The association between *MC1R* genotype and *BRAF* mutation status in cutaneous melanoma: findings from an Australian population

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Short Title: *MC1R* variants and *BRAF* mutations in melanoma

Abstract

There is increasing epidemiologic and molecular evidence that cutaneous melanomas arise through multiple causal pathways. The purpose of this study was to explore the relationship between germline and somatic mutations in a population-based series of melanoma patients to reshape and refine the divergent pathway model for melanoma. Melanomas collected from 123 Australian patients were analyzed for *MC1R* variants and mutations in the *BRAF* and *NRAS* genes. Detailed phenotypic and sun exposure data were systematically collected from all patients. We found that *BRAF*-mutant melanomas were significantly more likely from younger patients and those with high nevus counts, and were more likely in melanomas with adjacent neval remnants. Conversely, *BRAF*-mutant melanomas were significantly less likely in people with high levels of life-time sun exposure. We observed no association between germline *MC1R* status and somatic *BRAF* mutations in melanomas from this population. *BRAF*-mutant melanomas have different origins from other cutaneous melanomas. These data support the divergent pathways hypothesis for melanoma, which may require a reappraisal of targeted cancer prevention activities.

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Introduction

Cutaneous melanoma is a common form of cancer arising from the pigment cells of the skin. Risk factors for melanoma include large numbers of melanocytic nevi, fair skin and sunlight exposure (Siskind *et al.*, 2005). While solar ultraviolet radiation (UVR) is the principal environmental risk factor for these cancers, there is increasing evidence that the effect of sunlight on pigment cells is not the same for all people. Epidemiologic data support the concept that melanomas may develop through one of several pathways. Increasingly, it appears that the molecular profile (particularly for oncogenes *BRAF* and *NRAS)* of cutaneous melanomas reflects these causal pathways, typified by different patterns of associations with host and environmental risk factors (Curtin *et al.*, 2005; Thomas *et al.*, 2007; Whiteman *et al.*, 2006; 2003). For example, a recent study suggested that melanomas occurring in younger people with high early-life ambient UVR exposure have a high frequency of *BRAF* mutation, whereas melanomas arising in people with high levels of lifetime UVR exposure are associated with *NRAS* mutations (Thomas *et al.*, 2007).

The melanocortin-1 receptor (*MC1R*) gene is a key determinant of human pigmentation and is highly polymorphic with specific variants linked to red hair and melanoma risk (Palmer *et al.*, 2000; Sturm *et al.*, 2003). Recently, a synergistic relationship between germline *MC1R* variants and somatic *BRAF* mutations was suggested (2006), whereby *MC1R* variant genotypes conferred a significantly increased risk of developing *BRAF*-mutant melanoma in skin not damaged by sunlight. Recent work by Fargnoli et al (2008) further examined the role of *MC1R* in the Italian population and found patients with MC1R variants had a higher risk of carrying BRAF mutations in tumors from chronically sun-exposed sites (OR 13.9, 95% CI = 1.5-133.3) than intermittently sun exposed sites (OR 3.4, 95% CI = 0.8-14.0) although this was not

significantly different. They reported increased risks for *BRAF*–mutant melanoma associated with variants of *MC1R*, not only for *R* variants, but also for *r.*

Here, we present the findings of the first study to explore the relationship between germline *MC1R* status and somatic *BRAF* mutations in melanomas from a susceptible population exposed to very high levels of ambient UVR.

Results

Subject Characteristics

For this analysis (n=123 patients), mean age at diagnosis was 56.4 years and 48% of patients were females. The percentages of histological subtypes were 62% SSM, 1.6% NM, 28% LMM and 8.9% unclassified melanoma. No acral lentiginous melanoma, spitzoid or nevoid lesions were included in this study. Tumors were generally thin; 85% of the lesions were Clark level I or II, and 74% had Breslow thickness <0.75 mm.

BRAF and *NRAS* Mutational Frequencies

Mutually exclusive *BRAF*-mutant and *NRAS*-mutant tumors occurred at frequencies of 31.5% and 3% respectively. Detection of mutations was based on cut-offs imposed using DNA from whole blood buffy coat as wild-type controls (Figure S1) and previous studies which have demonstrated the sensitivity of the Sequenom MassArray platform to detect mutant alleles as low as 1.5–3% of the analyzed sample (Vivante et al, 2007). Due to the low number of tumors with *NRAS* mutations no further statistical analysis was performed using these samples.

Clinical and Pathologic Characteristics of *BRAF*-mutant Lesions

Overall, the mean age at diagnosis for patients carrying a *BRAF* V600 mutation was 47.6 years compared with 60.8 years for wild-type cases (t-test $p<0.001$) (Table 1), and similar patterns were observed within the pre-specified age groups (stratum <50 years: mean age *BRAF* V600 35.4 years vs wild-type 42.9 years; stratum 50+ years mean age *BRAF* V600 58.6 years vs wild-type 66.5 years). There was no association between gender and *BRAF* V600 mutation. The prevalence of *BRAF* V600 mutations differed by histological subtype, with only 12% of LMM carrying *BRAF* mutations

compared with 45% of SSM and 50% of NM (Fishers Exact p=0.001). While 20% of in situ melanomas carried *BRAF* V600 mutations compared with 50% of invasive lesions (Chi-Square p=0.02), we found no evidence that the prevalence of *BRAF* V600 mutation increased further with increasing depth of invasion.

Phenotypic and environmental factors associated with *BRAF* Mutations

Compared with patients who had 0-15 nevi, those with 16-60 nevi were 10-fold more likely to have *BRAF* V600 mutant melanoma and patients with >60 nevi had similarly increased risks of harboring a mutation (Table 2). Furthermore, melanomas with evidence of adjacent neval remnants were more likely to have a *BRAF* V600 mutation (OR 2.7, 95% CI = 1.2-6.2). The ability to develop a tan was also associated with tumors that carried *BRAF* V600 mutations (OR 4.3, 95% CI 1.8-10.4). Freckling, hair and eye color were not significantly associated with *BRAF* V600 mutational status.

We found no association between anatomic site (head and neck vs trunk and limbs) and *BRAF* V600 mutation, however we found that *BRAF* V600 mutant melanomas were statistically significantly less likely to occur in people in the highest groups of cumulative sun exposure or actinic keratosis counts (Table 2). Similarly, *BRAF* V600 mutant melanomas were less common among people who reported large numbers of sunburns as adults, although this was not statistically significant.

To assess which of these phenotypic and environmental factors was most predictive of *BRAF* V600 mutation status, we fitted multivariable logistic regression models using a variety of supervised algorithms. Regardless of the approach to model-fitting (forwards, backwards, stepwise), the final model included terms only for total nevus count and the presence of contiguous neval remnants (in addition to the sampling variables, age group and sex) as the best predictors of *BRAF* V600 mutation status for melanoma (Table 3).

We repeated all of the analyses restricted only to the patients with invasive melanomas, and found essentially similar patterns to those reported above, albeit with reduced precision (Table S1).

Frequency of MC1R Variants

74.5% of melanoma patients carried one of the eight common *MC1R* variants, consistent with previous reports in this population. The estimated allele frequency of measured variants in this population is presented in Table 4. There was no association of *MC1R* variants with gender, histological subtype and invasive classification.

Phenotypic and environmental factors associated with *MC1R* variants

Of a number of phenotypic characteristics for which we sought associations with *MC1R* (Table S2) the only characteristic statistically significantly associated with *MC1R* status was freckling density on both the face and arms (few facial freckles OR 2.1, 95%CI 0.8-5.5; many facial freckles OR 9.8, 95%CI 2.4-39.4 p=0.03). While not statistically significant due to small numbers, red hair was only observed in patients carrying *MC1R* variants. There was no increase in the number of total body nevi or actinic keratosis in patients carrying *MC1R* variants, nor was there any association with sun exposure (Table S2).

BRAF* and *MC1R

There was no association between germline *MC1R* variants and somatic *BRAF* V600 mutations in tumor samples (Table 5). Lesions were categorized into intermittently and chronically sun-exposed body sites but no difference in the rates of *BRAF* V600 mutations was observed. As we found a lower incidence of BRAF V600 mutations in LMM lesions we repeated the analysis excluding this sub-type, we still found no observed

difference in the prevalence of *BRAF* V600 mutations amongst patients carrying *MC1R* variants (Table S3). Due to the age structure of our cases we further investigated the relationships between MC1R and somatic *BRAF* V600 mutations overall and within broad strata of actinic keratosis and sun exposure history (Table 5). However, there was no observed difference in the prevalence of *BRAF* V600 mutations amongst patients carrying *MC1R* variants compared to patients with wild-type *MC1R.* We also excluded the possibility of the LMM subtype confounding the data by excluding them (Table S3). We further analyzed the relationship between germline *MC1R* variants and somatic *BRAF* mutations restricted only to patients with invasive melanomas and found no association between the prevalence of *BRAF* V600 mutations amongst patients carrying *MC1R* variants compared to patients with wild-type *MC1R* (Table S3).

Discussion

We have analyzed melanoma samples from an Australian population to further explore the 'divergent pathway model' for melanoma. This model proposes at least two different causal pathways to melanoma development, one arm pertaining to host susceptibility and nevus growth and another arm associated with chronic sun exposure. Our results accord with this model, since we found that melanomas with and without *BRAF* V600 mutations displayed significantly different associations with a range of phenotypic, histological and environmental factors. We found that melanomas harboring *BRAF* V600 mutations were more likely among younger patients and those with high nevus counts, and were more likely to occur in melanomas with adjacent neval remnants. Melanomas with *BRAF* V600 mutations were less likely to occur in people with evidence of high-levels of life-time sun exposure such as self-reported sun exposure history and nurse counts of actinic keratosis. In keeping with this observation, melanomas of the *lentigo maligna* subtype exhibited a lower frequency of *BRAF* V600 mutations. Patients with tumors carrying *BRAF* V600 mutations had the ability to develop a tan, suggesting intact pigmentation pathways. While *BRAF* V600 mutant melanomas were more likely to occur in younger people, it was notable that such lesions were more likely to be invasive compared to wild-type melanomas. It has been suggested that *BRAF* V600 mutations are induced in melanocytes as a result of childhood sun exposure (Thomas *et al.*, 2007). Presumably a proportion of these transformed cells progress to melanoma, accounting for the younger mean age of diagnosis.

Only a few studies to date have examined determinants for *BRAF* V600 mutations in melanoma (Fargnoli *et al.*, 2008; Landi *et al.*, 2006; Thomas *et al.*, 2007). Our findings are in substantial agreement with those of a study performed in North Carolina by Thomas et al (2007), although we observed no association between anatomic site and

prevalence of *BRAF* V600 mutations. It is important to note that the lack of association here most likely reflects the sampling strategy used in the parent study from which these samples were derived (Whiteman *et al.*, 2003). Patients in the parent study were frequency sampled within strata of age, sex and anatomic site to ensure similar numbers of younger and older patients for each body site. In our sample, the mean age of patients with melanoma of the head and neck was actually younger (55 years) than that of patients with melanoma of the trunk (58 years). This was entirely due to the sampling, as our previous studies in the same population have shown that on average, patients with head and neck melanomas are significantly older than those with melanomas of the trunk (Siskind *et al.*, 2005). Our findings differ from Fargnoli et al (2008), for which the published data (their Table 2) suggest no association between nevus count and *BRAF* mutation when nevus count was dichotomized at the median.

We sought to explore the biological differences between in situ and invasive melanomas by reanalyzing the dataset restricted only to invasive melanomas. The findings are essentially unchanged, although with the reduced sample size, the precision of risk estimates is less than the originally reported findings (Table S1). We sought to explore the effects of age and anatomical site in melanoma causation by intentionally sampling patients from within pre-defined strata. One consequence of this is that in Queensland at least, younger patients with melanocytic lesions tend to present for medical care early, especially for facial melanomas. As a result, most melanomas among young people are thin lesions, particularly on the head and neck.

Importantly, our findings differ from the studies by Landi and colleagues (Fargnoli *et al.*, 2008; Landi *et al.*, 2006), which were based on sequenced *MC1R* and *BRAF* genes and were restricted only to invasive melanomas. We observed no association between germline *MC1R* status and the prevalence of somatic *BRAF* V600 mutations in melanomas, even after classifying lesions into intermittent and chronic sun-exposed

sites. We further investigated the relationships between *MC1R* and *BRAF* V600 mutations using the number of actinic keratosis and self-reported sun exposure history as proxies for total sun exposure, however we found no evidence of any difference in the prevalence of *BRAF* V600 mutation by *MC1R* status. It is possible that the discordant study findings reflect underlying differences in the populations, although the small sample sizes for each study means that chance cannot be excluded. Clearly, further studies of substantially larger size are warranted to clarify the possible biological relationship between germline *MC1R* status and somatic *BRAF* mutations.

Strengths of our study include the population-base sampling frame and the detailed epidemiologic data (including nurse counts of nevi and actinic keratosis blind to genotype status) accompanying the tumor specimens. In particular, we intentionally over-sampled younger patients with melanoma to ensure that we could account for possible age-specific differences in associations between likely causal factors and site of melanoma. The prevalence of somatic *BRAF* V600 mutation-positive samples (31.5%) was consistent with previous reports (Thomas *et al.*, 2007), and the distribution of *MC1R* variants was very similar to earlier reports from Queensland (Duffy *et al.*, 2004).

A limitation of this study was the restricted number of samples for analysis, due to the use of tissue samples for earlier immunohistochemical investigations (Lee *et al.*, 2006; Richmond-Sinclair *et al.*, 2008). 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. The distributions were similar in each group of patients (data not shown).

A further limitation is that we did not perform full sequencing of the entire *MC1R* gene. However, our distribution of variants (Table 4) is very similar to the largest sequencing

effort so far completed in Caucasian populations (Kanetsky et al, 2006). The MC1R variants genotyped in the present study comprise over 93% of the non-synonymous changes observed in the Kanetsky study, which analyzed a far more ethnically diverse sample than our study (USA, Italy and Australia). Therefore we do not believe the rare *MC1R* variants not covered in our investigation would markedly affect our risk estimates; assessment in a large population-based sample is necessary to conclusively address this issue.

In light of these findings we have refined and extended the divergent molecular pathway model by explicitly incorporating mutational events as well as additional environmental and phenotypic data (Figure 1). The initiation event in this model is early-life sun exposure, which has previously been shown using migrant studies to hold the greatest risk for developing melanoma later in life (Whiteman *et al.*, 2001). Work by Bauer et al (2007) has shown that congenital nevi, which develop independently of sun exposure, lack *BRAF* V600 mutations, while acquired nevi are associated with sun exposure in early life (English *et al.*, 2006) and commonly harbor *BRAF* V600 mutations (Pollock *et al.*, 2003).

Several studies have speculated that host factors may underlie susceptibility for melanocyte proliferation and nevus formation. Work by Bataille et al (2007) explored telomere length in white blood cells as a possible predictor of nevus counts. Subjects with high nevus counts exhibited longer telomeres, and it has been inferred that such individuals may have increased cellular replicative potential. It is presumed that this is not just limited to melanocytes. Genome-wide linkage studies for nevus counts have identified several regions of linkage on chromosomes 2, 5, 8, 9, and 17. Of particular interest was the association of the *CDKN2A* locus with nevus formation (Falchi *et al.*, 2006; Zhu *et al.*, 2007). Mutations and loss of p16 (one product encoded by *CDKN2A*)

are well documented in melanoma, and it appears that p16 contributes to melanoma pathogenesis through pathways that escape routine senescence.

In conclusion, our work provides further support for the divergent pathway hypothesis for melanoma by demonstrating that *BRAF* V600 mutant melanomas occur more commonly in younger individuals and those with high nevus counts, and occur in melanomas with contiguous neval remnants. These findings suggest that a sub-group exists within the general population at risk of BRAF V600 mutant melanoma, and that these people may be characterized by distinct phenotypic attributes. Understanding the interacting roles of sunlight, susceptibility and *BRAF* mutation on melanoma development is the aim of our continuing research.

Materials & Methods

Subjects

We compared the prevalence of *BRAF* and *NRAS* mutations in formalin-fixed paraffin-embedded melanoma specimens from 123 patients ascertained from the Queensland Cancer Registry. Detailed description of subject selection and data collection for this study have been described previously (Whiteman *et al.*, 2003). Briefly, eligible patients were residents of greater Brisbane, Australia (latitude 27^{o}S) who were diagnosed with a histologically confirmed primary cutaneous melanoma between January 1, 1998, and December 31, 1999. Patients were intentionally sampled within pre-defined strata of age (<50 years, >50 years) and sex to ensure similar distributions for these variables in the ensuing epidemiologic analyses. Those with metastatic melanoma or a previous diagnosis of melanoma were not eligible. Of 452 eligible patients for the initial epidemiologic study, 387 (86%) completed questionnaires and 328 (73%) provided written informed consent to obtain specimens of archived melanoma tissue. This analysis was restricted to 123 patients for whom sufficient material was remaining for mutation analysis. The age and sex distribution of the 123 patients that were genotyped for BRAF was the same as for the 264 patients who were not.

Approval to perform the study was given by the Human Research Ethics Committee of the Queensland Institute of Medical Research and the Queensland Cancer Registry. The declarations of Helsinki protocols were followed and all participants gave their written consent to take part.

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. Sections (20 μm) were cut from each tumor block and deparaffinized in xylene and washed twice in absolute ethanol. DNA was isolated using Qiagen DNeasy Tissue Kit (Qiagen, Germany), with additional proteinase K digestion at 55^{o}C for 48 hours. DNA was extracted from whole blood buffy coat and melanoma cell lines using Qiagen DNeasy kits (Qiagen). DNA quantification was determined by spectrophotometry (Nanodrop, Wilmington, DE) and DNA quality was checked using 2% agarose gels (Amresco, Solon, OH).

MC1R, *BRAF* and *NRAS* Genotyping

Genotyping was performed using the MassArray platform (Sequenom Inc, San Diego, CA). An optimized multiplex assay of all common and a subset of rare known variants of *MC1R* were used as previously described (Duffy *et al.*, 2004). Only non-synonymous variants or insertions/deletions in *MC1R* were considered in this analysis.

BRAF V600 and *NRAS* Q61 mutations were detected with single base extension or allele-specific assays, using the iPLEX genotyping format (Sequenom) (see Table S4 for primer details). Samples were analyzed in duplicate with genotyping repeated three times to confirm mutation status. Melanoma cell lines previously characterized in (Stark and Hayward, 2007) were used in this study as positive controls for the *MC1R*, *BRAF* and *NRAS* genotyping assays. DNA from whole blood buffy coat was used as wild-type controls in the *BRAF* and *NRAS* genotyping assays (Figure S1). We have only examined the BRAF V600 mutations, other rare changes such as D594, L597, and L584 were not examined in this study.

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 (Whiteman *et al.*, 2003). In addition to background information, we asked participants to report their occupational history (including periods of study and unemployment) since leaving school. We asked participants to report how much time they spent outdoors in the sun in summer on work and non-work days for each period of employment. Participants were asked to report their nevus burden as a teenager and the number of previous treatments for keratinocyte cancers (basal cell carcinomas and squamous cell carcinomas). Finally, a single trained research nurse who was unaware of the study hypotheses examined each participant. The nurse recorded hair and eye color and counted melanocytic nevi and solar keratoses. Nevi were defined as pigmented macules or papules of any size and distinguished from freckles and seborrheic keratoses. Numbers of nevi were counted on the head and neck, the upper limbs, and the trunk and were classified according to size as less than 5 mm or greater than or equal to 5 mm in diameter by use of a transparent plastic stencil. Freckles were defined as irregular but sharply demarcated macules, usually small (<4 mm), uniformly pigmented (tan/light brown), and usually occurring in clusters on exposed body sites. The density of freckling on the face was categorized on a four-point scale. Solar keratoses, defined as superficial, rough scaly areas with erythematous background and ill-defined margins were counted separately on the dorsal surfaces of the hands, forearms, and face.

Statistical analysis

We calculated the amount of sun exposure received on working days (hereafter "occupational exposure") by multiplying the duration of each employment period (in weeks) by the number of days per week worked, and the number of hours per day spent outdoors in the sun on workdays. Ambient recreational exposure was calculated in a

similar manner using self-reported estimates of sun exposure on non-work days in each employment period. We summed occupational and recreational sun exposures across all employment periods after age 20 years up until age of diagnosis to derive cumulative totals for each pattern of exposure. Total ambient sun exposure for each participant was the sum of cumulative occupational and recreational sun exposure.

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 *BRAF* mutation status (V600 mutant vs V600 wild-type). We included terms for age stratum (<50 years, >50 years) and sex to control for possible confounding introduced by the study design. We conducted supervised model-fitting to identify the best model to predict *BRAF* V600 mutation status using forward, backward and stepwise elimination procedures. P-values less than or equal to 0.05 were considered as statistically significant and all such tests were 2-sided. All analyses were performed using the SAS 9.1 statistical software package (SAS institute, Cary, NC).

Conflict of Interest

The authors state no conflict of interest.

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Supplemental Material

Table S1. Association between phenotypic factors and *BRAF* V600 mutations in invasive melanoma only

Table S2. Phenotypic and environmental factors interacting with MC1R variants

Table S3. Association between *MC1R* and *BRAF* stratified by sun exposure and excluding LMM lesions

Table S4. Primer sequences used for *BRAF* and *NRAS* genotyping

Figure S1. Detection of *BRAF* V600 mutations used DNA from whole blood buffy coat as wild-type controls. Mutations were detected if the percent of primer extension was above control samples (above dotted line).

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Table 1. Clinical and pathologic characteristics of patients and lesions

Characteristic	BRAF V600 mutation n= 40	BRAF V600 wild-type n= 83	MC1R (any variant) n= 90	MC1R wild-type[#] n=33	MC1R (r/wt or r/r) n=33	MC1R (any R allele) n=57
Age at diagnosis (y) Mean + SD, y	47.5 + 14.1	60.7 + 13.4	56.1 + 14.7	57.3 + 15.8	56.3 + 15.8	56 + 14.2
Gender, n (%)						
Male	17 (27)	47 (73)	49 (76)	15 (23)	18 (37)	31 (63)
Female	23 (39)	36 (61)	41 (69)	18 (31)	15 (37)	26 (63)
Histological subtype,						
SSM	34 (45)	42 (55)	56 (74)	20 (26)	22 (39)	34 (61)
NM	1 (50)	1 (50)	1 (50)	1 (50)	0 (0)	1 (100)
LMM	4 (12)	30 (88)	24 (71)	10 (29)	8 (33)	16 (67)
Not stated	1 (9)	10 (91)	9 (82)	2 (18)	3 (33)	6 (67)
Pathological Classification,						
In situ	12 (21)	45 (79)	39 (68)	18 (32)	14 (36)	25 (64)
Invasive	28 (42)	38 (58)	51 (77)	15 (23)	19 (37)	32 (63)
Clark level						
1	13 (20)	52 (80)	46 (71)	19 (29)	15 (33)	31 (67)
2	18 (46)	21 (54)	29 (74)	10 (26)	11 (38)	18 (62)
>=3	8 (50)	8 (50)	12 (75)	4 (25)	7 (58)	5 (42)
not stated	1 (33)	2 (67)	3 (100)	0 (0)	0 (0)	3 (100)
Breslow thickness						
<0.75 mm	19 (46)	22 (54)	30 (73)	11 (27)	12 (40)	18 (60)
>=0.75 mm	7 (50)	7 (50)	11 (79)	3 (21)	6 (55)	5 (45)
not stated	14 (21)	54 (79)	49 (72)	19 (28)	15 (31)	34 (69)

[#]Wild-type here denotes an *MC1R* allele that does not carry any of the eight variants we measured (listed in Table 4).

Table 2. Association between phenotypic factors and *BRAF* V600 mutations in cutaneous melanoma

Characteristic	BRAF V600 mutation n= 40	BRAF V600 wild-type n= 83	OR (95%CI)*
Total nevus count			
0-15	2 (6)	33 (94)	1.0 (ref)
16-60	19 (41)	27 (59)	10.9 (2.3-51.6)
>60	17 (46)	20 (54)	11.9 (2.3-61.2)
missing	2 (40)	3	
Contiguous neval remnants			
No	17 (22)	59 (78)	1.0 (ref)
Yes	23 (49)	24 (51)	2.7 (1.2-6.2)
Propensity to tan			
Light or no tan	13 (21)	48 (79)	1.0 (ref)
Mod/Deep tan	27 (45)	33 (55)	4.3 (1.8-10.4)
missing		2	
Freckles Face			
None	14 (36)	25 (64)	1.0 (ref)
Few	14 (31)	31 (69)	0.7 (0.3-1.8)
Many	12 (32)	26 (68)	0.6 (0.2-1.6)
missing		1 (100)	
Eye Color			
Blue and green	23 (33)	47 (67)	1.0 (ref)
Black or Brown	15 (31)	33 (69)	0.8 (0.3-1.9)
missing	2 (40)	3 (60)	
Hair Color			
Black/Brown	30 (33)	60 (67)	1.0 (ref)
Blondes	9 (39)	14 (61)	1.1 (0.4-2.9)
Red	1 (11)	8 (89)	0.2 (0.0-1.9)
missing		1 (100)	
Tumor site			
Trunk and limbs	13 (30)	36 (70)	1.0 (ref)
Head & neck	27 (35)	47 (65)	1.4 (0.6-3.1)
Ambient Sun Exposure			
Low	17 (44)	22 (56)	1.0 (ref)
Medium	15 (37)	25 (63)	1.0 (0.4-2.7)
High	8 (19)	35 (81)	0.5 (0.2-1.4)
missing		1 (100)	
Total Number of Solar Keratosis			
None	22 (51)	21 (49)	1.0 (ref)
1-20	12 (34)	23 (66)	0.6 (0.2-1.7)
>20	4 (10)	36 (90)	0.1 (0.0-0.5)
missing	2 (40)	3 (60)	
Propensity to sun burn			
Rare/Never/Some	13 (38)	21 (62)	1.0 (ref)
Mostly Burn	18 (42)	25 (58)	0.9 (0.3-2.5)
Always Burn	9 (20)	35 (80)	0.3 (0.1-0.9)
missing		2 (100)	
Number of sun burns since school			
Never	5 (56)	4 (44)	1.0 (ref)
1-5	16 (30)	37 (70)	0.3 (0.1-1.4)
6-20	17 (36)	30 (64)	0.4 (0.1-2.0)
>20	2 (17)	10 (83)	0.2 (0.0-1.4)
missing		2 (2.5)	

*Odds ratio and 95% confidence interval, adjusted for age stratum (<50, 50+) and sex

Table 3. Association between risk factors and *BRAF* V600 mutations in cutaneous melanoma: stepwise logistic regression model

Characteristic	OR (95%CI)*
Total nevus count	
0-15	1.0 (ref)
16-60	11.8 (2.4-57.7)
>60	9.7 (1.8-52.1)
Contiguous neval remnants	
No	1.0 (ref)
Yes	3.1 (1.2-8.1)

*Odds ratio and 95% confidence interval, adjusted for age stratum (<50, 50+), sex and all other terms in table

Table 4. Estimated allele frequency of MC1R variants

Variant Allele	Frequency (%) in Tumors	Frequency (%) in South East Queensland population (Duffy *et al.*, 2004)	Frequency (%) in melanoma patients by direct sequencing (Kanetsky et al., 2006)
R142H	1.5	0.4	1.0
V60L	12.3	12.2	13.3
D84E	1.2	1.2	1.8
R151C	15.0	11.0	13.2
R160W	6.9	7.0	9.8
D294H	2.7	2.7	2.8
V92M	10.8	9.7	10.0
R163Q	5	4.7	4.2
All other variants	Not done	0.9	4.0
r	28.1	26.6	27.5
R	27.3	22.3	28.6

r =V60L, V92M, R163Q

R= R142H, D84E, R151C, R160W, D294H

Table 5. The lack of association between *MC1R* and *BRAF*

	BRAF V600		
MC1R	WT	Mutant	OR (95% CI)*
All lesions			
WT/WT	21 (64)	12 (36)	ref
Any variant	62 (69)	28 (31)	0.72 (0.28-1.82)
Total	**83**	**40**	
Intermittent sun exposed lesions (Trunk and upper limbs and lower limbs)			
WT/WT	7 (64)	4 (36)	ref
Any variant	28 (76)	9 (24)	0.4 (0.07-2.14)
Total	**35**	**13**	
Chronic sun exposed lesions (Head, neck)			
WT/WT	13 (62)	8 (38)	ref
Any variant	33 (63)	19 (37)	0.98 (0.31-2.93)
Total	**46**	**27**	
Lower category of sun exposure (0-5 actinic keratosis)			
WT/WT	4 (40)	6 (60)	ref
Any variant	17 (52)	16 (48)	0.68 (0.21-2.22)
Total	**21**	**22**	
High category of sun exposure (6+ actinic keratosis)			
WT/WT	16 (73)	6 (27)	ref
Any variant	43 (81)	10 (19)	0.69 (0.14-3.31)
Total	**59**	**16**	
Low sun exposure (low exposure history)			
WT/WT	9 (47)	10 (53)	ref
Any variant	38 (63)	22 (37)	0.76 (0.19-2.97)
Total	**47**	**32**	
High sun exposure (High exposure history)			
WT/WT	12 (86)	2 (14)	ref
Any variant	23 (79)	6 (21)	0.77 (0.21-2.84)
Total	**35**	**8**	

*Odds ratio and 95% confidence interval, adjusted for age and sex.
There were 2 lesions missing site location information, 5 lesions missing actinic keratosis information and 1 lesion missing sun exposure history.
#WT/WT here denotes an *MC1R* allele that does not carry any of the eight variants we measured (listed in Table 4).

Figure legend

Figure 1. The divergent molecular pathway model of melanoma development incorporates molecular, environmental and phenotypic data.