Multigene panel sequencing of established and candidate melanoma susceptibility genes in a large cohort of Dutch non-CDKN2A/CDK4 melanoma families

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Germline mutations in the major melanoma susceptibility gene CDKN2A explain genetic predisposition in only 10–40% of melanoma-prone families. In our study we comprehensively characterized 488 melanoma cases from 451 non-CDKN2A/CDK4 families for mutations in 30 established and candidate melanoma susceptibility genes using a custom-designed targeted gene panel approach. We identified (likely) pathogenic variants in established melanoma susceptibility genes in 18 families (n = 3 BAP1, n = 15 MITF p. E318K; diagnostic yield 4.0%). Among the three identified BAP1-families, there were no reported diagnoses of uveal melanoma or malignant mesothelioma. We additionally identified two potentially deleterious missense variants in the telomere maintenance genes ACD and TERF2IP, but none in the POT1 gene. MC1R risk variants were strongly enriched in our familial melanoma cohort compared to healthy controls (R variants: OR 3.67, 95% CI 2.88–4.68, p < 0.001). Several variants of interest were also identified in candidate melanoma susceptibility genes, in particular rare (pathogenic) variants in the albinism gene OCA2 were repeatedly found. We conclude that multigene panel testing for familial melanoma is appropriate considering the additional 4% diagnostic yield in non-CDKN2A/CDK4 families. Our study shows that BAP1 and MITF are important genes to be included in such a diagnostic test.

Key words: familial melanoma, genetic susceptibility, gene panel sequencing, BAP1, MITF, high-penetrance genes, candidate susceptibility genes, OCA2

Abbreviations: CM: cutaneous melanoma; MAF: minor allele frequency; UM: uveal melanoma

Additional Supporting Information may be found in the online version of this article.

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Introduction

Cutaneous melanoma is the most aggressive type of common skin cancers and incidence has been increasing worldwide over the past decades. With an age-standardized rate of 19.4 per 100,000, the Netherlands is among the countries with the highest incidence rates in the world, comparable to incidence rates in the northernmost European (Scandinavian) countries. Well-established personal and environmental risk factors for melanoma include a fair skin type, having (many) atypical nevi, a high level of ultraviolet radiation exposure, and a history of sunburns in childhood. A family history for the disease is also a significant risk factor and suggests a shared genetic predisposition among family members. This familial clustering occurs in approximately 5–10% of melanoma cases, and is referred to as familial melanoma.

The major high-risk susceptibility gene for familial melanoma is CDKN2A and germline mutations are identified in 10–40% of familial cases. In the Netherlands, a specific founder mutation in CDKN2A, known as p16-Leiden (c.225_243del, p.A76Cfs*64; RefSeq NM_000077.4), is the most frequent cause of familial melanoma (~80% of CDKN2A mutations). Carriers of this mutation show not only a markedly increased risk for (multiple) cutaneous melanomas, but also for other cancers, especially pancreatic cancer and cancers of the upper respiratory tract (larynx, pharynx, oral cavity). CDKN2A is an unusual gene in that it encodes two distinct...
proteins, p16INK4a and the alternatively spliced p14ARF, both of which are tumor-suppressors that act in two distinct pathways. The p16-retinoblastoma(Rb)-pathway controls cell-cycle G1-phase exit, while the p14ARF-p53 pathway induces cell cycle arrest or apoptosis.9 Despite the major role of these pathways in melanoma susceptibility, only one other gene in the p16-retinoblastoma(Rb)-pathway, the CDK4 gene, has been shown to be associated with familial melanoma, and only a small number of families with germline mutations in this gene have been identified to date.10

However, new melanoma susceptibility pathways have emerged in recent years.5,6 Several high penetrance genes involved in telomere lengthening (TERT) or telomere maintenance (Shelterin complex: POT1, ACD, TERF2IP) have been identified, and mutations in these genes each account for approximately 1% of familial melanoma predisposition.11–13 Furthermore, germline mutations in the BRCA1-associated protein (BAP1) gene cause a specific cancer predisposition syndrome mainly characterized by an increased susceptibility for uveal melanoma and malignant mesothelioma, but also including cutaneous melanoma, renal cancer, basal cell carcinoma and characteristic skin lesions called atypical Spitz tumors (AST) or melanocytic BAP1–mediated atypical intradermal tumors (MBAIT).14 The MITF gene is a medium penetrance melanoma susceptibility gene and shows incomplete co-segregation with the phenotype. MITF is a basic-helix-loop–helix-leucine zipper transcription factor that has a key function in melanocyte homeostasis. Loss-of-function mutations in this gene cause auditory-pigmentary syndromes, such as Waardenburg syndrome type 2A (MIM #193510). However, a specific missense variant (c.952G>A, p.E318K; RefSeq NM_000248.3) located in a small-ubiquitin-like modifier (SUMO) consensus site impairs the SUMOylation of MITF, which results in a gain-of-function increase in MITF transcriptional activity. Carriers of this variant have an approximately three- to fourfold increased risk for melanoma and are more likely to develop multiple primary melanomas.15 Several other cancers (renal cancer, pancreatic cancer) have also been reported in carriers of this variant.16,17 In addition to these known high- and medium penetrance melanoma susceptibility genes, there are several well-established (common) variants in the lower penetrance MCIR gene that are associated with an increased risk for melanoma in the general population. MCIR encodes the receptor for α-melanocyte stimulating hormone (α-MSH), which plays an important role in skin pigmentation.

Variants in MCIR that are most strongly associated with red hair color (RHC) confer an approximately twofold increased risk for melanoma (R variants), while other variants (r variants) show a weaker association with RHC (non-RHC) and confer a much smaller increase in risk for melanoma.18 It has also been shown that both R and r variants in MCIR act as modifiers of melanoma risk in families with a CDKN2A germline mutation.19 Furthermore, mutations in other cancer susceptibility genes have been recently reported in melanoma families in studies using mainly Whole Exome Sequencing (WES) technologies,20–22 but the exact role of these and other candidate melanoma susceptibility genes in the familial setting remains unclear and requires further evaluation.

Although Dutch melanoma families are well characterized for CDKN2A and CDK4 mutations,23 no large scale investigation has yet been performed to identify (potential) deleterious variants in other established or candidate melanoma susceptibility genes. In the current study, we therefore sequenced a comprehensive panel of 30 (candidate) melanoma susceptibility genes in a large cohort of Dutch melanoma-prone families without a known CDKN2A or CDK4 mutation. Our goal was to determine the frequency of pathogenic variants in established melanoma susceptibility genes and to investigate the role of a broad range of candidate susceptibility genes in familial melanoma.

Patients and Methods

Patient cohort

Both cutaneous melanoma (CM) and uveal melanoma (UM) patients were eligible for inclusion in the study if they had at least one other relative (up to third-degree) with CM and/or UM, and no previously identified pathogenic germline variant in the melanoma core genes CDKN2A or CDK4. Diagnostic sequencing of these two genes was performed at the Laboratory for Diagnostic Genome Analysis (LDGA) at the Department of Clinical Genetics of the Leiden University Medical Centre (LUMC), which has served as the primary sequencing facility for CDKN2A and CDK4 in the Netherlands since 1998. In a small minority of referred families, the CDKN2A gene was only partly sequenced and/or the CDK4 gene was not sequenced. Both genes were included in our research gene panel in order to exclude the presence of pathogenic variants in these genes. The study was approved by the LUMC Ethics Committee (#P15.341) and informed consent was obtained from all included individuals.

We initially selected 500 patients from 460 families for inclusion in the study. After critical re-evaluation of these
families, 11 samples were excluded from the analysis based on failure to meet above mentioned inclusion criteria. In one of these samples, a pathogenic variant in the 5’UTR region of CDKN2A (c.-34G>T) was identified. Another sample was excluded because sequencing was unsuccessful. In total, 488 samples from 451 families remained for analysis (Table 1). Most families had a proband with CM (n = 446) and the majority of these probands had at least one other relative with CM (n = 442 families; n = 478 samples). This familial CM subgroup included 208 two-case families (83% of which consisted of first-degree relatives), 182 three-case families and 52 families with four or more melanoma cases. An additional four probands with CM had one or more relatives with UM, but no CM. The remaining five families had a proband with UM and one or more relatives with UM and/or CM. A control cohort consisted of a total of 449 adult individuals sequenced at the LUMC for a nonmelanoma, nononcogenic indication (MODY; MIM #606391). MODY is an autosomal dominant syndrome, was performed using an in-house developed and stringent post-sequencing annotation pipeline (using BWA-GATK-VEP). Only variants that occurred with a minor allele frequency (MAF) of less than 5% in the 1,000 Genomes variant database were collected and annotated. Subsequent variant filtering and analysis was performed using a second in-house developed variant analysis tool called LOVDplus. Only variants that had an optimal Genotype Quality (GQ) score of 99 (range 0–99) were considered for further interpretation. The obtained sequencing data had an average depth of >1,000 (>99% at least 30x) with horizontal coverage >99%, and were aligned to human reference genome build GRCh37. Variants with an alternate read ratio of <0.2 were excluded.

**Variant selection and interpretation**

We used Alamut® Visual (V.2.9.0, Interactive Biosoftware, Rouen, France) as an *in silico* tool for interpretation of the variants. In the primary filtering step, we selected exonic variants and intronic variants up to 10 nucleotides from the exon-intron junction with a MAF of less than 0.01 in the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/) and Genome of the Netherlands (GoNL; http://nlgenome.nl) public variant databases. Synonymous variants without a possible effect on splicing were excluded. The functional effect of missense variants was predicted by the *in silico* tools SIFT (http://sift.jcvi.org/), Align GVGD (http://agvgd.hci.utah.edu/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and the CADD score (http://cadd.gs.washington.edu/). A further selection of variants of interest (secondary filtering) was based on the following criteria: 1) known pathogenic variants in literature, 2) truncating variants, 3) missense variants with a CADD score >15 and at least two out of three *in silico* protein prediction tools predicting a possible functional effect, 4) in-frame indels, and 5) variants that likely affect splicing (predicted by SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer and Human Splicing Finder, incorporated in Alamut®). Analysis of the POLE gene was confined to variants in the exonuclease-domain (exon 9–14), while analysis of CDK4, TERT, MITF and MC1R was restricted to specific variants known to be associated with an increased melanoma risk. This included the p.R24H and p.R24G variants in CDK4, the c.-57T>G promoter variant in TERT, the p.E318K variant in MITF, and the R and r variants in MC1R. Co-segregation analysis of the detected variants was possible for families in which more than one case was included in the study. Finally, all variants of interest were evaluated using a recently published *in silico* prediction tool, UMD-predictor (http://umdpredictor.eu/). This tool uses a combinatorial approach to predict pathogenicity of coding single nucleotide variants by pooling information at the nucleotide level, the protein level and at the mRNA level, and has an exceptionally good reported performance.

### Table 1. Characteristics of the cohort

| Proband history | Family history | No. of families | No. of samples |
|-----------------|----------------|-----------------|----------------|
| Cutaneous melanoma (CM) | Total no. of CM cases in family | 446 | 483 |
| 1 | 4 | 5 |
| 2 | 208 | 218 |
| 3 | 182 | 198 |
| 4+ | 52 | 62 |
| Total | 446 | 483 |
| Uveal melanoma (UM) | Total no. of UM cases in family | 451 | 488 |
| 1 | 2 | 2 |
| 2 | 3 | 3 |
| Total | 451 | 488 |

1Uveal melanoma was present in all four single-case families (one additional sample included), six two-case families, one three-case family and six families with four or more cases.

2Cutaneous melanoma was present in both single-case families and in one two-case family.

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**Gene selection and sequencing**

A total of 30 genes were selected by a multidisciplinary expert team (TP, RvD, NG, FH, NvdS; July 2016) and incorporated into a custom-designed targeted gene panel. This included nine established melanoma susceptibility genes and an additional 21 candidate genes identified in previous studies (Table 2). Sequencing of all coding exons, including exon-intron boundaries, was performed on the Illumina HiSeq4000 platform to yield 150 basepair, paired-end reads. Targets were captured using a custom-designed, gene panel-specific Agilent SureSelect XT Capture enrichment kit and sequenced using the 200 ng XT protocol. Capture, enrichment and sequencing were performed at the GenomeScan sequencing facility in Leiden (https://www.genomescan.nl/). Subsequent data analysis was performed using our in-house developed set-up for diagnostic next generation sequence (NGS) analysis. In brief, FastQ sequence data was analyzed using an in-house developed and stringent post-sequencing annotation pipeline (using BWA-GATK-VEP).
Results

In our cohort of 488 samples (451 families), a total of 171 variants passed our primary filtering criteria (see Supporting Information). These included 151 exonic variants, of which eight were truncating (four frameshift, four nonsense), 138 missense, three in-frame indels, and two synonymous variants with a possible effect on splicing. The remaining 20 variants were intronic. Of the 171 variants, 44 were novel (not reported in the reference databases ExAC and GoNL), 41 were extremely rare (MAF < 0.0001), 29 were very rare (MAF < 0.001), and the remaining 57 variants were rare (MAF < 0.01). Subsequent filtering resulted in 60 variants of interest in 20 genes (Tables 3–5). These selected variants were only detected in probands with CM and in none of the probands with UM. The MC1R risk variants were separately analyzed (Table 6).

Variants of interest in established melanoma susceptibility genes and shelterin complex genes

We detected two novel splice variants and one novel truncating variant in the BAP1 gene in three probands (0.7% of families) (Table 3). The c.122+1G>T, p.? and c.1730-1G>A, p.? variants are both located in a canonical splice site and are

| Gene        | Full Name                                | Alt. Name | MIM no. | Refs.                  |
|-------------|------------------------------------------|-----------|---------|------------------------|
| Established melanoma susceptibility genes | Reviewed in: Aoude et al.5, Read et al.6 |
| CDKN2A      | Cyclin-Dependent Kinase Inhibitor 2A     |           | 600160  |                        |
| CDK4        | Cyclin-Dependent Kinase 4                |           | 123829  |                        |
| BAP1        | BRCA1-Associated Protein 1               |           | 603089  |                        |
| POT1        | Protection of Telomeres 1                |           | 606478  |                        |
| ACD         | Adrenocortical Dysplasia Homolog         |           |         |                        |
| TERTF2IP    | TERF2-Interacting Protein                |           | 609377  |                        |
| TERT        | Telomerase Reverse Transcriptase         |           | 187270  |                        |
| MIF         | Microphthalmia-Associated Transcription Factor |       | 156845  |                        |
| Low to medium penetrance: |                                   |           |         |                        |
| MC1R        | Melanocortin 1 receptor                  |           | 155555  |                        |
| Shelterin complex candidate genes | Aoude et al.12 |
| TERF1       | Telomeric Repeat-Binding Factor 1        |           | 600951  |                        |
| TERF2       | Telomeric Repeat-Binding Factor 2        |           | 602027  |                        |
| TINF2       | TINF1-Interacting Nuclear Factor 2       |           | 604319  |                        |
| Candidate genes from WES/WGS and GWA studies | Tuominen et al.22, Wadt et al.21 |
| BRIP1       | BRCA1-Interacting Protein 1              |           | 605882  |                        |
| RAD51B      | RAD51 Paralog B                          | RAD51L1   | 602948  | Wadt et al.21          |
| POLE        | DNA Polymerase Epsilon                   |           | 174762  | Aoude et al.20         |
| NEK2        | NIMA-Related Kinase 2                    |           | 604043  | -                      |
| NEK4        | NIMA-Related Kinase 4                    |           | 601959  | -                      |
| NEK10       | NIMA-Related Kinase 10                   |           | -       | -                      |
| NEK11       | NIMA-Related Kinase 11                   |           | -       | -                      |
| DOT1L       | DOT1-Like Histone Lysine Methyltransferase |       | 607375  | -                      |
| PARP1       | Poly (ADP-Ribose) Polymerase 1           |           | 173870  | -                      |
| CENPS       | Centromere Protein 5                    | APITD1    | 609130  | -                      |
| CREBβL1     | CAMP Responsive Element Binding Protein 3 Like 1 |       | 616215  | -                      |
| MLT76       | Mixed-Lineage Leukemia, Translocated to, 6 |       | 600328  | -                      |
| ERCC3       | ERCC Excision Repair 3                   |           | 133510  | -                      |
| CBLB        | Cbl Proto-Oncogene B                     |           | 604491  | -                      |
| Other candidate genes |
| PTEN        | Phosphatase and Tensin Homolog           |           | 601728  | Bubien et al.48        |
| RASEF       | RAS and EF-Hand Domains-Containing Protein |       | 611344  | Moat et al.49          |
| POLH        | DNA Polymerase Eta                      |           | 603968  | Di Lucca et al.50      |
| OCA2        | OCA2 Melanosomal Transmembrane Protein |           | 611409  | Hawkes et al.55        |

Abbreviation: MIM, Mendelian Inheritance in Man (http://www.omim.org).
| Gene | Variant | Type       | Allele count | MAF (AN = 976) | MAF in ExAC1/GoNL | CADD | SIFT | Align GVGD2 | PolyPhen-23 | UMD-Predictor | FD | CoS4 |
|------|---------|------------|--------------|----------------|--------------------|------|------|-------------|--------------|---------------|----|------|
| ACD  | c.871A>G, p.(Thr291Ala) | Missense | 1            | 0.0010025      | 0.0012/0.0011      | 23.2 | Delet. | C55        | Prob. Damaging | Polymorphism | Y  |     |
| BAP1 | c.122+1G>T, p.? | Splicing | 1            | 0.0010025      | –/-                | –/-  | –/-  | –/-        | –/-          | –/-           | Y  |     |
| BAP1 | c.1730-1G>A, p.? | Splicing | 1            | 0.0010025      | –/-                | –/-  | –/-  | –/-        | –/-          | –/-           | Y  |     |
| BAP1 | c.1936_1937insTT, p.(Tyr646Phefs*10) | Frameshift | 1            | 0.0010025      | –/-                | –/-  | –/-  | –/-        | –/-          | –/-           | Y  |     |
| MITF | c.952G>A, p.(Glu318Lys) | Missense | 15           | 0.015369       | 0.0025/0.007       | 27.9 | Tol.  | C0         | Prob. Damaging | Prob. Polymorphism | Y  |     |
| TERF2IP | c.398G>A, p.(Arg133Gln) | Missense | 1            | 0.0010025      | 0.00022/-          | 23.4 | Delet. | C35        | Benign        | Polymorphism | Y  |     |
| TERF1 | c.186_188dup, p.(Glu62dup) | In-frame Duplication | 2            | 0.0002049      | 0.0005/-           | –/-  | –/-  | –/-        | –/-          | –/-           | Y  |     |
| TERF1 | c.212_217dup, p.(Glu71_Ala72dup) | In-frame Duplication | 1            | 0.0010025      | 0.00014/-          | –/-  | –/-  | –/-        | –/-          | –/-           | Y  |     |
| TERF1 | c.1193A>G, p.(Tyr398Cys) | Missense | 1            | 0.0010025      | 0.000009/-         | 24.7 | Delet. | C25        | Prob. Damaging | Pathogenic | Y  |     |
| TERF2 | c.566G>A, p.(Asp19Gly) | Missense | 1            | 0.0010025      | 0.00012/-          | 16.35| Delet. | C0         | Pos. Damaging  | N.a.         | N  |     |
| TERF2 | c.794G>A, p.(Arg265His) | Missense | 1            | 0.0010025      | 0.000027/-         | 28.3 | Delet. | C0         | Pos. Damaging  | Prob. Polymorphism | N  |     |
| TERF2 | c.1492G>A, p.(Glu498Lys) | Missense | 4            | 0.004098       | 0.0022/0.003       | 34   | Delet. | C55        | Pos. Damaging  | Prob. Polymorphism | Y  |     |
| TINF2 | c.380G>T, p.(Arg131Glu) | Missense | 1            | 0.0010025      | –/-                | 27   | Delet. | C0         | Prob. Damaging | Pathogenic | Y  |     |
| TINF2 | c.734C>A, p.(Ser245Thr) | Missense | 3            | 0.003074       | 0.00073/-          | 22.7 | Delet. | C15        | Benign        | Polymorphism | N  |     |

Gene reference sequences: ACD: NM_001082486.1, BAP1: NM_004656.3, MITF: NM_000248.3, TERF2IP: NM_018975.3, TERF1: NM_017489.2, TERF2: NM_005652.4, TINF2: NM_001099274.1. Gene reference sequences: ACD: NM_001082486.1, BAP1: NM_004656.3, MITF: NM_000248.3, TERF2IP: NM_018975.3, TERF1: NM_017489.2, TERF2: NM_005652.4, TINF2: NM_001099274.1.

Abbreviations: AN, allele number; MAF, minor allele frequency; CADD, Combined Annotation Dependent Depletion; FD, in known functional domain; CoS, co-segregation with melanoma in one or more families; Y, yes; N, no; Delet, deleterious; Pos, possibly; Prob, probably.

1In European (non-Finnish) population.

2Possible classifications in Align GVGD are C0, C15, C25, C35, C55 and C65. Variants in class C0 have the least probability of being pathogenic, variants in class C65 have the highest probability of being pathogenic. See also http://agvgd.hci.utah.edu/classifiers.php.

3HumVar trained PolyPhen-2 model used for prediction.

4Co-segregation analyses of variants with melanoma phenotype: TERF1 p.E62dup: 2/2.

5Common variant (MAF > 1%) in one or more non-European populations.
Table 4. Selected variants of interest in candidate melanoma susceptibility genes \(BRIP1\), \(POLE\) and \(OCA2\)

| Gene | Variant | Type       | Allele count | MAF (AN = 976) | MAF in ExAC \(^1\) / GoNL | CADD | SIFT | Align GVGD \(^2\) | PolyPhen-2 \(^3\) | UMD-Predictor | FD | CoS \(^6\) |
|------|---------|------------|--------------|----------------|--------------------------|------|------|----------------|-----------------|---------------|-----|---------|
| \(BRIP1\) | c.517C>T, p.(Arg173Cys) | Missense | 9            | 0.009221       | 0.0047 / 0.0047          | 27.6 | Delet. | C55 | Prob. Damaging | Pathogenic | Y   |
| \(BRIP1\) | c.790C>T, p.(Arg264Trp)  | Missense | 1            | 0.0010025      | 0.0012 / 0.003           | 32   | Delet. | C0  | Prob. Damaging | Pathogenic | Y   |
| \(BRIP1\) | c.894C>A, p.(Cys298*)    | Nonsense | 1            | 0.0010025      | -/-                      | 36   |       |    | Pathogenic    |           |     |
| \(BRIP1\) | c.1198G>T, p.(Asp400Tyr) | Missense | 2            | 0.002049       | 0.000027 / -/-           | 33   | Delet. | C35 | Prob. Damaging | Pathogenic | Y   |
| \(BRIP1\) | c.1255C>T, p.(Arg419Trp) | Missense | 1            | 0.0010025      | 0.000046 / 0.001         | 33   | Delet. | C35 | Prob. Damaging | Pathogenic | Y N |
| \(BRIP1\) | c.2069G>A, p.(Gly690Glu) | Missense | 1            | 0.0010025      | -/-                      | 32   | Delet. | C65 | Prob. Damaging | Pathogenic | Y   |
| \(BRIP1\) | c.2582C>G, p.(Ser861Cys) | Missense | 1            | 0.0010025      | -/-                      | 28.5 | Delet. | C65 | Prob. Damaging | Pathogenic | Y   |
| \(BRIP1\) | c.2593C>T, p.(Arg865Trp) | Missense | 1            | 0.0010025      | 0.000027 / -/-           | 34   | Delet. | C25 | Prob. Damaging | Pathogenic | Y N |
| \(POLE\)  | c.861T>A, p.(Asp287Glu)  | Missense | 9            | 0.009221       | 0.0017 / 0.004           | 25.7 | Delet. | C35 | Prob. Damaging | Pathogenic | Y   |
| \(POLE\)  | c.893A>G, p.(Tyr298Cys)  | Missense | 1            | 0.0010025      | -/-                      | 28.3 | Delet. | C65 | Prob. Damaging | Pathogenic | Y   |
| \(OCA2\)  | c.163del, p.(Ala55Leufs*47) | Frameshift | 1            | 0.0010025      | 0.00019 / -/-             | 18.24 | Delet. | C0  | Pos. Damaging | Prob. Polymorphism | N   |
| \(OCA2\)  | c.796C>T, p.(Arg266Trp)  | Missense | 1            | 0.0010025      | 0.0018 / 0.003           | 16.24 | Delet. | C0  | Pos. Damaging | Prob. Polymorphism | N   |
| \(OCA2\)  | c.1255C>T, p.(Arg419Trp) | Missense | 1            | 0.0010025      | 0.00011 / -/-             | 32   | Delet. | C0  | Prob. Damaging | Pathogenic | Y   |
| \(OCA2\)  | c.1261C>T, p.(Arg421Trp) | Missense | 1            | 0.0010025      | 0.00065 / -/-             | 28   | Delet. | C0  | Prob. Damaging | Pathogenic | Y N |
| \(OCA2\)  | c.1327G>A, p.(Val443Ile) | Missense | 18           | 0.018443       | 0.0051 / 0.008           | 34   | Tol.   | C0  | Prob. Damaging | Polymorphism | Y N |
| \(OCA2\)  | c.1441G>A, p.(Ala489Asp) | Missense | 1            | 0.0010025      | 0.0026 / 0.001           | 27.6  | Tol.   | C0  | Pos. Damaging | Prob. Polymorphism | Y   |
| \(OCA2\)  | c.1465A>G, p.(Asn489Asp) | Missense | 7            | 0.007172       | 0.0007 / 0.003           | 28.2  | Delet. | C0  | Prob. Damaging | Pathogenic | Y Y |
| \(OCA2\)  | c.1592A>G, p.(Tyr531Cys) | Missense | 1            | 0.0010025      | 0.00011 / 0.001          | 25.3  | Delet. | C0  | Prob. Damaging | Pathogenic | Y   |
| \(OCA2\)  | c.2037G>C, p.(Trp679Cys) | Missense | 1            | 0.0010025      | 0.00015 / -/-             | 34   | Delet. | C0  | Prob. Damaging | Pathogenic | Y   |

Gene reference sequences: \(BRIP1\): NM_032043.2, \(POLE\): NM_006231.2, \(OCA2\): NM_000275.2.

Abbreviations: AN, allele number; MAF, minor allele frequency; CADD, Combined Annotation Dependent Depletion; FD, in known functional domain; CoS, co-segregation with melanoma in one or more families; Y, yes; N, no; Delet, deleterious; Pos, possibly; Prob, probably.

\(^1\)In European (non-Finnish) population.

\(^2\)Possible classifications in Align GVGD are C0, C15, C25, C35, C45, C55 and C65. Variants in class C0 have the least probability of being pathogenic, variants in class C65 have the highest probability of being pathogenic. See also http://agvgd.hci.utah.edu/classifiers.php

\(^3\)HumVar trained PolyPhen-2 model used for prediction.

\(^4\)Co-segregation analyses of variants with melanoma phenotype: \(BRIP1\) p.R419W: 1/2, \(BRIP1\) p.R865W: 1/2, \(OCA2\) p.R443I: 1/2 (two families), \(OCA2\) p.W449D: 3/3 (one family), \(POLE\) p.W410*: 1/2.

\(^5\)Variants reported in patients with oculocutaneous albinism type 2.

\(^6\)Common variant (MAF > 1%) in one or more non-European populations.
| Gene       | Variant                  | Type            | Allele count | MAF (AN = 976) | MAF in ExAC1 / GoNL | CADD | SIFT | Align GVGD2 | PolyPhen-23 | UMD-Predictor | FD | CoS4 |
|------------|--------------------------|-----------------|--------------|----------------|---------------------|------|------|-------------|-------------|---------------|----|------|
| CBLB       | c.770A>T, p.(His257Leu)  | Missense        | 1            | 0.0010025      | --/--               | 33   | Delet. | C0          | Prob. Damaging | Pathogenic    | Y  |      |
| CBLB       | c.1402CG, p.(Arg468Gly)  | Missense        | 1            | 0.0010025      | 0.000018/--         | 23.6 | Delet. | C0          | Pos. Damaging  | Pathogenic    | Y  |      |
| ERCC3      | c.496GGA, p.(Val166Leu)  | Missense        | 1            | 0.0010025      | --/--               | 24.6 | Delet. | C25         | Benign        | Prob. Polymorphism | Y |      |
| ERCC3      | c.847CT, p.(Arg283Cys)   | Missense        | 5            | 0.005123       | 0.0014/0.002        | 34   | Delet. | C65         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.1421dup, p.(Asp474Glufs*2) | Frameshift | 1            | 0.0010025      | 0.000016/--         | 33   | Delet. | C0          | Prob. Damaging | Pathogenic    | Y  |      |
| ERCC3      | c.1776T>G, p.(Arg468Gly) | Missense        | 1            | 0.0010025      | 0.000018/23.6       | 23.6 | Delet. | C0          | Prob. Damaging | Prob. Pathogenic | Y |      |
| ERCC3      | c.496GGA, p.(Val166Leu)  | Missense        | 1            | 0.0010025      | --/--               | 24.6 | Delet. | C25         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.847CT, p.(Arg283Cys)   | Missense        | 5            | 0.005123       | 0.0014/0.002        | 34   | Delet. | C65         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.1421dup, p.(Asp474Glufs*2) | Frameshift | 1            | 0.0010025      | 0.000016/--         | 33   | Delet. | C0          | Prob. Damaging | Pathogenic    | Y  |      |
| ERCC3      | c.1776T>G, p.(Arg468Gly) | Missense        | 1            | 0.0010025      | 0.000018/23.6       | 23.6 | Delet. | C0          | Prob. Damaging | Prob. Pathogenic | Y |      |
| ERCC3      | c.496GGA, p.(Val166Leu)  | Missense        | 1            | 0.0010025      | --/--               | 24.6 | Delet. | C25         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.847CT, p.(Arg283Cys)   | Missense        | 5            | 0.005123       | 0.0014/0.002        | 34   | Delet. | C65         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.1421dup, p.(Asp474Glufs*2) | Frameshift | 1            | 0.0010025      | 0.000016/--         | 33   | Delet. | C0          | Prob. Damaging | Pathogenic    | Y  |      |
| ERCC3      | c.1776T>G, p.(Arg468Gly) | Missense        | 1            | 0.0010025      | 0.000018/23.6       | 23.6 | Delet. | C0          | Prob. Damaging | Prob. Pathogenic | Y |      |
| ERCC3      | c.496GGA, p.(Val166Leu)  | Missense        | 1            | 0.0010025      | --/--               | 24.6 | Delet. | C25         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.847CT, p.(Arg283Cys)   | Missense        | 5            | 0.005123       | 0.0014/0.002        | 34   | Delet. | C65         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.1421dup, p.(Asp474Glufs*2) | Frameshift | 1            | 0.0010025      | 0.000016/--         | 33   | Delet. | C0          | Prob. Damaging | Pathogenic    | Y  |      |
| ERCC3      | c.1776T>G, p.(Arg468Gly) | Missense        | 1            | 0.0010025      | 0.000018/23.6       | 23.6 | Delet. | C0          | Prob. Damaging | Prob. Pathogenic | Y |      |
| ERCC3      | c.496GGA, p.(Val166Leu)  | Missense        | 1            | 0.0010025      | --/--               | 24.6 | Delet. | C25         | Benign        | Pathogenic    | Y  | N    |
| ERCC3      | c.847CT, p.(Arg283Cys)   | Missense        | 5            | 0.005123       | 0.0014/0.002        | 34   | Delet. | C65         | Benign        | Pathogenic    | Y  | N    |

Gene reference sequences: **CBLB**: NM_170662.4, **ERCC3**: NM_000122.1, **MLLT6**: NM_005937.3, **NEK2**: NM_001618.3, **NEK10**: NM_152534.4, **NEK11**: NM_024800.4, **POLH**: NM_005937.3, **RASEF**: NM_152573.3.

Abbreviations: AN, allele number; MAF, minor allele frequency; CADD, Combined Annotation Dependent Depletion; FD, in known functional domain; CoS, co-segregation with melanoma in one or more families; Y, yes; N, no; Delet, deleterious; Pos, possibly; Prob, probably.

1 In European (non-Finnish) population.
2 Possible classifications in Align GVGD are C0, C15, C25, C35, C45, C55 and C65. Variants in class C0 have the least probability of being pathogenic, variants in class C65 have the highest probability of being pathogenic. See also http://agvgd.hci.utah.edu/classifiers.php
3 HumVar trained PolyPhen-2 model used for prediction.
4 Co-segregation analyses of variants with melanoma phenotype: **ERCC3** p.R283C: 1/2 (one family), **ERCC3** p.S704L: 1/2 (one family), **NEK2** p.E46G: 1/2, **NEK10** p.R365Q: 1/2 (one family).
5 Common variant (MAF > 1%) in one or more non-European populations.
6 The proband with the **POLH** p.W297* variant had a father with the recessively inherited disease xeroderma pigmentosum (MIM #278750) and he is therefore highly likely to have carried this variant as well.

**Table 5. Selected variants of interest in candidate melanoma susceptibility genes (excluding BRIP1, POLE and OCA2)**
predicted to inactivate the splice donor site of intron 3 and splice acceptor site of intron 13, respectively, likely resulting in a prematurely truncated protein. The c.1936_1937insTT, p. splice acceptor site of intron 13, respectively, likely resulting in the inactivation of the splice donor site of intron 3.

In two families, possible BAP1-associated nevi (Spitz nevi) were reported in first-degree relatives, and in one of these families, multiple relatives were also diagnosed with (one or several) basal cell carcinomas. No other BAP1-specific tumors, such as UM, malignant mesothelioma or renal cell carcinoma, were reported in these families. Interestingly, in the proband who carried the BAP1 c.122+1G>T, p.? variant we also identified a novel nonsense variant in the BRIP1 gene (c.894C>A, p.(C298*)) in silico tools, although UMD-predictor classified both variants as polymorphisms. Remarkably, we did not detect any potentially deleterious variants in the POT1 gene. In the other shelterin complex subunit genes TERT, TERF2 and TINF2, we identified eight potentially deleterious variants (six missense, two in-frame dup) (Table 3). These included a novel variant in the ACD/TERF2 binding motif domain of the TINF2 gene (c.38G>T, p.(R13L)) and two extremely rare variants in the TERT1 gene (c.1193A>G, p.(Y398C); MyB DNA binding domain) and the TERF2 gene (c.794G>A, p.(R265H)). An in-frame duplication in the TERT1 gene (c.186_188dup, p.(E62dup); telomeric repeat binding factor homology domain) was shared among two third-degree relatives with CM in one family, but as this is a common variant in Asian and African populations (MAF ~2% in ExAC) it is unlikely to be pathogenic. None of the patients in our cohort carried the known melanoma susceptibility variant in the TERT promoter region (c.-57T>G).

Since we were particularly interested in the frequency of MC1R risk variants in familial CM cases, we only analyzed the MC1R gene in the ‘familial CM’ subgroup (n = 478 individuals). In this cohort, we observed a substantial enrichment of R variants compared to controls (OR 3.67, 95% CI 2.88–4.68, p < 0.001) (Table 6). The frequency of p.D84E was most strikingly increased in our cohort (OR 5.66, 95% CI 3.18–10.88, p < 0.001).

Table 6. Association of MC1R risk variants with familial cutaneous melanoma

| All R variants | OR     | 95% CI   | p value2 |
|----------------|--------|----------|----------|
| 0.342          | 3.67   | 2.88–4.68| <0.001   |
| c.252CA, p.D84E| 0.017  |          | 0.001    |
| c.425GA, p.R142H| 0.008  | 0.004    | 0.001    |
| c.451CT, p.R151C| 0.145  | 0.058    | 0.031    |
| c.478CT, p.R160W| 0.150  | 0.059    | 0.018    |
| c.880GC, p.D294H| 0.022  | 0.011    | 0.005    |
| All r variants | 0.252  | 0.248    | 0.001    |
| c.178GT, p.V60L| 0.105  | 0.104    | 0.008    |
| c.274GA, p.V92M| 0.082  | 0.081    | 0.021    |
| c.464T>C, p.I155T| 0.006  | 0.006    | 0.540    |
| c.478C>T, p.R160W| 0.150  | 0.059    | 0.018    |
| c.880GC, p.D294H| 0.022  | 0.011    | 0.005    |

MC1R reference sequence: NM_002386.3.
Abbreviation: AN, allele number.
1Minor allele frequency (MAF).
2Using Fisher’s exact test (two-sided).
3Number of alleles without any R or r variant.
1.88–17.06, \( p = 0.001 \), followed by \( p.R160W \) (OR 3.82, 95% CI 2.72–5.37, \( p < 0.001 \)) and \( p.R151C \) (OR 3.78, 95% CI 2.68–5.34, \( p < 0.001 \)). Although less prominent, \( r \) variants were also enriched in familial CM cases (any \( r \) variant: \( OR = 1.53, 95\% \text{ CI} 1.22–1.91, p < 0.001 \)).

**Variants of interest in candidate melanoma susceptibility genes**

In addition to the novel, truncating variant in the *BRIP1* gene (c.894C>A, p.(C298*)) found in one of the *BAP1*-families, an additional seven potentially deleterious missense variants were identified in *BRIP1* (Table 4). This included one novel variant (c.2069G>A, p.(G690E)) and two extremely rare variants (c.2582C>G, p.(S861C) and c.2593C>T, p.(R865W)) located in the DNA helicase domain and predicted to be damaging by all in silico tools including UMD-predictor. However, the latter variant did not co-segregate with the phenotype in a two-case family. In this same domain, a different missense variant was previously reported to co-segregate in a three-case melanoma family.2

The remaining four variants were located in the ATPase/helicase core domain, and included an extremely rare variant (c.1198G>T, p.(D400Y)) in two probands and a very rare variant (c.1255C>T, p.(R419W)) in one proband. Currently, little is known from literature about the effect of these missense variants and no functional testing has been performed.

We further identified two missense variants in the exonuclease domain of the *POLE* gene: one novel variant (c.893A>G, p.(Y298C)) in a single proband and a rare variant (c.861T>A, p.(D287E)) in nine other probands (Table 4). Both variants were predicted to be damaging by all in silico tools including UMD-predictor. In another proband, we identified a novel truncating variant in *POLE* (c.1230G>A, p.(W410*)), but this variant did not co-segregate with the phenotype in a two-case family.

In the *OCA2* gene, we identified nine (potentially) deleterious variants, of which six were previously reported in patients with the recessively inherited condition oculocutaneous albinism type 2 (MIM #203200) (Table 4). Two of these established pathogenic variants, c.1327G>A, p.(V443I) and c.1465A>G, p.(N489D), were detected in multiple individuals \( (n = 17 \text{ and 7 , respectively}) \) and the frequency of these variants was more than twice that found in the Dutch GoNL reference database (MAF: 0.018 and 0.0071; GoNL: 0.008 and 0.003, respectively). Co-segregation analysis was, however, ambiguous: the c.1465A>G, p.(N489D) variant co-segregated with the phenotype in a three-case family (all first-degree relatives), but the c.1327G>A, p.(V443I) variant did not co-segregate in two two-case families. Interestingly, one proband was homozygous for the c.1327G>A, p.(V443I) variant. This proband had a medical history of three primary melanomas from age 57 and a first-degree relative (sibling) with melanoma. Although the proband was reported to have a fair skin type and reddish hair, no other physical signs of albinism were reported. Another proband, with a medical history of three primary melanomas from age 48 and a first-degree relative (child) with melanoma at age 32, carried two pathogenic variants in the *OCA2* gene (c.1327G>A, p.(V443I) and c.2037G>C, p.(W679C)). Since physical signs of albinism were not reported in the proband, it is possible that these variants are located on the same allele, but this could not be confirmed because co-segregation data was unavailable.

The other included candidate melanoma susceptibility genes, largely derived from whole exome/genome sequencing studies by both our own research group and other research groups, we detected four truncating variants (in *ERCC3*, *NEK2*, *POLH*, *RASEF*), two canonical splice site variants (in *NEK2*, *NEK4*) and several potentially deleterious missense variants (in *CBLB*, *ERCC3*, *MLLT6*, *NEK2*, *NEK4*, *NEK10*, *NEK11*, *PARP1*, *POLH*, *RASEF*) (Table 5). All of these variants occurred in only one proband and co-segregation data was only occasionally available. UMD-predictor classified the majority of these variants as (probably) pathogenic.

**Discussion**

In our study, we performed multigene panel testing of 30 (candidate) melanoma susceptibility genes in 451 Dutch melanoma-prone families without a *CDKN2A* or *CDK4* mutation. We identified (likely) pathogenic variants in established high- and medium penetrance melanoma susceptibility genes in 4.0% of these families (18/451; \( n = 3 \) *BAP1*, \( n = 15 \) *MITF*). In addition, two potentially deleterious missense variants were detected in important functional domains of the *ACD* and *TERF2IP* genes (0.4%) and, surprisingly, none of the 451 families carried a variant of interest in the *POT1* gene.

The frequency of *BAP1* mutations in our cohort \( (n = 3; 0.7\%) \) is in line with a reported frequency of ~1% among melanoma-prone families worldwide.2 BAP1 is a deubiquitinating hydrolase that acts as a tumor suppressor and is involved in the regulation of key pathways including cell proliferation, cell differentiation, cell survival and the DNA damage response. Germline *BAP1* mutations have been reported in patients with several types of tumors, but particularly in UM and malignant mesothelioma.14 Interestingly, these two major cancers were not present in our three families. Although CM itself is relatively common in *BAP1*-associated cancers. A recent population-based study reported only three loss-of-function *BAP1* mutations in CM cases (<0.2%), and all these cases had relatives with *BAP1*-associated cancers, although none had

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Our study demonstrates that BAP1 mutations can indeed be detected in some CM families without UM or malignant mesothelioma and it is therefore important to incorporate the BAP1 gene in a diagnostic (cutaneous) melanoma gene panel test. However, it should be noted that basal cell carcinoma and (atypical) Spitz nevi, features also associated with BAP1 mutations, were reported in two of the families.

Fifteen probands in our familial CM cohort (15/442; 3.4%) carried the MITF p.E318K risk variant, which is among the highest frequencies reported in familial non-CDKN2A cases. Only one small study from Switzerland reported a higher frequency, 7.7% (2/26), in melanoma-prone families. A similar frequency, 3.4% (19/558) in familial cases, was found in a study from the United States, although it is unclear if these patients were all pre-screened for CDKN2A mutations. Frequencies in various other cohorts range from 0 to 3%, with the lowest frequency (1%) reported in familial cases from Italy. In the Netherlands, diagnostic testing for the MITF p.E318K risk variant is now included in the default genetic work-up for familial CM and all carriers are offered regular dermatologic surveillance (regardless of the familial burden for CM). This regular surveillance is recommended because carriers are at increased risk for developing subsequent (multiple primary) melanomas that might also be fast-growing and/or amelanotic, a subtype less easily recognized by the dermatologist. Hence, knowledge about MITF p.E318K mutation status can be relevant for both the patient and the dermatologist. Surveillance for other cancers such as renal- or pancreatic cancer is not (yet) offered because the actual risk for these cancers is insufficiently established and surveillance methods are more challenging.

Germline mutations in the telomere maintenance pathway genes in melanoma families have been described in several studies. The present study demonstrates that mutations in these genes are probably very rare in the Dutch familial melanoma population. We identified only two potentially deleterious missense variants in ACD and TERF2IP (0.4%) and none in POT1 or the promoter region of TERT. In the ACD and TERF2IP genes, both nonsense and pathogenic missense variants have been previously reported in familial melanoma kindreds. Interestingly, the TERF2IP p.(R133Q) variant that we detected in a two-case melanoma family was previously reported in a three-case chronic lymphocytic leukemia (CLL) family (without melanoma). Because the variant co-segregated with only two of the cases, the authors concluded that this is a medium penetrance variant for CLL. Leukemia was not reported in relatives of the proband in our cohort. Of the eight potentially deleterious missense variants detected in the TERF1, TERF2 and TINF2 genes, co-segregation analysis was only possible for one of these variants. There is no additional evidence for pathogenicity of these missense variants, and as yet no protein truncating variants have been reported in these latter genes. Therefore, their role in melanoma susceptibility remains uncertain.

We identified several variants of interest in the known cancer susceptibility genes BRIP1 and POLE, including a nonsense variant in BRIP1. BRIP1 (BRCA1-interacting protein C-terminal helicase 1) is a Fanconi anemia group protein and is required for the double-strand break repair function of BRCA1. Heterozygous protein truncating variants in BRIP1 have mainly been associated with an increased susceptibility for ovarian cancer, but there were no diagnoses of ovarian cancer in family members of the proband with the nonsense BRIP1 variant in our study. Interestingly, this variant co-occurred with a canonical splice site variant in BAP1 in the same proband, the latter presumably being the predominant melanoma susceptibility factor in this family. We additionally identified several potentially deleterious missense variants in BRIP1, some novel or extremely rare, and most of which were predicted to be damaging by all in silico tools used. In a recent study from Sweden, an extremely rare missense variant in the DNA helicase domain of BRIP1 was found to co-segregate in a three-case melanoma family. Three missense variants in our cohort were located in this same functional domain. Based on these findings, the BRIP1 gene might be involved in melanoma susceptibility, but more research is needed to clarify this, in particular replication studies in other melanoma cohorts and functional studies to address the pathogenicity of missense variants. The POLE gene is a polymerase gene involved in DNA repair and replication and is primarily associated with colorectal cancer. It appears that only missense variants in the exonuclease domain confer an increased susceptibility for cancer through impaired proofreading, which results in tumors with a high mutation burden. Therefore, we restricted our analysis of variants to this specific exonuclease domain and, consequently, all reported variants in POLE are located within this domain. Recently, a novel missense variant in the exonuclease domain of POLE was reported in a seven-case melanoma family and showed near-complete co-segregation. Although we were not able to perform co-segregation analysis for the novel missense variant (c.893A>G, p.(Y298C)) detected in our cohort, functional analysis of melanoma tissue (mutation burden test) might provide more insight. Of note, colorectal cancer was not reported in this family.

Biallelic germline mutations in OCA2 cause oculocutaneous albinism type 2 (MIM #203200). OCA2 encodes the P-protein which has multiple functions in the biosynthesis of melanin. Loss-of-function of the P-protein results in hypopigmentation of the skin, hair and iris and an increased risk for sun-induced skin cancers, in particular basal cell carcinoma and squamous cell carcinoma. Although melanoma is not known to be a common cancer type in patients with OCA2-related albinism, families with multiple members with albinism and melanoma have been reported. In our cohort, one proband with a possible subclinical phenotype of albinism carried a homozygous pathogenic OCA2 variant. Additionally, we observed an increased frequency of rare heterozygous variants in the OCA2 gene, in particular the known pathogenic variants c.1327G>A, p.(V443I) and c.1465A>G, p.(N489D). The association with
melanoma predisposition of the c.1327G>A, p.(V443I) variant in combination with another OCA2 variant was also studied by Hawkes et al.55 in one albinism-melanoma family. They concluded that these variants might be high penetrance loci for melanoma in this family (OR 6.3). In a recent study by Goldstein et al.,56 the OCA2 gene was included in a multigene panel test of 42 (candidate) melanoma susceptibility genes that were sequenced in 144 melanoma cases from 76 American families. Comparable to our study, numerous rare variants in OCA2 were found. The frequency of rare variants in other albinism genes (TYR, TYRP1) was also significantly increased in the Goldstein study. Interestingly, a nonsense variant in TYR showed near-complete co-segregation in a large family with six melanoma cases. The precise role of OCA2 (and other albinism genes) in melanoma predisposition remains to be determined, but based on these findings a medium penetrance or modifier effect can be hypothesized. The albinism genes are therefore good candidates for further investigation.

There is extensive literature on the association between MCIR R and r variants and sporadic melanoma in population-based cohorts.18 In our ‘familial CM’ cases, we observed a high frequency of MCIR R variants in particular, a finding comparable to the results of a Danish high-risk melanoma cohort.28 This suggests that these common risk variants also play a significant role in the familial setting. Since some of the familial occurrence of melanoma might be explained by the aggregation of common risk variants in a family, we are currently incorporating all MCIR R and r variants in a polygenic risk score (PRS) model that also includes approximately 40 other common risk variants derived from large melanoma GWAS. PRS models have already been shown to improve risk stratification in other familial cancer cohorts, in particular familial breast cancer.27

A major strength of our study is cohort size. With the inclusion of 451 families lacking a mutation in the CDKN2A or CDK4 genes, of which 442 families had at least two cases of CM, to our knowledge this is the largest melanoma gene panel study to date. Although our inclusion criteria were not highly stringent, most families had at least two close relatives with melanoma (for instance, 83% of the two-case families consisted of first-degree relatives). Furthermore, our panel included all eight currently known high- and medium penetrance melanoma susceptibility genes and therefore our reported 4% diagnostic yield for these genes (excluding CDKN2A and CDK4) is probably very accurate. As a custom-designed targeted gene panel was used, filtering of variants was less strict compared to most reported WES studies. It is therefore very unlikely that potential pathogenic variants in the selected genes were missed in our study. A limitation is that co-segregation analysis of variants was not possible in many families. This was primarily due to Ethics Committee restrictions that prohibited us from re-contacting patients when variants of uncertain significance (VUS) or variants in nonestablished genes were detected. However, co-segregation analysis of (likely) pathogenic variants in known cancer susceptibility genes (BAP1, MITF, BRIP1) is currently being initiated.

To conclude, we demonstrate that multigene panel testing for familial melanoma results in an additional 4% diagnostic yield in non-CDKN2A/CDK4 families. The identification of several families with pathogenic variants in the BAP1 and MITF genes suggests a significant role of these genes in melanoma predisposition and it is therefore important to include these in a diagnostic test. Conversely, variants in the telomere maintenance genes, especially POT1, seem to be (very) rare in the Dutch population. When including these genes in a panel test, one should be aware of identifying variants of uncertain significance, as we did in the current study. In view of the relatively high frequency of (potential) pathogenic variants in the OCA2 gene in both our own and in a recently published American familial melanoma cohort, further elucidation of the role of heterozygous OCA2 variants in melanoma predisposition appears to be of particular interest. In the future, candidate susceptibility genes such as OCA2 could potentially be added to routine germline diagnostics, given sufficient evidence for their pathogenicity in melanoma predisposition. This will in turn enhance the diagnostic yield of the panel and improve tumor risk assessment in melanoma families.

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