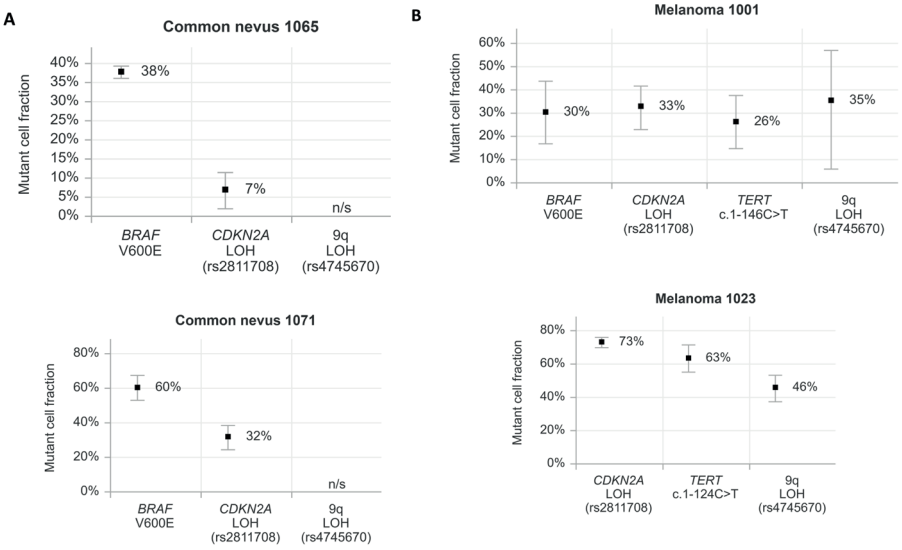

Figure 5 Summary of genetic events in common melanocytic nevi and primary melanomas. All values represent estimates on the fraction of cells having a certain alteration, relative to the complete sample (all cells). This is either a mutation of *BRAF*^{V600E} and p*TERT* (c.1-146C>T or c.1-124C>T) or loss-of-heterozygosity (LOH) for *CDKN2A* (rs2811708) and 9q (rs4745670) **A)** In common melanocytic nevus samples 1065 and 1071 **B)** In primary melanoma samples 1001 and 1023. Error bars represent 95% CIs based on Poisson statistics.

DISCUSSION

The genetic evolution of melanoma is characterized by key oncogenic drivers mainly involving the *BRAF, NRAS, TERT, CDKN2A* and *PTEN* genes [15]. Bi-allelic inactivation of *CDKN2A* is a common event in sporadic melanomas, with deletion being the main genetic mechanism [19]. Studies in primary human melanocytes have also revealed that engineering of *CDKN2A* deletions confer migratory and invasive phenotypes [15]. Homozygous deletion of *CDKN2A* has been reported in a small proportion of sporadic dysplastic nevi but never in common melanocytic nevi [15, 25]. In familial melanoma, *CDKN2A* LOH has been previously reported in primary and metastatic lesions of germline *CDKN2A* mutation carriers [20, 21].

Digital PCR assays targeting SNPs in close proximity to the *p16-Leiden* mutation site were developed to provide precise quantification of allelic imbalance in melanoma and common melanocytic nevus FFPE tissue samples to study LOH of the *CDKN2A* gene. In all informative cases, the fractional abundance of the SNP, located downstream of *CDKN2A*, confirmed the allelic imbalance detected using the SNP located upstream this locus.

Loss of the *CDKN2A* wild-type allele was observed in 9 of 14 (64%) melanomas in FAMMM syndrome. Bi-allelic *CDKN2A* inactivation may be more prevalent, as intragenic mutation and promoter hypermethylation may result in functional inactivation in addition to LOH that was investigated here. The dPCR 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 *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 *CDKN2A*, *BRAF* and *TERT*.

Remarkably, *CDKN2A* LOH was detected in 7 of 13 (54%) common melanocytic nevi of FAMMM syndrome patients. In melanoma, allelic imbalance at the *CDKN2A* locus occurred in all cells carrying a $BRAF^{V600E}$ mutation. By contrast, in common nevi, loss of the wild-type *CDKN2A* allele occurred in a subclone of cells that had acquired $BRAF^{V600E}$ mutation at an earlier stage of its development. Subclonal loss of the wild-type *CDKN2A* allele in melanocytic nevi was not associated with histopathological alterations; the lesions showed no tissue architectural or cytonuclear atypia. It is plausible that nevi containing subclones with bi-allelic inactivation of *CDKN2A* might be at higher risk to develop into melanoma. *TERT* promoter mutations could be detected in a subset of melanoma samples of FAMMM syndrome patients, but were not found in the few melanocytic nevi that were investigated.

In a proportion of melanocytic nevi of patients with *BAP1* tumor predisposition syndrome, immunohistochemical analysis shows loss of *BAP1* expression, commonly in a regional part of the lesion [26]. These *BAP1*-deficient melanocytic nevi, also termed melanocytic *BAP1*-associated intradermal tumors (MBAITs) or BAP-omas usually show clinical and histopathological atypia [26]. Due to the fact that $p16^{INK4A}$ is not uniformly expressed in nevi and truncated $p16^{INK4A}$ protein encoded by mutant *CDKN2A* is recognized by most antibodies, at the same level as $p16^{INK4A}$ wild-type protein, confirmation of $p16^{INK4A}$ -loss was not possible at the protein level.

This study presents for the first time, *CDKN2A* LOH as an early event in melanoma evolution of FAMMM syndrome patients, relative to other driver events (*BRAF, TERT*). Our quantitative data suggest that chromosome 9 disruption in hereditary melanoma consists of two steps; firstly by focal deletion of the *CDKN2A* locus and secondly by a deletion of chromosome 9 in melanomas. In conclusion, the absolute quantification of allelic imbalance using digital PCR has wider applications in determining the genomic evolution of melanoma and other tumor types. In contrast to sporadic melanoma, *CDKN2A* LOH may occur in a subclone of melanocytes that have acquired a mutation in *BRAF* during nevogenesis. We speculate that subclones of nevus cells with *CDKN2A* LOH are prone to progress to melanoma.

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SUPPLEMENTAL MATERIAL

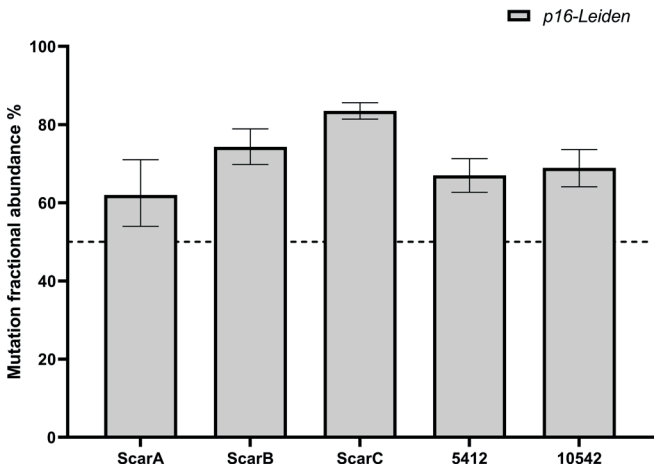
Supplemental Table 1 Summary of estimates on the fraction of cells having a certain alteration, relative to the complete sample (all cells). These include *BRAF*^{V600E} mutation, *CDKN2A* LOH (rs2811708), *CDKN2A* LOH (rs3731257), 9q LOH (rs4745670) and p*TERT* mutation (c.1-146C>T or c.1-124C>T). Benign nevi (n=13) and primary melanomas (n=14) (wild-type- WT, not significant- n/s, Not informative or homozygous for SNP- n/i, Not reliably measurable i.e <=5 droplets positive- n/m, copy number amplification- CNA).

Group	Clinical no	BRAF V600E	rs2811	rs3731	rs4745	TERT C250T	TERT C228T
SCAR	4049	WT	n/s	n/i	n/s	n/a	n/a
SCAR	3749	WT	n/s	n/i	n/i	n/a	n/a
BENIGN NEVUS	1063	28%	n/s	n/i	n/s	WT	n/a
BENIGN NEVUS	1065	38%	7%	16%	n/s	n/a	n/a
BENIGN NEVUS	1072	44%	20%	n/i	n/s	WT	n/a
BENIGN NEVUS	1061	47%	18%	n/i	n/s	n/a	n/a
BENIGN NEVUS	1071	60%	32%	31%	n/s	WT	n/a
BENIGN NEVUS	10542	12%	n/s	n/i	n/i	n/a	n/a
BENIGN NEVUS	1067	42%	29%	37%	n/s	WT	n/a
BENIGN NEVUS	1068	n/m	n/s	n/s	n/m	n/a	n/a
BENIGN NEVUS	1058	n/m	n/s	n/i	n/m	WT	n/a
BENIGN NEVUS	1060	n/m	n/s	n/m	n/m	n/a	n/a
BENIGN NEVUS	1066	n/m	51%	n/i	n/s	n/a	n/a
BENIGN NEVUS	1064	n/m	46%	n/i	n/i	n/a	n/a
BENIGN NEVUS	5412	9%	n/s	n/i	n/s	n/a	n/a
MELANOMA	1002	120% (CNA)	80%	n/i	72%	156% (CNA)	n/a
MELANOMA	1004	21%	20%	n/i	n/i	28%	WT
MELANOMA	1001	30%	33%	51%	35%	n/m	26%
MELANOMA	1012	31%	35%	n/i	n/s	WT	34%
MELANOMA	1005	36%	9%	7%	12%	WT	n/a
MELANOMA	1019	36%	42%	n/i	n/i	n/m	26%
MELANOMA	1016	87%	71%	n/i	55%	WT	n/m
MELANOMA	1007	n/m	n/s	n/m	n/m	n/m	n/m
MELANOMA	1017	WT	n/s	n/i	n/i	n/a	WT
MELANOMA	1006	WT	n/s	n/m	n/s	n/m	n/m
MELANOMA	1000	WT	n/s	n/m	n/s	n/m	n/m
MELANOMA	1010	WT	n/s	n/m	n/i	n/m	n/m
MELANOMA	1023	WT	73%	71%	46%	WT	63%
MELANOMA	1062	WT	14%	n/i	n/i	n/a	n/a

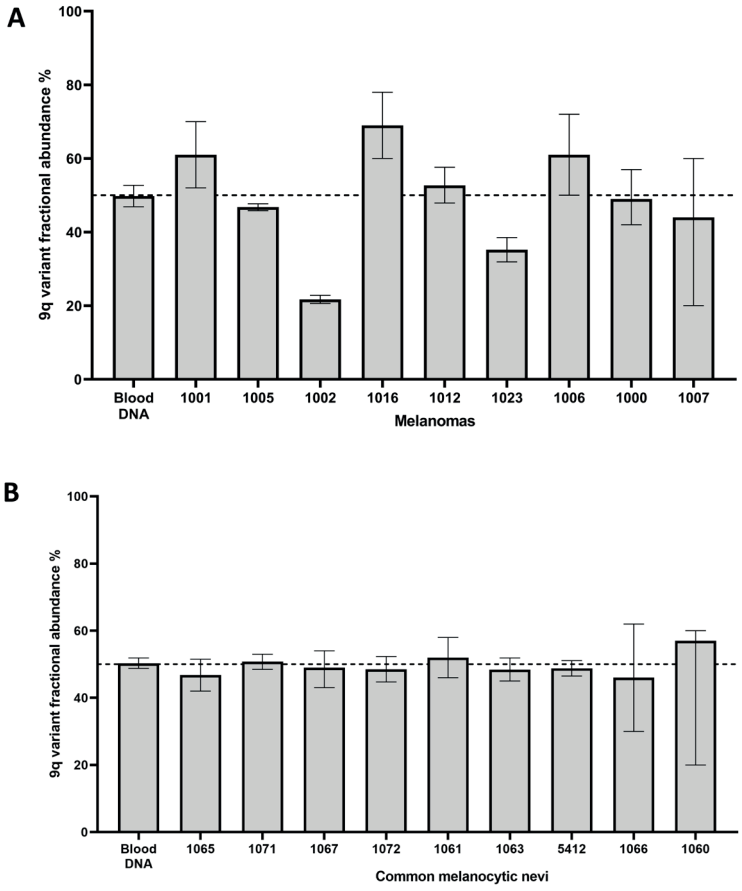
Supplemental Table 2 Clinical and pathological characteristics of benign nevi (BN) (n=13) and melanomas (M) (n=14)

Group (BN/M)	Clinical no	Age	Gender	Diameter (BN)/ Breslow's depth (M)	Anatomical location	Histological type
BN	1063	50	female	6mm	lower back	dermal nevus. No dysplasia.
BN	1065	38	male	7mm	abdomen	compound nevus. No dysplasia.
BN	1072	47	male	7mm	back	junction nevus. No dysplasia.
BN	1061	35	female	6mm	scalp	dermal nevus. No dysplasia.
BN	1071	47	female	7mm	abdomen	compound nevus. No dysplasia.
BN	10542	54	male	6mm	lower back	compound nevus. No dysplasia.
BN	1067	37	female	7mm	flank	junction nevus. No dysplasia.
BN	1068	43	male	8mm	scalp	dermal nevus. No dysplasia.
BN	1058	54	male	6mm	back	compound nevus. No dysplasia.
BN	1060	85	female	9mm	chest	junction nevus. No dysplasia.
BN	1066	55	female	6mm	chest	junction nevus. No dysplasia.
BN	1064	70	female	7mm	upper leg	junction nevus. No dysplasia.
BN	5412	47	male	7mm	back	nevus with junction activity. No dysplasia.
M	1002	43	male	1.4mm	Lower back	nodular melanoma
M	1004	66	female	0.7mm	Upper arm	superficial spreading melanoma
M	1001	58	male	1.0mm	Chest	superficial spreading melanoma
M	1012	45	male	1.4mm	Head	nodular melanoma
M	1005	37	male	0.9mm	Shoulder	superficial spreading melanoma
M	1019	58	female	0.8mm	Upper leg	superficial spreading melanoma
M	1016	52	female	1.2mm	Abdomen	superficial spreading melanoma

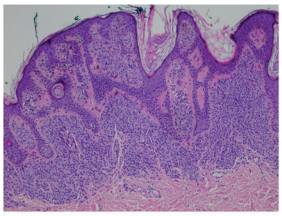
M	1007	57	female	0.8mm	Elbow	superficial spreading melanoma
M	1017	71	male	0.6mm	Upper arm	superficial spreading melanoma
M	1006	35	female	0.8mm	Lower back	superficial spreading melanoma
M	1000	51	female	0.8mm	Shoulder	superficial spreading melanoma
M	1010	44	male	0.8mm	Scalp	superficial spreading melanoma
M	1023	48	female	2.6mm	Lower leg	nodular melanoma
M	1062	69	female	3mm	upper arm	superficial spreading melanoma



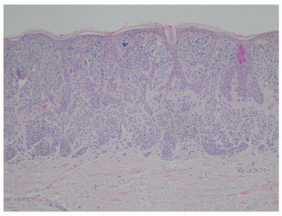
Supplemental figure 1 Absolute quantification of the *p16-Leiden* mutation in scar and common nevi from FAMMM syndrome patients. Fractional abundance of *p16-Leiden* mutation in three scar tissue samples and common nevi 5412 and 10542 from FAMMM syndrome patients. The fractional abundance was significantly higher than 50% in all benign lesions suggesting that this technique is not informative to precisely quantify the mutant cell fraction. Error bars represent 95% CIs based on Poisson statistics. Forward primer: CTGCTGCTGCTCCACG Reverse primer: ACCAGCGTGTCCAGGAAG *p16-Leiden* probe: ACTGCGCCCGACCCGT wild-type probe: ACTCTCACCCGACCCGTG.



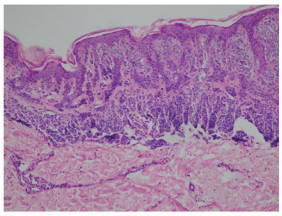
Supplemental Figure 2 Absolute quantification of rs4745670-[A] in melanomas and common melanocytic nevi from FAMMM syndrome patients. Absolute quantification of a copy number control SNP, rs4745670, located within the intronic region of 9q and fractional abundance of [A] allele in informative (heterozygous for SNP) **A)** Primary melanoma lesions and **B)** Common melanocytic nevi. Error bars represent 95% CIs based on Poisson statistics.



Common nevus 1065



Common nevus 1071



Common nevus 1067

Supplemental Figure 3 Histological examination of common melanocytic nevi from *p16-Leiden* mutation carriers. H&E staining of common melanocytic nevi (1065, 1071 and 1067) from *p16-Leiden* mutation carriers that showed loss of wild-type *CDKN2A* allele (magnification= 100x).