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Prevalence of Germline *BAP1*, *CDKN2A*, and *CDK4* Mutations in an Australian Population-Based Sample of Cutaneous Melanoma Cases

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Mutations in Cyclin-Dependent Kinase Inhibitor 2A (*CDKN2A)* and Cyclin-Dependent Kinase 4 (*CDK4*) contribute to susceptibility in approximately 40% of high-density cutaneous melanoma (CMM) families and about 2% of unselected CMM cases. BRCA-1 associated protein-1 (*BAP1)* has been more recently shown to predispose to CMM and uveal melanoma (UMM) in some families; however, its contribution to CMM development in the general population is unreported. We sought to determine the contribution of these genes to CMM susceptibility in a population-based sample of cases from Australia. We genotyped 1,109 probands from Queensland families and found that approximately 1.31% harbored mutations in *CDKN2A*, including some with novel missense mutations (p.R22W, p.G35R and p.I49F). *BAP1* missense variants occurred in 0.63% of cases but no *CDK4* variants were observed in the sample. This is the first estimate of the contribution of *BAP1* and *CDK4* to a population-based sample of CMM and supports the previously reported estimate of *CDKN2A* germline mutation prevalence.

■ **Keywords:** BAP1, CDK4, CDKN2A, cutaneous melanoma, germline mutation

Many environmental and genetic factors play a part in melanomagenesis. While exposure to ultraviolet radiation plays a significant role in melanoma development, an underlying genetic predisposition also contributes to an individual's risk. Studies have shown that ~10% of cutaneous malignant melanoma (CMM) cases occur in people that have a family history of melanoma (Gruis et al., 1995; Hussussian et al., 1994; MacGeoch et al., 1994; Soufir et al., 1998; Walker et al., 1995; Zuo et al., 1996). Known high-risk genes account for susceptibility in a proportion of these families. The major CMM predisposition locus *CDKN2A* encodes two tumor suppressors, p16INK4A and p14ARF, that inhibit progression of cancer cells by inducing senescence or apoptosis, respectively (de Snoo & Hayward, 2005; Palmieri et al., 2009). *CDKN2A* is thus involved in two of the most important tumor suppressor pathways, the p53 and the retinoblastoma (RB) pathways.

The largest study of germline mutations in *CDKN2A* in families to date was conducted by the International Melanoma Genetics Consortium (GenoMEL), in which 466 families from North America, Europe, Asia, and Australia were genotyped for mutations in *CDKN2A* and *CDK4* (Goldstein et al., 2006). They found 41% of high-density families (defined by case-load depending on the region of origin) carried germline mutations in either p16 (38%), p14 (1.5%) or *CDK4* (1%). Overall, 57 unique mutations in p16 were attributed to increased CMM risk. In contrast, only two variants in *CDK4* have been attributed to CMM risk; p.R24C and p.R24H (Soufir et al., 1998; Zuo et al., 1996). A study into the prevalence of *CDKN2A* and *CDK4* mutations in CMM cases from a Greek hospital-based sample found that 5% of cases (16 of 320) harbored a mutation in one of these genes (Nikolaou et al., 2011). But, to date, there are only two published studies determining the prevalence of high-risk predisposition loci in population-based samples of CMM cases. The Genes Environment and Melanoma (GEM) study genotyped probands from nine different geographical regions in the USA, Canada, Italy, and Australia

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TABLE 1

Number of Samples Sequenced for *CDKN2A* and *BAP1* Variants

Study	Total no. of samples collected per study	No. of samples sanger sequenced from each study	No. of samples ion torrent sequenced from each study
High	91	87~	54^
Intermediate	414	194~	243
Low	1,392	201~	565
Men over 50	178	none	65
Childhood	101	31*	48
Adolescents	298	none	105
Twins	125	none	29

Note: ~ Aitken et al., (1999); *Whiteman et al. (1997); ^*BAP1* sequencing only.

(Begg et al., 2005). They discovered 65 *CDKN2A* mutation carriers in a sample of 3,550 affected individuals, equating to a population frequency of ~2%. A second study looked at the contribution of *CDKN2A* to melanoma in a population-based sample of cases ($N = 482$) from Queensland (Aitken et al., 1999), from which it was estimated that *CDKN2A* mutations occur in 0.2% of population-based Queensland CMM cases (Aitken et al., 1999).

BAP1 is a tumor suppressor gene located on chromosome 3 that has also been associated with predisposition to CMM. In a seminal study, somatic *BAP1* mutations were first observed in a panel of sporadic UMM cases; notably, a single UMM case also carried a germline *BAP1* mutation (Harbour et al., 2010). Since then, *BAP1* has been linked to predisposition of a spectrum of cancer types extending beyond UMM to include CMM, mesothelioma, renal cell carcinoma, basal cell carcinoma, as well as a distinct type of benign melanocytic tumor (Abdel-Rahman et al., 2011; Aoude et al., 2013; Carbone et al., 2012; Cheung et al., 2013; de la Fouchardiere et al., 2014; Harbour et al., 2010; Hoiom et al., 2013; Njauw et al., 2012; Popova et al., 2013; Testa et al., 2011; Wadt et al., 2014; Wiesner et al., 2011). Population-based and clinic-based prevalence studies show that *BAP1* germline mutations contribute to only a small proportion of UMM cases overall (3–4%; Aoude et al., 2013; Njauw et al., 2012) but the contribution of such mutations to a population based-sample of CMM cases has not been reported.

The primary aim of this study was to more fully quantify the contribution of constitutional *CDKN2A* and *CDK4* mutations in an Australian population-based sample of CMM cases. A secondary aim was to determine the prevalence of germline *BAP1* mutations in this sample.

Materials and Methods

Ethics

Written consent was obtained for each participant in this study. Ethics approval was obtained from the QIMR Berghofer Human Research Ethics Committee (HREC).

Study Cohort

Samples were ascertained as part of the Q-MEGA project, a population-based study from Queensland investigating the associations between genes and environment in CMM development (Baxter et al., 2008). Q-MEGA is made up of four distinct CMM case sample collections: childhood, adolescent, men over 50 years, and the Queensland Familial Melanoma Project (QFMP; Aitken et al., 1996). Individuals who presented with histologically confirmed CMM and were reported to the Queensland Cancer Registry between the years 1982 and 1990 were approached to participate in the QFMP study ($N = 12,006$). This accounted for approximately 95% of CMM cases diagnosed in Queensland over this period (Aitken et al., 1996). Cases were asked to fill out a questionnaire pertaining to family history, pigmentation, freckling, mole count, and likelihood of sunburn. In the instance where an individual had a family history of melanoma, the first degree relatives and affected cases were also ascertained. A follow-up study in 2002–2005 collected updated data and additional blood samples. A total of 1,897 individual families were sampled and stratified into three categories according to a standardized family risk index, previously described (Aitken et al., 1994). Generally, although there were a few exceptions, individuals with no family history of CMM were categorized as low-risk ($N = 1,392$), two-case families were categorized as intermediate-risk ($N = 414$) and families with three or more cases were categorized as high-risk ($N = 91$). Additionally, twins with CMM collected in Queensland and New South Wales were included (Shekar et al., 2009). For the current study a random selection of 1,109 cases from across the 2,599 probands in Q-MEGA was used (Table 1). Selection was based on the order in which DNA samples were replated into 384 well plates.

Sample Preparation

Blood samples were obtained from 2,599 probands (Table 1) ascertained through the Q-MEGA studies (Baxter et al., 2008). Genomic DNA was extracted using a standard salting out method (Miller et al., 1988).

Targeted Sequencing of CDKN2A and BAP1 using the Ion Torrent PGM

Melanoma cases were assessed for variants in *BAP1* and *CDKN2A* in a targeted sequencing approach. Using Ion AmpliSeq library kits (Life Technologies, CA, USA), 10 ng

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of genomic DNA from each proband were amplified using custom-designed primer pools. The panel was designed to generate coverage of 40X across all regions with amplicon lengths of 150 bp to 250 bp. *BAP1* and *CDKN2A* had coverage of 96% and 97%, respectively. Ion Xpress barcode adapters 1–64 were used to pool samples. Unamplified libraries were purified using Agencourt Ampure XP reagent (Beckman Coulter, CA, USA) in order to eliminate fragments < 100 bp and increase the proportion of on-target reads. Libraries were equalized to ~100 pM using Ion Library Equalizer kits then combined into a single sample. A portion of the library (4 μL) was then diluted in 21 μL nuclease-free water to create a working stock. Using OT2 200 Kits (Life Technologies, CA, USA), clonal amplification and template enrichment of the Ion Sphere particles was performed. The template quality was assessed using a Qubit 2.0 (Life Technologies, CA, USA). Finally, Ion 318v2 chips were run on a Personal Genome Machine (Life Technologies, CA, USA) with 500 run flows per chip.

Overall, 1,109 samples had mean sequence coverage of 30X and therefore the sequencing of these samples was considered to be of sufficient depth to give accurate reads. Sequence data were analyzed using Torrent Suite software (Life Technologies, CA, USA). In order to minimize the false positive rates, filtering criteria were applied to the output. First, variants were required to have minimum of four reads for the reference and four reads for the alternative alleles. The variant allele also had to comprise at least 20% of the total read count. Quality score had to be > 40. Synonymous variants were excluded. Variants occurring commonly in the NHLBI Exome Sequencing Project (ESP6500; minor allele frequency (MAF) > 0.01) were also excluded as they are unlikely to be high-risk predisposition variants, but rather, common population polymorphisms. This sculpted the final list of variants, which were verified using Sanger sequencing. As a previous study has reported the *CDKN2A* mutations in the QFMP high-risk cohort (Aitken et al., 1999), *CDKN2A* genotyping of these samples was not repeated here, but the published results were included in the overall prevalence statistics. Sanger sequencing was used to validate the *CDKN2A* and *BAP1* variants identified through targeted sequencing. See Supplementary Table S1 for the list of primers used. Supplementary material is available on the Cambridge Journals Online website.

CDK4 Genotyping using the Sequenom MassArray

The two known familial melanoma variants in *CDK4* (p.R24C and p.R24H) were genotyped in the QFMP probands using a Sequenom iPLEX gold assay (Sequenom Bioscience, CA, USA). The MassArray designer software was used to design the forward, reverse and extension primers for p.R24C (acgttggatgagtggctgaaattggtgtcg, acgttggatgtcacactcttgagggccac, and gcactgtggggatcac) and p.R24H (acgttggatgagtggctgaaattggtgtcg, acgttggatgtcacactcttgagggccac, and ttggccactgtggggatca). IPLEX Gold PCR amplification reactions were set up according to standard manufacturer protocols. Cluster plots were analyzed using Typer Analyzer software 4.0.

Results

Targeted sequencing of an unselected population of CMM cases from Queensland, Australia revealed 6 of 1,055 cases harbor a missense variant in *CDKN2A* (Table 2). We report a single incidence of each of the following variants occurring in p16 (NM˙000077): p.L16P, p.R22W, p.G35R, and p.I49F. We also report two incidences of p.G101W (rs104894094), a common founder mutation in European melanoma populations (Goldstein et al., 2006). We found a recurrent mutation, p.A121T (rs199888003), in p14 (NM˙058195) in two cases. As this residue is not overly conserved in primates (Rhesus, Figure 1), this is likely to be a non-deleterious mutation and is therefore not included in the overall prevalence statistics. Seventy-two cases carried the well-documented *CDKN2A* p.A148T polymorphism, giving it a MAF of 0.0353. The MAF reported for this variant in the European American cohort ($N = 4{,}300$) of the ESP6500 is 0.0225, which correlates well with the occurrence in our sample. *CDKN2A* p.A148T has also been omitted from the prevalence statistics. Of the variants seen here, three are novel and have not been previously reported to the Leiden Open Variation Database (p.R22W, p.G35R, and p.I49F). All occur at evolutionarily conserved amino acids (Figure 1), and although none of these variants have been reported as somatic mutations in CMM in the COSMIC (catalogue of somatic mutations in cancer) database, p.G35R has been seen in ovarian and pancreatic cancers (Table 3).

The incidence of *CDKN2A* mutation in the QFMP high-risk probands has been reported previously (Supplementary Table S2; Aitken et al., 1999). We did not re-genotype *CDKN2A* in these samples here, as the results for over 95% of the cases are in the report by Aitken et al. (1999). In that study, Sanger sequencing found that 9 of 87 probands had a *CDKN2A* mutation. When these results are combined with those of the current study, we find that 1.31% (15 of 1,142) of the Australian population-based CMM sample carries a *CDKN2A* mutation affecting p16. The childhood cohort has also been partly reported on previously, with the p.L16P mutation being found in a case presenting with multiple primary melanomas by the age of 12 (Whiteman et al., 1997), the results of which have been replicated here.

Genotyping of *CDK4* p.R24C and p.R24H showed that no proband in this population-based sample is a carrier of a melanoma-associated *CDK4* mutation.

Targeted sequencing of an unselected population of CMM cases from Queensland, Australia found 7 out of 1,109 cases harbor a missense variant in *BAP1* (Table 2). Two novel variants occurred in the ubiquitin carboxy-terminal hydrolase domain (p.G121R and p.R150C), one novel variant occurred in the BARD1

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TABLE 2
Variants in *BAP1* and *CDKN2A* Identified Through Targeted Sequencing

Sample	Study	Gene	Exon	Position	Reference	NT change	AA change	rsID	ESP6500MAF*	Sift	Polyphen 2	Gerp ++
006090	Low	BAP1	5	52441988	NM_004656	c.361G>C	p.G121R	–	–	Damaging	Damaging	5.14
002203	Low	BAP1	7	52441322	NM_004656	c.448C>T	p.R150C	–	–	Damaging	Damaging	5.38
009341	Intermediate	BAP1	9	52440388	NM_004656	c.664C>A	p.P222T	–	–	Damaging	Damaging	5.69
011937	High	BAP1	13	52437824	NM_004656	c.1337A>T	p.N446I	–	–	Tolerated	Damaging	4.76
052809	Intermediate	BAP1	13	52437606	NM_004656	c.1555C>G	p.P519A	–	–	Damaging	Damaging	5.7
050611	Intermediate	BAP1	14	52437234	NM_004656	c.1810G>A	p.V604M	–	0.000154	Tolerated	Benign	1.49
051964	Low	BAP1	14	52437234	NM_004656	c.1810G>A	p.V604M	–	0.000154	Tolerated	Benign	1.49
006528	Low	CDKN2A (p14)	2	21971040	NM_058195	c.361G>A	p.A121T	rs199888003	0.000154	Tolerated	NA	2.33
002507	Low	CDKN2A (p14)	2	21971040	NM_058195	c.361G>A	p.A121T	rs199888003	0.000154	Tolerated	NA	2.33
012039	Childhood	CDKN2A (p16)	1	21974780	NM_000077	c.47T>C	p.L16P	–	–	Damaging	Damaging	4.14
050731	Intermediate	CDKN2A (p16)	1	21974763	NM_000077	c.64C>T	p.R22W	–	–	Damaging	Damaging	-0.43
010255	Low	CDKN2A (p16)	1	21974724	NM_000077	c.103G>A	p.G35R	–	–	Damaging	Probably damaging	4.84
051362	Intermediate	CDKN2A (p16)	1	21974682	NM_000077	c.145A>T	p.I49F	–	–	Damaging	Damaging	4.22
008197	Low	CDKN2A (p16)	2	21971057	NM_000077	c.301G>T	p.G101W	rs104894094	–	Damaging	Probably damaging	5.49
061542	Adolescent	CDKN2A (p16)	2	21971057	NM_000077	c.301G>T	p.G101W	rs104894094	–	Damaging	Probably damaging	5.49

Note: *European American population; NA = not available; GERP++ is an estimate of the constrained elements in the human genome.

interacting domain (p.P222T), two novel variants occurred outside of any known domain (p.N446I and p.P519A) and a recurrent variant (p.V604M) was seen in two probands in the BRCA1 interacting domain (Figure 2). In our study, the MAF for p.V604M is 0.0018, compared to the MAF reported in the ESP6500 of 0.000154. Overall, 0.63% of cases harbored a missense variant in *BAP1*.

Since some missense mutations in BAP1 have been shown to affect alternative splicing (Popova et al., 2013; Wadt et al., 2012), the Automated Splice Site and Exon Definition Analyses (ASSEDA) online tool (https://splice.uwo.ca), was used to assess whether *BAP1* mutations in this study might create cryptic acceptor/donor sites (Rogan et al., 2003). No splice site alterations were predicted. Mutations in BAP1 occurred in highly conserved residues across species (Figure 1) and the five novel mutations (p.G121R, p.R150C, p.P222T, p.N446I, and p.P519A) were predicted to be damaging by SIFT and/or Polyphen 2 (Table 2).

Discussion

We identified *CDKN2A* missense mutations in 0.57% of melanoma cases in the intermediate, low, twin, childhood, adolescent, and men over 50 cohorts. When this data is combined with that for the QFMP high-risk group published by Aitken et al. (1999), 1.31% of the overall Queensland population-based sample of CMM cases carried a *CDKN2A* mutation. The estimate for *CDKN2A* we report here is in keeping with that reported (~2%) in the GEM study (Begg et al., 2005). As might be expected, since *CDKN2A* mutation has been associated with an increased risk of development of cancer in general (Mukherjee et al., 2012), *CDKN2A* mutation-positive families in this study are enriched for other cancers (Tables 3 and 4), although absolute numbers are too low to show statistically significant association with any particular cancer type other than melanoma. Furthermore, because the number of mutations we observe is low, there is no statistical difference between the individual Q-MEGA population groups.

The *CDKN2A* p.G35R mutation has been reported to occur somatically in the COSMIC database, but here we report for the first time its occurrence in the germline of an individual presenting with melanoma and colorectal cancer. A study using both functional and computational prediction of p16 mutations has found that the pathogenicity of this mutation is uncertain (Scaini et al., 2014). The p.A148T variant was observed at a frequency of 6.8% in the Queensland melanoma cases. This substitution is generally not thought to be deleterious, but the functional effect of this variant has been debated in the literature (Debniak et al., 2005; Spica et al., 2006). It has been shown to occur more frequently in Celtic populations and therefore its potential association with CMM risk may be due to its prevalence in a more melanoma-prone population (Aitken et al., 1999).

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TABLE 3

***CDKN2A* Mutations Reported in Publically Available Databases LOVD and COSMIC**

AA change	Present in LOVD	Present in COSMIC CMM	Present in COSMIC in other cancers
p.L16P	yes	no	biliary tract carcinoma, upper aerodigestive tract
p.R22W	no	no	no
p.R24P	yes	yes	CNS (glioma), soft tissue sarcoma
p.G35R	no	no	ovary, pancreas
p.I49F	no	no	no
p.G101W	yes	yes	lung carcinoma

Note: LOVD = Leiden open variation database 3.0, which lists published germline *CDKN2A* variants in cancer; COSMIC = catalogue of somatic mutations in cancer v68; CMM = cutaneous malignant melanoma; CNS = central nervous system.

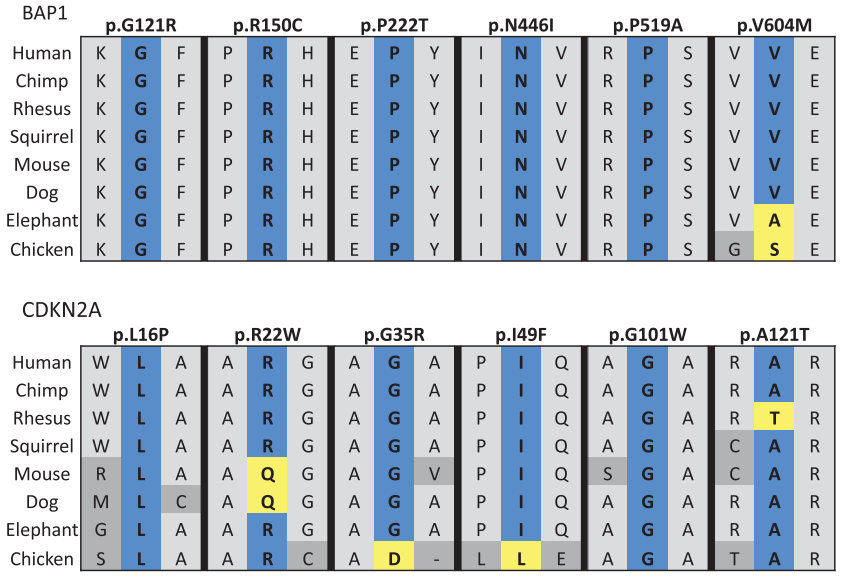

FIGURE 1

(Colour online) Conservation across species.
Note: Conservation of protein altering missense variants in BAP1 and CDKN2A.

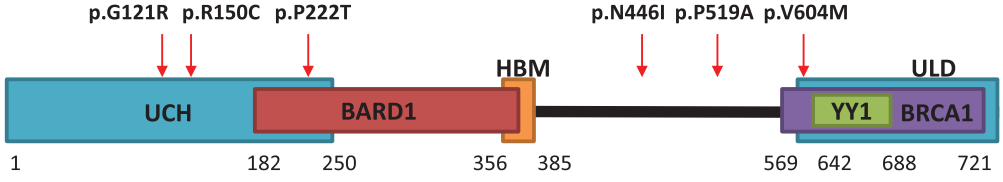

FIGURE 2

(Colour online) Germline variants in functional domains and protein interaction regions of BAP1.
Note: All germline variants found in the population-based sample of cutaneous melanoma cases are shown in relation to their position in the protein. The arrows show the position of germline variants. UCH is the ubiquitin carboxy-terminal hydrolase domain; HBM is the HCFC1 binding motif; ULD is the UCH37-like domain. Binding sites for genes BARD1, BRCA1 and YY1 are depicted by their gene symbols.

We found that 0.63% of CMM cases harbored germline missense mutations in *BAP1*. This mutation rate is similar to that reported by Njauw et al. (2012) when they screened a hospital-based sample of 193 CMM families for *BAP1* mutation and reported one truncating mutation (0.5%). To date, all disease-associated variants in *BAP1* have been shown to truncate the protein. The variants we report here are all missense mutations that occur at highly conserved residues in mammals (Figure 2). None were predicted to alter splicing; thus, at this stage it is not clear whether they are responsible for melanoma susceptibility in these individuals, or if they represent rare, benign polymorphisms.

We did not observe any mutations in *CDK4*. This result is in keeping with the low frequency of mutations of this

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TABLE 4

Summary of Cancers in Families With Germline *BAP1* and *CDKN2A* Mutations

Proband	Gene	AA change	Study	Ages of onset of CMM	Other cancers in proband (age)	Other cancers in family (age)
006090	BAP1	p.G121R	Low	45, 72		
002203	BAP1	p.R150C	Low	32, 57	breast (57)	
009341	BAP1	p.P222T	Intermediate	36		CMM (37)
011937	BAP1	p.N446I	High	53	lung (65), lung (68)	CMM (60)
052809	BAP1	p.P519A	Intermediate	41		CMM (36)
050611	BAP1	p.V604M	Intermediate	64		CMM (33)
051964	BAP1	p.V604M	Low	59		lung (65)
006528	CDKN2A (p14)	p.A121T	Low	69		
002507	CDKN2A (p14)	p.A121T	Low	38		lung (65), colorectal (64)
012039	CDKN2A (p16)	p.L16P	Childhood	12, 18, 22		CMM (19), CMM (21), CMM (23), CMM (25), CMM (43), CMM (44), Lung (40)
050731	CDKN2A (p16)	p.R22W	Intermediate	45,		CMM (26), pancreas (52), oesophageal (76), prostate (58), stomach (63)
010255	CDKN2A (p16)	p.G35R	Low	78	colorectal (86)	stomach (45), breast (66), oesophagus (60)
051362	CDKN2A (p16)	p.I49F	Intermediate	26, 26, 27, 29, 45		CMM (38), breast (38), breast (48), lung (84)
008197	CDKN2A (p16)	p.G101W	Low	47, 71		CMM (28), CMM (44)
061542	CDKN2A (p16)	p.G101W	Adolescent	18, 30, 30, 35		CMM (36), cervix (31), lung (69)

Note: CMM = cutaneous malignant melanoma.

gene reported in the literature. To date, only 17 families worldwide have been documented to carry *CDK4* mutations (Puntervoll et al., 2013; Soufir et al., 1998; Zuo et al., 1996). All reported pathogenic mutations in p14 have been splice mutations, whole gene deletions or insertions (Harland et al., 2005; Mistry et al., 2005; Randerson-Moor et al., 2001; Rizos et al., 2001). Currently, no pathogenic missense mutations are known to occur in p14.

In summary, we report three novel variants in *CDKN2A* and three previously reported mutations, along with seven novel missense variants in *BAP1*. Germline mutations that we observe for the two genes combined (~2%) thus account for only a small proportion of all CMM cases in this population. This suggests that genes other than the high-penetrance familial melanoma loci are responsible for the bulk of CMM susceptibility in the general population.

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Supplementary Material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/thg.2015.12

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