A novel recurrent mutation in *MITF* predisposes to familial and sporadic melanoma

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Abstract

So far, two familial melanoma genes have been identified, accounting for a minority of genetic risk in families. Mutations in *CDKN2A* account for approximately 40% of familial cases[1], and predisposing mutations in *CDK4* have been reported in a very small number of melanoma kindreds[2]. To identify other familial melanoma genes, here we conducted whole-genome sequencing of probands from several melanoma families, identifying one individual carrying a novel germline variant (coding DNA sequence c.G1075A; protein sequence p.E318K; rs149617956) in the melanoma-lineage-specific oncogene microphthalmia-associated transcription factor (*MITF*). Although the variant co-segregated with melanoma in some but not all cases in the family, linkage analysis of 31 families subsequently identified to carry the variant generated a log odds ratio (lod) score of 2.7 under a dominant model, indicating E318K as a possible intermediate risk variant. Consistent with this, the E318K variant was significantly associated with melanoma in a large Australian case–control sample. Likewise, it was similarly associated in an independent case–control sample from the United Kingdom. In the Australian sample, the variant allele was significantly over-represented in cases with a family history of melanoma, multiple primary melanomas, or both. The variant allele was also associated with increased naevus count and non-blue eye colour. Functional analysis of E318K showed that MITF encoded by the variant allele had impaired sumoylation and differentially regulated several MITF targets. These data indicate that *MITF* is a melanoma-predisposition gene and highlight the utility of whole-genome sequencing to identify novel rare variants associated with disease susceptibility.

Cutaneous malignant melanoma is predominantly a disease of fair-skinned individuals. Aetiology is complex, with environmental (mainly ultraviolet radiation exposure) and genetic factors affecting disease risk. Phenotypic risk factors, which are largely heritable, include pigmentation (fair skin, blue or green eyes, blonde or red hair), sun sensitivity, an inability to tan[3–6], high number of melanocytic naevi[7,8], or the presence of clinically atypical naevi[7]. Candidate-gene studies and genome-wide association studies (GWAS) for melanoma and these melanoma-associated phenotypes have identified several variants associated with melanoma risk in the general population[9–13]. Family studies, on the other hand, have identified only two high-penetrance melanoma genes, *CDKN2A* (ref. 1) and *CDK4* (ref. 2), accounting for a minority of genetic risk in melanoma families.

As part of a larger sequencing effort to identify novel melanoma risk genes, we sequenced the genome of an affected individual from an eight-case melanoma family negative for alterations in *CDKN2A* or *CDK4* (Fig. 1, FAM1) using a nanoarray-based short-read sequencing-by-ligation strategy[14]. From among the 410 novel variants predicted to affect protein structure, we prioritized for follow-up a single nucleotide polymorphism (SNP) resulting in a glutamic acid to lysine substitution in MITF (E318K, codon numbering based

on the melanocyte-specific MITF-M isoform; c.G1075A, NCBI accession NM_000248.3; p.E318K, NCBI accession NP_000239.1; rs149617956). Although linkage[15] and GWAS studies[9,10] have not provided evidence implicating *MITF* in either predisposition to melanoma or the melanoma-associated phenotypes of pigmentation and naevogenesis[11,12,16–19], MITF is known to regulate a broad repertoire of genes whose functions in melanocytes range from development, differentiation, survival, cell-cycle regulation and pigment production. *MITF* is somatically amplified[20,21] or mutated[22] in a subset of melanomas, and strongly overexpressed in others[20], making it an attractive candidate despite the lack of prior evidence for involvement in germline risk.

We evaluated whether *MITF* E318K is a high-penetrance melanoma susceptibility variant in Family 1 by genotyping the remaining affected individuals available for study. The *MITF* variant allele was found in 3/7 melanoma cases assessed in this family (Fig. 1), consistent with it being a medium-penetrance melanoma risk variant. To assess further this possibility, we genotyped two large Australian melanoma case–control samples for *MITF* E318K. The variant was found in 14/1,953 controls (carrier frequency = 0.0072) and thus represents a rare population variant (Table 1). We observed a significantly higher frequency (34/2,059) in cases (carrier frequency = 0.0165) than controls (Fisher exact $P = 0.008$, odds ratio (OR) 2.33, 95% confidence interval (CI) 1.21–4.70), indicating that the variant correlates with increased melanoma risk in the general population. The effect size for E318K is larger than those reported for variants from melanoma GWAS[9,10] and similar to that observed for red-hair-colour-associated variants of the melanocortin 1 receptor (*MC1R*) gene (OR for most populations ~2.4)[23]. Among cases, the *MITF* E318K variant was enriched in those with multiple primary melanomas (OR 4.22, 95% CI 1.52–10.91), a family history of melanoma (OR 2.95, 95% CI 1.23–6.92), or both (OR 8.37, 95% CI 2.58–23.80), but not in cases with earlier age of onset (comparing diagnosis before age 40 versus after 40 years) (Table 2).

We replicated these findings in two independent population-based case–control samples from the United Kingdom. In the combined UK sample, the variant allele frequency was also significantly higher in cases (carrier frequency = 0.0176) than controls (carrier frequency = 0.0085, $P = 0.012$, OR 2.09, 95% CI 1.14–3.94, Table 1). The association with melanoma in the pooled UK and Australian data was highly significant (combined $P = 0.0003$, OR 2.19, 95% CI 1.41–3.45). In the UK cases there were also trends towards family history, earlier age of onset, and the occurrence of more than one primary melanoma in variant carriers (Table 2).

To extend assessment of the MITF variant in melanoma-prone families, we screened for E318K in 182 UK families with at least two melanoma cases and 88 Australian families with at least three cases, all of which are negative for mutations in *CDKN2A* or *CDK4*. Six families (2.2%) were found to carry the variant. In the UK, E318K was enriched in the more melanoma-dense families; 4/54 (7.4%) families with at least three melanoma cases versus 1/128 (0.8%) families with two melanoma cases (Fisher's exact $P = 0.013$). We subsequently evaluated whether *MITF* E318K co-segregated with melanoma in these as well as additional multiple-case families identified from the case–control sample. In total, we identified 31 unrelated cases carrying *MITF* E318K from Australia and the UK with at least one first- or second-degree relative diagnosed with melanoma (listed in Supplementary Table 1; Supplementary Fig. 3), 22 of which had DNA available from additional affected family members for genotyping. In 9/31 families (five three-case and four two-case families) the variant was found in all affected individuals (Supplementary Fig. 3a; non-segregating families shown in Supplementary Fig. 3b), whereas in 12 additional families, the variant co-segregated with melanoma in the available cases, but DNA from all cases was not available for screening (Supplementary Fig. 3c). To test more formally for linkage of melanoma with

MITF E318K in these families, we calculated a lod score of 2.7 under a dominant model, again consistent with an incompletely penetrant medium risk variant.

To assess whether the *MITF* variant is related to known melanoma-associated risk phenotypes of pigmentation and naevus count, we tested for association both in cases and controls from the Australian and British populations. The *MITF* variant allele is significantly associated with increased naevus count (combined *P* = 0.002, OR 2.54, 95% CI 1.42–4.55; Supplementary Table 2) and non-blue eye colour (combined *P* = 0.018, OR 2.01, 95% CI 1.11–3.81; Supplementary Table 3). It was not associated with skin colour, hair colour, or freckling (Supplementary Table 4). Reassessing the case–control analysis accounting for naevus count and eye colour gave a slightly reduced effect size for association of the variant with melanoma (OR 1.82, 95% CI 0.85–3.92), suggesting that the risk of melanoma attributable to *MITF* E318K may be mediated at least in part via one or both of these phenotypes, but that there is a substantial residual risk conferred by the variant through an as yet undetermined mechanism.

We next sought to evaluate whether the E318K mutation alters MITF function. The E318K variant is located within one of two IKXE consensus sites on MITF previously shown to be post-translationally modified by the addition of the small ubiquitin-like-modifier SUMO[24]. Mutation of the residue to which SUMO is covalently attached in this motif (K316R) has previously been shown to abrogate MITF sumoylation and significantly increase MITF transcriptional activity *in vitro*[24,25]. We thus hypothesized that E318K would similarly alter sumoylation and transcriptional activity of MITF. To test this we constructed a cDNA encoding His-tagged MITF carrying the E318K mutation. We evaluated the effects of E318K on sumoylation in comparison to the wild type and previously characterized synthetic mutations of the two known MITF sumoylation sites (K316R and K182R) by co-transfecting with haemagglutinin (HA)-tagged SUMO1 in COS-7 cells (Fig. 2a). Wild-type MITF shows two SUMO1-modified forms, whereas MITF mutants K182R or K316R each show only one modified form (Fig. 2a). Similar to the synthetic K316R and K182R mutants, E318K abrogates sumoylation, resulting in complete loss of the doubly sumoylated form of MITF and reducing the mono-sumoylated form. When the second site is mutated (K182R) simultaneously with E318K, MITF sumoylation is completely abolished. Immunoprecipitation of endogenously expressed MITF E318K from melanoma cells homozygous for E318K (NAE) when transfected with SUMO similarly revealed only bands corresponding to mono- and non-sumoylated isoforms of MITF on western blot (Fig. 2b).

We then looked for differences between mutant and wild-type MITF transcriptional activity using a reporter construct containing the MITF-responsive *TRPM1* promoter[25]. At two concentrations tested, the E318K mutant exhibited 1.34–1.40 fold induction of the *TRPM1* luciferase reporter relative to wild-type MITF (Fig. 2c). This fold induction is similar to that observed previously on multiple MITF target promoters using single or double artificial sumoylation-site MITF mutants[24,25] and suggests that the E318K variant found in melanoma changes the transcriptional potency of MITF. To study this in greater detail, we determined the effect of the E318K mutation on global MITF target gene transcription. We developed a tetracycline-inducible system for expression of wild-type MITF or the E318K variant in melanoma cell lines with constitutively low or undetectable levels of endogenous MITF (HT144 and C32, respectively[26], Fig. 2d). At the phenotypic level, induction of wild-type or E318K MITF led to increased proliferation compared to uninduced controls for each of the cell lines, although there was no significant difference in growth rate between the cells expressing the different isoforms (data not shown). We examined whole-genome expression profiles in these cells following induction of either wild-type or E318K MITF for 48 h. Of the 37 genes commonly regulated by wild-type and E318K MITF in both cell lines (Supplementary Fig. 1a, b; see Methods for analysis details), 28 (76%) had previously been

identified as MITF target genes (Supplementary Table 5)[27,28], and 17 showed ≥1.25-fold differences in expression between the wild-type and E318K isoforms (Supplementary Fig. 1b). We also identified two gene products that were uniquely differentially regulated compared to uninduced cells by the induction of wild-type MITF but not MITF E318K in both parental cell lines, and 16 gene products after induction of MITF E318K but not wild-type MITF (Supplementary Table 6). Of these, 61% (11/18) have previously been reported as MITF targets (Supplementary Table 6)[27,28]. Collectively, these data indicate that the MITF E318K mutant exhibits differential transcriptional activity against some, although not all, target genes. In agreement with the reporter assays (Fig. 2c), we identified transcriptional differences in gene products known to be involved in pigmentation (*DCT*, *MLANA*), in which the differences were more marked with expression of the E318K variant in comparison to wild-type MITF. These were validated by quantitative polymerase chain reaction with reverse transcription (qRT–PCR) in the cell lines used for microarray analysis (Fig. 2e), as well as in an additional melanoma cell line constitutively expressing wild-type or E318K mutant MITF (Fig. 2f and Supplementary Fig. 2). In keeping with the increase in expression of these pigmentation genes, we detected a 22% increase in melanin content in HT-144 melanoma cells 72 h after induction of MITF E318K compared to wild-type MITF (data not shown). This is also consistent with our observation that carriers are more likely to have darker (that is, non-blue) eye colour (Supplementary Table 3) but, notably, these data contrast with other previously reported 'fair-skin-associated' melanoma risk variants, such as those in *MC1R* or *TYR*. It is uncertain whether the enhanced expression of pigment genes may contribute to melanomagenesis, perhaps by increasing oxidative stress and an increase in oxidative DNA damage[29], or alternatively may simply reflect increased MITF activity, which (separately) promotes tumorigenesis, as *MITF* is a previously recognized amplified melanoma oncogene[20].

We adopted the approach of whole-genome sequencing of patients from melanoma families and identified a novel germline mutation of *MITF*. This mutation was found to be present in numerous melanoma families, as well as the general population, in which its association with melanoma has an effect size similar to red-hair-causing variants of *MC1R*[23]. The melanoma susceptibility genes discovered through GWAS so far account for only a minority of inherited disease risk. A proportion of this 'missing heritability' may be due to rare sequence variants, which are poorly detected by GWAS using SNP arrays. The new *MITF* variant reported here shows reasonably strong linkage to melanoma (lod score 2.7) but crucially not a high enough signal to be clearly visible in previous genome-wide linkage scans. We also provide *in vitro* data supporting a functional mechanism by which this mutation may mediate melanoma risk, specifically abrogation of MITF sumoylation and differential transcription of select MITF target genes. Although the individual changes in transcription induced by the mutant E318K MITF in comparison to wild-type MITF are modest, the orchestrated change in the levels of multiple MITF target genes is likely to be biologically important, especially over the lifetime of a person. This study offers a rare glimpse of a complex functionality whereby a risk-conferring SNP affects the post-translational processing of a crucial lineage-specific survival and differentiation gene. This study demonstrates the utility of performing whole-genome and exome resequencing in appropriate affected individuals to identify such novel rare disease-specific variants and functionally characterize variants associated with complex disease not otherwise detectable via GWAS or linkage approaches.

METHODS SUMMARY

The collection of the Australian melanoma families used for the study, as well as the Queensland and AMFS case–control sets are described elsewhere and in Methods. Likewise the UK studies from Leeds and Cambridge as well as the panel of melanoma cell lines.

Whole-genome sequencing, assembly and variant calling were performed by Complete Genomics, as described previously[14]. Genotyping of *MITF* E318K was performed using the Sequenom MassArray system (Australian studies) or a custom TaqMan assay (UK studies), with DNA from the affected family member in which E318K was identified included multiple times as a positive control. Statistical analyses are described in detail in Methods. Co-segregation analyses were performed in melanoma families via Sanger sequencing using the primers: forward, 5′-CAGGCTCGAGCTCATGGA-3′; reverse, 5′-TGGGGACACTATAGGCTTGG-3′. MITF sumoylation and *TRPM1* reporter assays were performed as previously described[25].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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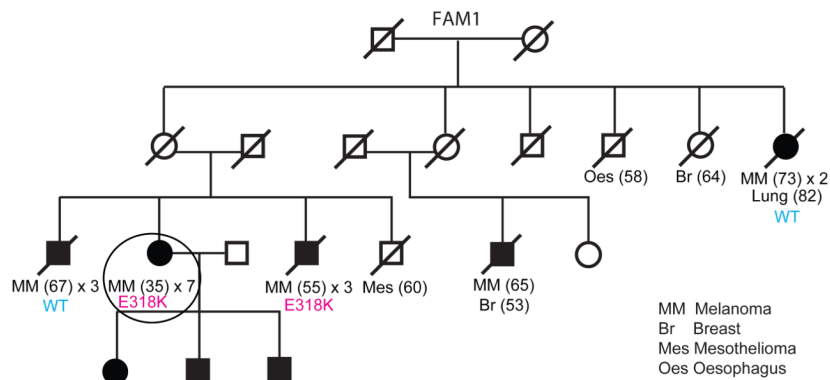
Figure 1. Co-segregation analysis of the *MITF* E318K variant in the family in which it was identified

The pedigree shows individuals that have had melanoma (shaded circles or boxes), with the age of first melanoma diagnosis indicated in brackets and the number of melanomas that have occurred in the individual so far (for example, × 2 indicates two primary melanomas). If the number of melanomas is not stated, the individual has had a single melanoma. A diagonal line through the symbol indicates that the person is deceased. The genotype for the *MITF* E318K variant for individuals with an available DNA sample for testing is annotated 'E318K' if a carrier or wild type 'WT'. A presumed obligate carrier is designated '(E318K)'. A presumed wild-type individual with melanoma is designated '(WT)'. Other cancer types are also indicated with the age of first diagnosis indicated in brackets if known. Br, breast; Mes, mesothelioma; MM, melanoma; Oes, oesophagus. The individual circled in Family 1 (FAM1) is the melanoma case in which the *MITF* E318K variant was discovered through whole-genome sequencing. See Supplementary Fig. 3 for pedigrees of all other families identified as carrying E318K.
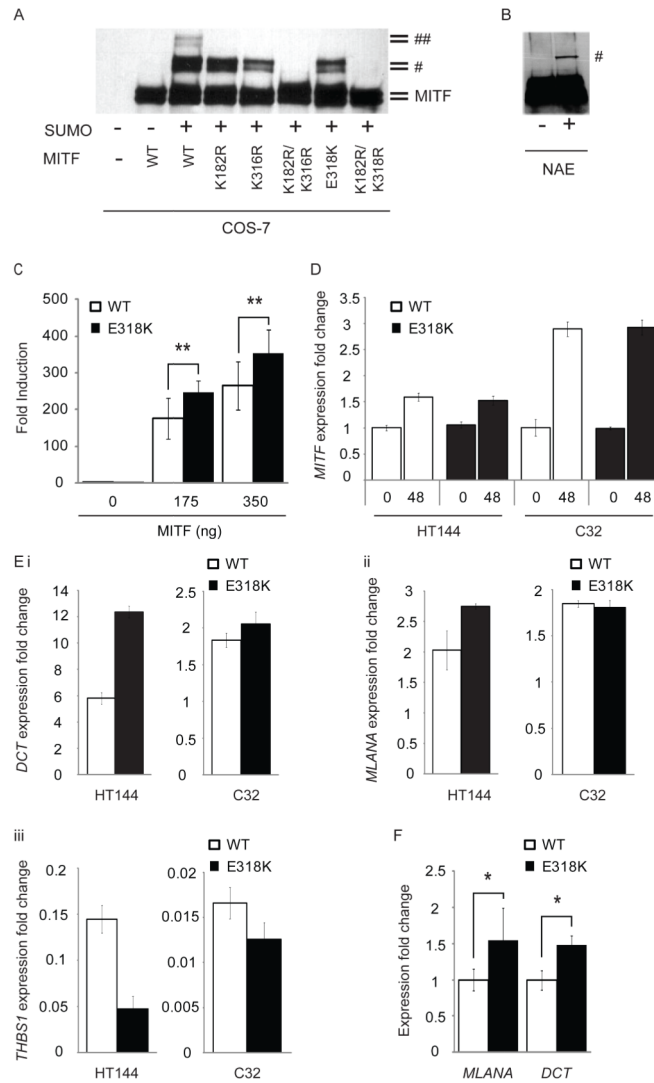
Figure 2. E318K prevents MITF sumoylation and results in differential expression of MITF target genes

a, His-tagged wild-type MITF or the indicated single or double point mutants were co-transfected with HA–SUMO1 in COS-7 cells or **b**, HA–SUMO was transfected alone into homozygous mutant E318K MITF melanoma cells (NAE). Single- and double-sumoylated forms of MITF are indicated by a dagger and double dagger, respectively. The doublet bands are caused by MAPK-mediated phosphorylation at serine 73 (ref. 30). **c**, UACC62 human melanoma cells were transfected with *TRPM1*-promoter constructs with indicated amounts of expression vector encoding wild-type or mutant forms of MITF. Fold induction is shown as the ratio to the average of no MITF transfection (0 ng). Data are mean ± s.d. of at least four independent experiments. **d**, Expression of *MITF* in two melanoma cell lines (HT144 and C32) engineered to inducibly express wild-type (WT) or mutant (E318K) MITF after treatment with tetracycline for 48 h (48), as determined by qRT–PCR. Performed in triplicate, error bars depict s.d. **e**, Expression of MITF target genes *DCT* (top left), *MLANA* (top right) and *THBS1* (bottom left) determined by qRT–PCR in melanoma cell lines 48 h after induction of wild-type or E318K MITF. Gene expression is normalized to *GAPDH* and shown as fold change compared to uninduced cells. Performed in triplicate, error bars denote s.d. **f**, qRT–PCR analysis of total RNA isolated from UACC62 human melanoma cells,

which were transfected with expression vector encoding wild-type or mutant forms of MITF. The expression level of each target gene was normalized to *MITF* mRNA. Fold induction is shown as the ratio to each mRNA expression with wild-type *MITF*. Data are mean ± s.d. of at least three independent experiments. *$P < 0.05$, **$P < 0.01$.

Table 1

MITF E318K association with melanoma

Population	Group	No. of individuals with variant	No. of individuals without variant	Variant carrier frequency	OR	95% CI	One- sided exact *P*	Two- sided exact *P*
Australia	Cases	34	2,025	0.0165	2.33	1.21–4.70	0.0045	0.0083
	Controls	14	1,939	0.0072				
UK	Cases	34	1,895	0.0176	2.09	1.14–3.94	0.0074	0.0115
	Controls	18	2,097	0.0085				
Australia + UK	Cases	68	3,920	0.0171	2.19	1.41–3.45	0.0001	0.0003
	Controls	32	4,036	0.0079				

It should be noted that the reported allele frequencies for *MITF* E318K in the population-based samples are without removing individuals with *CDKN2A* or *CDK4* mutations, as screening for these genes was not routinely performed.

Table 2

Association of *MITF* E318K with melanoma-associated variables

Case set	Australia				UK			
	Carrier frequency	Carrier counts (carrier/wild type)	OR vs Aus controls	95% CI	Carrier frequency	Carrier counts (carrier/wild type)	OR vs UK controls	95% CI
All cases	0.0165	34/2,025	2.33	1.21–4.70	0.0176	34/1,895	2.09	1.14–3.94
Age of onset <40 years	0.0139	14/996	1.95	0.86–4.42	0.0174	5/283	2.10	0.60–5.91
Family history of melanoma	0.0209	12/563	2.95	1.23–6.92	0.0273	3/107	3.36	0.62–11.77
Multiple primary melanomas	0.0296	8/262	4.22	1.52–10.91	0.0225	2/87	2.74	0.30–11.74
Multiple melanomas and family history	0.0571	6/99	8.37	2.58–23.80	0.0000	0/10	-	-

Aus, Australia.