Accepted Manuscript

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PII: S0022-202X(16)00351-1

DOI: 10.1016/j.jid.2015.12.035

Reference: JID 140

To appear in: *The Journal of Investigative Dermatology*

Received Date: 14 October 2015

Revised Date: 26 November 2015

Accepted Date: 18 December 2015

Please cite this article as: Hacker E, Olsen CM, Kvaskoff M, Pandeya N, Yeo A, Green AC, Williamson RM, Triscott J, Wood D, Mortimore R, Hayward NK, Whiteman DC, Histologic and phenotypic factors and *MC1R* status associated with *BRAF*^{V600E}, *BRAF*^{V600K} and *NRAS* mutations in a community-based sample of 414 cutaneous melanomas, *The Journal of Investigative Dermatology* (2016), doi: 10.1016/j.jid.2015.12.035.

Histologic and phenotypic factors and *MC1R* status associated with *BRAF*^{V600E}, *BRAF*^{V600K} and *NRAS* mutations in a community-based sample of 414 cutaneous melanomas

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Short Title: Factors associated with *BRAF* and *NRAS* mutations in cutaneous melanoma

Abstract

Cutaneous melanomas arise through causal pathways involving interplay between exposure to ultraviolet radiation and host factors, resulting in characteristic patterns of driver mutations in *BRAF, NRAS* and other genes. To gain clearer insights into the factors contributing to somatic mutation genotypes in melanoma, we collected clinical and epidemiologic data, performed skin examinations, and collected saliva and tumor samples from a community-based series of 414 patients aged 18 to 79, newly diagnosed with cutaneous melanoma. We assessed constitutional DNA for 9 common polymorphisms in *MC1R.* Tumor DNA was assessed for somatic mutations in 25 different genes. We observed mutually exclusive mutations in $BRAF^{V600E}$ (26%), $BRAF^{V600K}$ (8%), $BRAF^{other}$ (5%), and *NRAS* (9%). Compared to patients with *BRAF* wild-type melanomas, those with $BRAF^{V600E}$ mutants were significantly younger, had more nevi, fewer actinic keratoses, were more likely to report a family history of melanoma and their tumors were more likely to harbor neval remnants; $BRAF^{V600K}$ mutations were also associated with high nevus counts. Both $BRAF^{V600K}$ and *NRAS* mutants were associated with older age but not with high sun exposure. We also found no association between *MC1R* status and any somatic mutations in this community sample of cutaneous melanomas, contrary to earlier reports.

WORD COUNT: **198**

Introduction

Melanoma is a potentially lethal cancer arising from the pigment cells, melanocytes. While ultraviolet (UV) radiation from sunlight is the principal environmental cause for these cancers, there is increasing evidence that the effect of UV radiation on melanocytes is not the same for all people (1). Epidemiologic observations originally led to the concept that melanomas may arise through one of several pathways under a 'divergent pathway model' for melanoma (2). This model suggested at least two different causal pathways to melanoma development, one pertaining to host susceptibility and nevus prevalence and the other associated with chronic sun exposure. Subsequent investigations strongly suggested that the molecular profile of tumors for several oncogenes including *BRAF* and *NRAS* reflected these causal pathways (2-5). Several studies have now illustrated that melanomas arising on the trunk tend to occur in younger individuals and are associated with adjacent melanocytic nevi and *BRAF* mutations, and these appear biologically distinct from melanomas arising on chronically sun-exposed sites, such as the head and neck, which tend to occur in older individuals carrying other mutation profiles including *NRAS* mutations (2-4, 6-11). More recent data have emerged suggesting that different genotypes exist within *BRAF*-mutant melanoma, and that melanomas harboring $BRAF^{V600K}$ mutations are associated with older age, male sex, higher levels of sun exposure, and poorer prognosis than $BRAF^{V600E}$ melanomas (12-14). Thus, there appear to be marked differences in the associations between sun exposure, melanocyte susceptibility and host characteristics with a suite of melanoma mutations, strongly suggestive of different causal pathways to melanoma development.

The melanocortin-1 receptor (*MC1R*) gene is a key determinant of human pigmentation with specific variants linked to red hair and melanoma risk (15, 16). An interaction between germline *MC1R* variants and somatic *BRAF* mutations was reported in tumors from US and Italian populations (5, 17), suggesting that people carrying

germline *MC1R* variants had a greater risk of developing a melanoma harboring a *BRAF* mutation in skin not damaged by sunlight. Analyses of Spanish and Austrian samples found no association between germline *MC1R* variants and somatic *BRAF* mutations across all tumor samples, but did observe a modest trend between germline *MC1R* status and somatic *BRAF* mutations in melanomas of the trunk [OR 1.8 (0.8-4.1, p=0.1] but an inverse association between *MC1R* and *BRAF* for melanomas of the head and neck [OR 0.3 (0.1-0.8), p=0.02] (9). However, the association between germline *MC1R* variants and somatic *BRAF* mutations has not been replicated in other populations, including studies from the USA (18), Australia (6) and Germany (19). Indeed, Scherer and colleagues (19) observed significantly lower frequencies of somatic *BRAF* mutations in carriers of *MC1R* variants. These conflicting findings across different populations underscore the complexity of gene-environment interactions for melanoma.

Given the emergence of novel therapies targeting somatic mutations in melanoma, coupled with the desire to develop evidence-based primary prevention programs, there is a need to catalog the frequency of mutations in large samples of melanoma patients and to understand the mechanisms through which they arise. Here, we present the findings of an investigation into the epidemiologic, histologic and genotypic associations with melanoma mutations, comprising a large, community-based sample of 414 primary cutaneous invasive melanoma patients arising in a high-incidence population exposed to very high levels of ambient UV radiation.

Results

Subject characteristics

A total of 766 patients with primary invasive melanoma were recruited for the parent epidemiological study (20), (32% female) with a mean age of 58 years. The majority of melanomas were classified histologically as SSM (72%), with the remainder classified as LMM (13%), nodular (5%) and unclassified (10%). Tumors were generally thin; 65% were Clark level II, and 82% had Breslow thickness ≤1 mm. The analyses presented here were restricted to 414 patients for whom sufficient material was remaining for somatic mutation analysis (Figure S1). There were no significant differences between those genotyped (n=414) and those not (n=352), in terms of sex (71% vs 65% males) or melanoma thickness distribution (84% vs 81% ≤1mm) but participants not genotyped were slightly older (56.3 years vs 59.8, p=0.04) and were more likely to have melanomas of the head and neck (9.2% vs 17.6%, p <0.001) and of the lentigo maligna subtype (15.7% vs 24.7%, p=0.005).

Mutation Frequencies

Mutations were identified using the MelaCarta multiplex assay (Agena Bioscience); mutually exclusive *BRAF*-mutant and *NRAS*-mutant tumors occurred at frequencies of 38.7% (*V600E* 67%, *V600K* 31%, Other 12%) and 9.2% (Q61H 5%, Q61K 37%, Q61L 24%, Q61R 34%), respectively (Table 1 and Table S1). Further statistical analysis was performed for the *BRAF* and *NRAS* mutant samples, due to the low frequencies of mutations in other genes.

Clinical and Pathologic Characteristics of lesions

Overall, patients older than 70 years were significantly less likely to have $BRAF^{V600E}$ mutant melanomas than *BRAF* wild-type melanomas (OR 0.08, 95% CI 0.03-

0.19, but were more likely to have melanomas harboring *BRAF*^{V600K} or *NRAS* mutations (Table 2 and Table S2). *BRAF*^{V600E} mutations were significantly more frequent in melanomas from women (p=0.01), whereas *NRAS* mutations were more common in melanomas from men (p=0.01). We observed that *BRAF*^{V600E} (p=0.01) and *BRAF*^{V600K} (p=0.047) mutant melanomas were more likely to harbor somatic mutations in other genes on the 'melanoma panel' than *NRAS* mutant melanomas (Table 2 and Table S2). Although numbers were small, melanomas carrying somatic mutations in *KRAS* or *EPHB6* were more likely to carry additional mutations than melanomas without these mutations (Table S3). While the prevalence of *BRAF* and *NRAS* mutations differed somewhat by histological subtype and anatomic site, the differences were not statistically significant. We found no statistical evidence that the risks of *BRAF*^{V600E} or *BRAF*^{V600K} mutations differed by Clark level or tumor thickness, but somewhat against expectation, we found melanomas with *NRAS* mutations were significantly less likely to have marked dermal elastosis (OR 0.26, 95%CI 0.07-0.95; p=0.03). Melanomas with *BRAF*^{V600E} mutations were significantly more likely to have contiguous neval remnants than wild-type melanomas (OR 1.94, 95% CI 1.14-3.31; p=0.02), but melanomas carrying other *BRAF* or *NRAS* mutations were not significantly associated with this feature.

Phenotypic and environmental factors associated with BRAF and NRAS Mutations

We observed strong positive associations between increasing nevus count and risk of *BRAF*^{V600E} (p-trend=0.03) or *BRAF*^{V600K} (p-trend=0.02) mutations (Table 3 and Table S4), but no associations with *NRAS* mutations. In contrast, there were inverse associations between the numbers of excised skin cancers and *BRAF*^{V600E} mutational status (p-trend=0.04); numbers of skin cancers were also inversely associated with *BRAF*^{V600K} mutations although the trend was of marginal significance (p-trend=0.06). The measure of cumulative sun exposure (summed from a matrix capturing recreational and

occupational sun exposure for all career episodes since leaving high school) showed an unusual pattern of association with *BRAF*^{V600E} and *BRAF*^{V600K} mutations. Although not statistically significant, relative risks of *BRAF*-mutant melanoma were higher for patients with intermediate categories of cumulative sun exposure than for those with the highest levels of sun exposure. No consistent associations between markers of cumulative sun exposure and risks of *NRAS* mutant melanoma were observed, although it was notable that risk estimates were less than unity for all categories of solar keratosis counts and for having 3 or more skin cancers excised (Table 3). There were no consistent associations between hair or eye color and risks of any type of *BRAF* mutations or *NRAS* mutations, however patients with *BRAF*^{V600K} mutant melanomas were significantly more likely to have blue/gray eye color (OR 2.38, 95% CI 1.00-5.65; p=0.049). In addition, patients with *BRAF*^{V600E} mutant melanomas were significantly less likely (p-trend=0.02) to report having any extent of facial freckling as a teenager compared with patients with *BRAF* wild-type melanomas; associations between freckling and other mutation types were not significant. A family history of melanoma was associated with *BRAF*^{V600E} mutation status (OR 1.85, 95% CI 1.06-3.21; p=0.03). Other characteristics were assessed for associations with *BRAF/NRAS* mutational status, but in the main these were unremarkable (Table S5).

Somatic mutations and *MC1R* Variants

84% of melanoma patients in this series carried one of the nine common *MC1R* variants, with 53% carrying red hair color variants (RHC) and 31% carrying non red hair variants (NRHC) (Table S6 and S7). As expected, *MC1R* status was associated with red hair color (p<0.001) and skin type determinants such as susceptibility to burn (p<0.001), propensity to tan (p<0.001) and was inversely associated with nevus counts (p=0.02) (Table 4). There was no association between germline *MC1R* variants and somatic *BRAF* or *NRAS* mutations in melanoma samples overall (Table 5 and S8). In site-specific

analyses (trunk melanoma; head and neck melanoma), we found no evidence that the risks of *BRAF* mutations were associated with *MC1R* variants, regardless of the type of variant. We repeated the analyses by excluding patients with LMM subtype but this made no material difference to our conclusions (Table S9). We also observed no association between the number of *MC1R* polymorphisms and either *BRAF* (p=0.38) or *NRAS* (p=0.83) mutation status.

Discussion

We assessed the frequency of somatic mutations in 25 putative 'melanoma genes' in a large community-based series of 414 primary cutaneous invasive melanomas. Mutation prevalences were less than 2.5% for all genes except *BRAF* and *NRAS*, which occurred mutually exclusively at frequencies of 39% and 9%, respectively. Previous community-based series of primary cutaneous melanomas have reported *BRAF* mutations at frequencies ranging from 32% to 39% in Australian populations, 20-22% in Spanish, Austrian and German populations, 44% and 64% in Italian populations, and 43% in the USA (4, 6, 9, 19, 21). In all prior series, $BRAF^{V600E}$ mutations were at least 3 to 4-fold more common than $BRAF^{V600K}$ mutations, as we found. Importantly, we found that *BRAF*-mutant melanomas were significantly more likely than wild-type *BRAF* melanomas to carry mutations in other genes on the melacarta panel, whereas *NRAS* mutant melanomas were not.

As we expected, we found that the somatic mutation status of melanomas was correlated with a number of clinical and phenotypic features. $BRAF^{V600E}$ mutant melanomas were more likely than wild-type *BRAF* melanomas among women, younger patients, and those with high nevus counts, contiguous neval remnants adjacent to the tumor, and a family history of melanoma. These findings accord with previous studies examining *BRAF* status and characteristics of patients with cutaneous melanoma (4, 22). In addition, patients with $BRAF^{V600E}$ tumors were less likely than those with wild-type *BRAF* tumors to have phenotypic features indicative of high cumulative sun exposure such as high numbers of actinic keratoses or a history of prior skin cancer excisions,.

Earlier reports have suggested that melanomas carrying $BRAF^{V600K}$ mutations have been exposed to higher levels of cumulative sun exposure than other melanomas, but we found no evidence to support that conclusion (12-14). In our large series, patients with $BRAF^{V600K}$ melanomas were significantly less likely than patients with wild-type

BRAF melanomas to report prior history of non-melanoma skin cancer, and were not significantly different in terms of self-reported lifetime sun exposure, numbers of actinic keratoses or dermal elastosis adjacent to the melanoma. Even though the series reported here is the largest and most comprehensively annotated to date, the number of cases with $BRAF^{V600K}$ mutations was still modest (n=33) and so our study suffers from lack of statistical power to explore these associations fully. Pooling data from comparable studies to increase the sample size would permit more definitive assessments of the role of cumulative sun exposure in the development of $BRAF^{V600K}$ melanomas. We note with interest however, that $BRAF^{V600K}$ melanomas were even more strongly associated with total nevus count than $BRAF^{V600E}$ melanomas, providing strong evidence that these tumors arise through a 'nevus-prone' pathway.

A synergistic relationship between germline *MC1R* variants and somatic *BRAF* mutations was suggested by Landi and colleagues (5), whereby people with *MC1R* variant genotypes carried a significantly increased risk of developing *BRAF*-mutant melanoma in skin not damaged by sunlight. Analyses of Spanish and Austrian samples found a modest trend between germline *MC1R* status and somatic *BRAF* mutations in melanomas from trunk sites with an inverse association between *MC1R* and *BRAF* for melanomas of the head and neck (9), other studies conducted in North Carolina (18), Australia (6) and Germany (19) have not observed associations between *MC1R* status and increased risk of somatic *BRAF* mutations. This latest investigation, comprising a community-based sample of 414 patients with cutaneous melanoma of predominantly northern European and Anglo-Celtic ancestry exposed to high levels of ambient UV radiation, also found no association between germline *MC1R* variants and somatic *BRAF* mutations. These conflicting findings across different populations highlight the complexity of gene-environment interactions in the development of melanoma. The model proposed by Thomas et al 2010 to explain this discordance illustrated opposing effects of MC1R

status and highlighted a role for pigmentation in photo-protection and generation of oxidative stress (23). The allele frequencies of seven common nonsynonymous *MC1R* variants (V60L, D84E, V92M, R151C, R160W, R163Q, and D294H) differ significantly between Northern European (France, Netherlands, Britain/Ireland) and Southern European populations (Italy and Greece) (24). We also observed this difference in our cancer cohorts with over 60% of the Australian, USA and German cohorts carrying one of the *MC1R* RHC variants, while the Spanish, Austrian and Italian cohorts *MC1R* RHC carries only accounted for less than 45% (Table S6). The downstream effects of *MC1R* on cellular function appears to vary depending on the polymorphisms, and thus it is possible that the discordance between studies could relate to the variation in *MC1R* allele frequencies in the different populations as well as the differences in environmental conditions and patterns of UVR exposure.

Melanoma risk is intricately associated with pigmentation characteristics, and genome-wide association studies have revealed a number of genetic variants involved in pigmentation, including *MC1R*, *ASIP*, *OCA2*, *SLC45A2*, *TYRP1* and *TYR* (25, 26). The discordant results across studies examining solely *MC1R* status as a determinant for developing somatic *BRAF* mutant melanoma may also be due to the confounding role of other pigmentation genes. It must also be noted that the relatively small sample size of all studies examining the association of *MC1R* variants and *BRAF*-mutant melanoma, we cannot rule out the possibility that the differences in results are attributable to chance alone. To expand this work, our future focus needs to be on modeling the complex regulation of pigmentation as a factor of genetic interactions and through larger studies or meta-analyses.

Strengths of our study include the population-based sampling frame and the detailed epidemiologic data (including physician counts of nevi and actinic keratoses, blinded to genotype status) accompanying the tumor specimens. The call rate for somatic

mutations was high using the MelaCarta platform, with mutation status determined for 98% of samples genotyped. Although we did not fully sequence the entire *MC1R* gene, the variants genotyped in this study comprise over 95% of the non-synonymous changes observed (27). We do not believe that further sequencing to identify rare *MC1R* variants could materially alter our null findings. A potential weakness was the relatively limited number of samples for analysis due to insufficient tumor material remaining for mutation analysis after sections had been cut for diagnostic purposes. This is to be expected from a community-based study conducted in Queensland, Australia where the vast majority of patients present with thin melanomas (<1mm). To assess possible selection bias, we compared the prevalence of phenotypic (including skin type, hair and eye color, freckling density and counts of nevi and actinic keratosis) and histological (contiguous neval remnants, thickness, anatomic site) characteristics as well as the age and sex among those participants with tumor blocks available for analysis and those without. We found that those participants whose tumors were not genotyped were slightly older and were more likely to have melanomas of the head and neck and of the lentigo maligna subtype than those who were genotyped, but in other respects were not significantly different. Given these features, it is possible that our sample may have had a higher prevalence of *BRAF* mutations than melanomas arising in the general population, although there is no reason to conclude that the associations between *BRAF* mutation statues and phenotype or other factors would differ.

In conclusion, these data from a large, well-characterized, community-based sample of cutaneous melanomas provide robust estimates of the somatic mutation frequencies of putative 'melanoma genes'. The study confirmed that *BRAF*-mutant melanomas differ from wild-type melanomas for associations with sun exposure, nevus propensity and host characteristics, with largely similar patterns of association for $BRAF^{V600E}$ and $BRAF^{V600K}$ melanomas. There was no evidence that *MC1R* status

conferred particular risks of mutations in *BRAF, NRAS* or other. Taken together, these findings highlight the diversity of mutation profiles in melanoma and the heterogeneity of pathways through which these cancers arise.

Materials & Methods

Subjects

We compared the prevalence of *BRAF* and *NRAS* mutations in formalin-fixed paraffin-embedded melanoma specimens from 414 patients ascertained from southern Queensland (latitude 27^{o}S), Australia. Detailed descriptions of subject selection and data collection for this study have been described previously (20, 28). Briefly, eligible patients were residents of greater Brisbane, Australia who were diagnosed between April 1, 2007 and September 30, 2010 with a histologically confirmed primary invasive cutaneous melanomaarising on the head, neck or trunk. Those with metastatic melanoma or a previous diagnosis of melanoma were not eligible. No acral lentiginous melanoma, spitzoid or nevoid lesions were included in this study. Of 1456 eligible patients for the initial epidemiologic study, 808 (55%) completed questionnaires and 766 (53%) provided written informed consent to obtain specimens of archived melanoma tissue, and 414 (28%) patients had sufficient tissue remaining for mutation analysis (Figure S1). The age, sex, site and histology subtype distribution of the 414 patients that were genotyped differed from the 352 patients who were not, as described above.

Approval to perform this study was given by the Human Research Ethics Committee of the QIMR Berghofer Medical Research Institute. The study adhered to The Declaration of Helsinki and all participants gave their informed written consent to take part.

Histological assessment

At the time of histological diagnosis, collaborating dermatopathologists assessed the extent of solar elastosis in the skin adjacent to the melanoma using a scale of four categories (nil, mild, moderate and marked,) as previously described (20, 28). In addition, they assessed each tumor's histological type, tumor thickness, and the presence of neval

remnants adjacent to the tumor. The anatomical site of each melanoma was abstracted from the pathology report and was confirmed directly with the patient.

DNA Isolation

Hematoxylin and eosin stained sections of each patient's melanoma were assessed for areas of normal and tumor tissue, and the percentage of tumor cells was recorded. Formalin-fixed paraffin-embedded tissue sections were dissected to select areas where melanoma cells dominated over stromal cells. Punch biopsy's (2 mm) were taken from each tumor block and deparaffinized in xylene and washed twice in absolute ethanol. DNA was isolated using Qiagen GeneRead DNA FFPE Kit (Qiagen, Germany), with additional proteinase K digestion at 56^{o}C for 3 hours. DNA quantification was determined by spectrophotometry Qubit (Life Technologies, Carlsbad, CA). Saliva samples were also collected and DNA was extracted for *MC1R* genotyping from saliva samples using Oragene saliva kits (DNA genotek, Ottawa, ON, Canada) following the manufacturer's instructions.

Genotyping

Genotyping was performed on the mass spectrometric genotyping platform using an optimized multiplex assay of 25 common mutations found in melanomas (MelaCarta Panel, Agena Bioscience, San Diego, CA), which includes *AKT3, BRAF, CDK4, CXCR4, CTNNB1, EPHA10, EPHB6, ERBB4, GNA1, GNAQ, KIT, KRAS, MEK, MET, NEK10, NRAS, PDGFRA, PIK3CA, PTK2B, ROR2, EGFR, IDH1, JAK2, ATK1 and ABL1*. An optimized multiplex assay of all nine common variants of *MC1R* (I155T, R142H, D84E, R160W, D294H, V92M, R163Q, V60L, R151C) were used as previously described (29). Participants with none of the *MC1R* variants listed above were classified as wild-type 'WT' for these analyses. People carrying one or more of the red hair color 'RHC' alleles

(R142H, D84E, R160W, D294H, R151C) were classified as 'RHC' variants, and people carrying one or more non red hair color 'NRHC' alleles (I155T, V92M, R163Q, V60L) were classified as 'NRHC' variants (Table S6 and S7). People carrying both 'RHC' and 'NRHC' alleles were classified as 'RHC' variants (Table S7).

Phenotypic characteristics and sun exposure history

Relevant exposure data (including sun exposure history and skin sensitivity) were collected from study participants through a self-completed, structured questionnaire as described previously (28). After completing the questionnaire, each participant was examined by the same dermatologist, who recorded hair and eye color and counted the number of melanocytic nevi (defined as brown to black pigmented macules or papules of any size which are darker than the surrounding skin). Using a standard international protocol (30), nevi were counted on the back, neck, face and upper limbs (left and right) using a transparent plastic stencil. The numbers of actinic keratoses (defined as superficial, rough scaly areas with erythematous background and ill-defined margins) were counted on the dorsum of hands and forearms, and on the face.

Statistical analysis

We performed simple cross-tabulations and calculated Pearson's chi-square and/or Fischer's exact test (for cells with expected count of less than 5) as a measure of statistical association. We used multivariable logistic regression to calculate odds ratios (ORs) and 95% confidence intervals (CIs) as measure of association between patient/tumor characteristics and mutation status. We included terms for age stratum (<40, 40-49, 50-59, 60-69, 70+ years) and sex to control for possible confounding introduced by the study design. P-values less than 0.05 were considered as statistically significant and all such tests were 2-sided. We tested for trend by including each

category as an ordinal variable in the multivariable model, with category values taken as the midpoint of the range. All analyses were performed using the SAS 9.4 statistical software package (SAS institute, Cary, NC).

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

The authors would like to thank all participants for their time and generosity as well as Sullivan and Nicolaides Pathology, Queensland Medical Laboratory and IQ Pathology for their support and assistance in performing this study. This work was funded by a Project Grant (#442960) and a Program Grant (#552429) and AG, NH and DW are supported by fellowships from the National Health and Medical Research Council (NHMRC) of Australia.

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Table 1. Spectrum and frequency of mutations in primary cutaneous melanoma samples

Gene	No	Freq (%)
BRAF	160	38.7
V600E		66.9
V600K		20.6
Other		12.5
CDK4	5	1.2
CTNNB1	1	0.2
EPHB6	10	2.4
ERBB4	6	1.5
GNA11	2	0.5
GNAQ	1	0.2
KIT	4	1.0
KRAS	10	2.4
MEK	3	0.7
MET	1	0.2
NRAS	38	9.2
Q61H		5.3
Q61K		36.8
Q61L		23.7
Q61R		34.2
PDGFRA	2	0.5
PIK3CA	6	1.5
PTK2B	3	0.7
JAK2	1	0.2
ABL1	2	0.5

No mutations were observed in AKT3, CXCR4, EPHA10, NEK10, ROR2, EGFR, IDH1 and ATK1.

Table 2. Association between clinical and pathologic characteristics with BRAF/NRAS mutation status in cutaneous melanoma

Characteristic	Age and sex adjusted OR (95% CI)			
	BRAF V600E* (n=107)	BRAF V600K* (n=33)	Other BRAF mutation* (n=20)	Any NRAS mutation# (n=38)
Age (years)				
<50	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
50-59	0.28 (0.15-0.51)	0.94 (0.27-3.28)	0.66 (0.19-2.28)	5.81 (1.59-21.22)
60-69	0.14 (0.08-0.28)	0.90 (0.28-2.92)	0.75 (0.25-2.30)	6.28 (1.78-22.18)
≥70	0.08 (0.03-0.19)	2.24 (0.75-6.66)	0.13 (0.02-1.15)	3.75 (0.94-14.96)
Age (continuous)	0.93 (0.91-0.95)	1.02 (0.99-1.05)	0.98 (0.95-1.02)	1.03 (1.00-1.05)
Sex				
Female	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Male	0.53 (0.33-0.86)	1.68 (0.66-4.24)	2.11 (0.60-7.44)	3.89 (1.35-11.21)
Number of other somatic mutations				
1	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
>1	5.94 (1.46-24.21)	4.71 (1.02-21.74)	1.82 (0.27-12.52)	0.56 (0.15-2.07)
Histological type of melanoma				
SSM	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
NM	0.70 (0.21-2.36)	0.38 (0.05-3.03)	0.68 (0.08-5.72)	2.08 (0.64-6.79)
LMM	0.40 (0.12-1.41)	0.67 (0.19-2.40)	-	0.83 (0.27-2.54)
Not stated (n=41)				
Clark level				
2	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
≥3	0.71 (0.42-1.23)	0.70 (0.32-1.53)	0.51 (0.18-1.47)	3.01 (1.49-6.09)
Tumor thickness (mm)				
≤1.0	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
>1.0	0.74 (0.36-1.53)	0.71 (0.26-1.97)	0.48 (0.10-2.18)	0.67 (0.41-2.67)
Anatomical site of melanoma				
Trunk	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Head or neck	1.00 (0.53-1.90)	1.64 (0.72-3.72)	0.44 (0.10-1.98)	0.61 (0.24-1.54)
Dermal elastosis				
Nil or mild	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Moderate	0.84 (0.41-1.73)	2.49 (0.90-6.97)	0.30 (0.06-1.44)	1.09 (0.47-2.52)
Marked	0.95 (0.44-2.05)	1.53 (0.50-4.67)	0.37 (0.08-1.82)	0.26 (0.07-0.95)
Missing (n=50)				
Contiguous neval remnants				
No	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Yes	1.94 (1.14-3.31)	1.20 (0.54-2.65)	1.47 (0.54-3.94)	1.52 (0.74-3.10)
Not stated (n=9)				

*Comparison group were samples wild-type for BRAF
Comparison group were samples wild-type for NRAS

Table 3. Association between phenotypic and environmental factors with BRAF/NRAS mutation status in cutaneous melanoma

Characteristic	Age and sex adjusted OR (95% CI)			
	BRAF V600E* (n=107)	BRAF V600K* (n=33)	Other BRAF mutation* (n=20)	Any NRAS mutation# (n=38)
Total nevus count (quartiles)				
0-29	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
30-59	1.80 (0.72-4.48)	2.25 (0.73-6.96)	0.45 (.10-2.05)	0.54 (0.20-1.47)
60-119	2.26 (0.91-5.59)	2.36 (0.67-8.34)	1.04 (0.29-3.76)	0.73 (0.27-1.97)
120+	2.90 (1.16-7.29)	5.03 (1.38-18.38)	0.74 (0.18-3.15)	0.93 (0.33-2.62)
Total number of solar keratoses				
0	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
1-4	0.64 (0.33-1.26)	1.00 (0.31-3.25)	1.61 (0.40-6.69)	0.44 (0.17-1.13)
5-9	0.52 (0.19-1.40)	0.43 (0.08-2.44)	0.51 (0.05-5.22)	0.51 (0.16-1.63)
10+	0.45 (0.18-1.12)	1.43 (0.43-4.80)	2.57 (0.57-11.58)	0.45 (0.17-1.21)
Skin cancers excised				
0	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
1-2	0.68 (0.34-1.35)	0.65 (0.37-1.15)	1.01 (0.32-3.17)	1.33 (0.57-3.14)
3+	0.48 (0.24-0.97)	0.45 (0.26-0.79)	0.35 (0.09-1.35)	0.76 (0.33-1.79)
Cumulative sun exposure (adult yrs)				
<1.6	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
1.6-2.8	1.98 (0.93-4.20)	2.23 (0.63-7.85)	1.59 (0.36-6.96)	0.98 (0.31-3.11)
2.8-4.5	2.17 (0.95-4.97)	2.01 (0.55-7.27)	1.89 (0.45-7.97)	0.70 (0.21-2.35)
>4.5	1.74 (0.67-4.48)	1.04 (0.25-4.28)	1.10 (0.21-5.80)	1.42 (0.45-4.44)
Hair color as a teenager				
Black/dark brown	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Light brown	0.95 (0.50-1.80)	1.88 (0.81-4.35)	1.23 (0.31-4.82)	1.03 (0.47-2.27)
Red/auburn	0.90 (0.40-2.03)	-	3.46 (0.89-13.48)	0.68 (0.21-2.22)
Blond	1.69 (0.85-3.37)	1.23 (0.39-3.92)	2.44 (0.61-9.83)	0.65 (0.22-1.93)
Eye color				
Brown	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Blue/grey	1.33 (0.70-2.54)	2.38 (1.00-5.65)	1.05 (0.28-3.98)	0.75 (0.27-2.09)
Green/hazel	0.74 (0.37-1.48)	0.55 (0.15-1.94)	0.66 (0.18-2.45)	1.24 (0.52-2.96)
Freckling as a teenager				
None	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
A few	0.50 (0.27-0.93)	0.69 (0.29-1.65)	1.49 (0.47-4.75)	0.94 (0.42-2.06)
Some	0.47 (0.22-1.03)	0.79 (0.26-2.45)	1.06 (0.23-4.88)	1.83 (0.72-4.65)
Many	0.34 (0.12-0.97)	0.83 (0.21-3.31)	2.56 (0.49-12.41)	-
Family history of melanoma				
No	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Yes	1.85 (1.06-3.21)	1.24 (0.51-3.03)	0.56 (0.17-1.84)	0.44 (0.16-1.23)

*Comparison group were samples wild-type for BRAF

Comparison group were samples wild-type for NRAS

Table 4. Association between MC1R and phenotypic characteristics

	Any MC1R variant (n=637) n (%)	RHC (n=399) n (%)	NRHC (n=238) n (%)	WT (n=120) n (%)
Hair color as a teenager				
Black/dark brown	194 (30.5)	99 (24.8)	95 (39.9)	54 (45.0)
Light brown	209 (32.8)	120 (30.1)	89 (37.4)	40 (30.3)
Red/auburn	97 (15.2)	91 (22.8)	6 (2.5)	4 (3.3)
Blond	136 (21.4)	88 (22.1)	48 (20.2)	22 (18.3)
missing (n=1)				
	p=0.001	p<0.001	p=0.76	ref
Eye color				
Brown	411 (64.5)	255 (63.9)	156 (65.6)	68 (56.7)
Blue/grey	136 (21.4)	82 (20.6)	54 (22.7)	28 (23.3)
Green/hazel	90 (14.1)	62 (15.5)	28 (11.8)	24 (20.0)
	p=0.18	p=0.33	p=0.09	ref
Freckling as a teenager				
None	201 (31.6)	98 (24.6)	103 (43.3)	65 (54.2)
A few	256 (40.2)	165 (41.5)	91 (38.2)	37 (30.8)
Some	118 (18.5)	83 (20.8)	35 (14.7)	12 (10.0)
Many	62 (9.7)	53 (13.3)	9 (3.8)	6 (5.0)
	p<0.001	p<0.001	p=0.18	ref
Total nevus count (quartiles)				
0-29	152 (23.9)	105 (26.3)	47 (19.8)	14 (11.7)
30-59	169 (26.5)	114 (28.6)	55 (23.1)	33 (27.5)
60-119	145 (22.8)	81 (20.3)	64 (26.9)	34 (28.3)
120+	171 (26.8)	99 (24.8)	72 (30.3)	39 (32.5)
	p=0.02	p=0.004	p=0.28	ref
Propensity to burn				
Never/Rarely	46 (7.2)	19 (4.8)	27 (11.3)	20 (16.7)
Sometimes	174 (27.3)	102 (25.6)	72 (30.3)	49 (40.8)
Mostly	187 (29.4)	128 (32.1)	59 (24.8)	29 (24.2)
Always	230 (36.1)	150 (37.6)	80 (33.6)	22 (18.3)
	p<0.001	p<0.001	p=0.01	ref
Propensity to tan				
Never	74 (11.6)	63 (15.8)	11 (4.6)	5 (4.2)
Lightly	199 (31.2)	137 (34.4)	62 (26.1)	18 (15.0)
Moderately	283 (44.4)	166 (41.6)	117 (49.2)	61 (50.8)
Deeply	81 (12.7)	33 (8.3)	48 (20.2)	36 (30.0)
	p<0.001	p<0.001	p=0.05	ref

RHC-red hair color (MC1R variants- R142H, D84E, R151C, R160W, D294H)

NRHC- non red hair color (MC1R varaints-V60L, V92M, R163Q, I155T)

All p-values are from multivariable logistic regression models adjusted for age and sex

Table 5. Association between *MC1R* variants and somatic *BRAF* and NRAS mutations in cutaneous melanoma

MC1R	Age and sex adjusted OR (95% CI)			
	BRAF V600E (n=107)	BRAF V600K (n=33)	Any BRAF mutation (n=160)	Any NRAS mutation (n=38)
All melanomas				
WT/WT	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Any variant	1.13 (0.58-2.20)	0.80 (0.32-2.02)	1.32 (0.75-2.32)	1.13 (0.47-2.74)
RHC variant	1.08 (0.53-2.20)	0.49 (0.17-1.43)	1.17 (0.64-2.14)	1.01 (0.39-2.59)
NRHC variant	1.23 (0.58-2.59)	1.30 (0.48-3.55)	1.53 (0.82-2.86)	1.37 (0.56-3.40)
Trunk melanomas				
WT/WT	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	
Any variant	1.38 (0.65-2.92)	0.68 (0.25-1.88)	1.46 (0.77-2.77)	
RHC variant	1.20 (0.53-2.70)	0.42 (0.13-1.41)	1.24 (0.63-2.47)	
NRHC variant	1.67 (0.72-3.86)	1.14 (0.38-3.39)	1.80 (0.89-3.65)	
Head and neck melanomas				
WT/WT	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	
Any variant	0.40 (0.08-1.94)	1.88 (0.18-19.42)	0.87 (0.24-3.13)	
RHC variant	0.51 (0.09-2.85)	0.94 (0.08-11.08)	0.87 (0.22-3.42)	
NRHC variant	0.16 (0.01-2.13)	5.26 (0.48-58.0)	0.61 (0.10-3.63)	

RHC-red hair color (MC1R variants- R142H, D84E, R151C, R160W, D294H)
NRHC- non red hair color (MC1R varaints-V60L, V92M, R163Q, I155T)