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

Elke Hacker[1,2], Catherine M. Olsen[1], Marina Kvaskoff[1], Nirmala Pandeya[1], Abrey Yeo[1], Adèle C. Green[1,3], Richard M. Williamson[4], Joe Triscott[5], Dominic Wood[5], Rohan Mortimore[6], Nicholas K. Hayward[1] and David C. Whiteman[1]

Cutaneous melanomas arise through causal pathways involving interplay between exposure to UV 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 nine common polymorphisms in melanocortin-1 receptor gene (*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 but fewer actinic keratoses, were more likely to report a family history of melanoma, and had tumors that 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.

Journal of Investigative Dermatology (2016) **136,** 829–837; doi:10.1016/j.jid.2015.12.035

INTRODUCTION

Melanoma is a potentially lethal cancer arising from the pigment cells, melanocytes. Although 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 (Whiteman et al., 2011). Epidemiologic observations originally led to the concept that melanomas may arise through one of several pathways under a divergent pathway model for melanoma (Whiteman et al., 2006). 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 (Curtin et al., 2005; Landi et al., 2006; Thomas et al., 2007; Whiteman et al., 2006). Several studies have now shown 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 (Curtin et al., 2005; Hacker et al., 2010, 2013; Lee et al., 2011; Maldonado et al., 2003; Thomas et al., 2007; van Elsas et al., 1996; Whiteman et al., 2003, 2006). 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 (Jewell et al., 2012; Mar et al., 2015; Menzies et al., 2012). Thus, there appear to be marked differences in the associations between sun exposure, melanocyte susceptibility, and host characteristics

[1]Cancer Division, QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia; [2]Queensland University of Technology, Institute of Health and Biomedical Innovation, Brisbane, Queensland, Australia; [3]Cancer Research UK Manchester Institute & University of Manchester, Manchester, UK; [4]Anatomical Pathology, Sullivan Nicolaides Pathology, Taringa, Queensland, Australia; [5]Anatomical Pathology, IQ Pathology, West End, Queensland, Australia; and [6]Anatomical Pathology, Queensland Medical Laboratory, Murarrie, Queensland, Australia

Correspondence: David Whiteman, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, Q4006, Queensland, Australia. E-mail: David.Whiteman@qimrberghofer.edu.au

Abbreviations: CI, confidence interval; MC1R, melanocortin-1 receptor; NRHC, non-red hair color; OR, odds ratio; RHC, red hair color

Received 14 October 2015; revised 26 November 2015; accepted 18 December 2015; accepted manuscript published online 22 January 2015

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 (Palmer et al., 2000; Sturm et al., 2003). An interaction between germline *MC1R* variants and somatic *BRAF* mutations was reported in tumors from US and Italian populations (Fargnoli et al., 2008; Landi et al., 2006), 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 they did observe a modest trend between germline *MC1R* status and somatic *BRAF* mutations in melanomas of the trunk (odds ratio [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], $P = 0.02$) (Hacker et al., 2013). However, the association between germline *MC1R* variants and somatic *BRAF* mutations has not been replicated in other populations, including studies from the United States (Thomas et al., 2010b), Australia (Hacker et al., 2010), and Germany (Scherer et al., 2010). Indeed, Scherer and colleagues (2010) 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 (32% female, mean age 58 years) were recruited for the parent epidemiologic study (Kvaskoff et al., 2013). The majority of melanomas were classified histologically as superficial spreading melanoma (72%), with the remainder classified as lentigo maligna melanoma (13%), nodular (5%), or 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 (see Supplementary Figure S1 online). There were no significant differences between those genotyped (n = 414) and those who were not (n = 352) in terms of sex (71% vs. 65% males) or melanoma thickness distribution (84% vs. 81% ≤1 mm), but participants not genotyped were slightly older (56.3 years vs. 59.8 years, $P = 0.04$) 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, San Diego, CA). 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 Supplementary Table S1 online). Further statistical analysis was performed for the *BRAF* and *NRAS* mutant samples because of 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% confidence interval (CI) 0.03–0.19] but were more likely to have melanomas harboring $BRAF^{V600K}$ or *NRAS* mutations (Table 2 and Supplementary Table S2 online). $BRAF^{V600E}$ 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 Supplementary Table S2). Although numbers were small, melanomas carrying somatic mutations

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

Gene	No.	Frequency (%)
BRAF	160	38.7
V600E	107	66.9
V600K	33	20.6
Other	20	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	2	5.3
Q61K	14	36.8
Q61L	9	23.7
Q61R	13	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 odds ratio (95% confidence interval)			
	BRAF V600E[1] (n = 107)	BRAF V600K[1] (n = 33)	Other BRAF mutation[1] (n = 20)	Any NRAS mutation[2] (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)
No. 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)
Histologic type of melanoma				
Superficial spreading melanoma	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
Nodular melanoma	0.70 (0.21–2.36)	0.38 (0.05–3.03)	0.68 (0.08–5.72)	2.08 (0.64–6.79)
Lentigo maligna melanoma	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)
Anatomic 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)				

[1]Comparison group were samples wild type for BRAF.
[2]Comparison group were samples wild type for NRAS.

in *KRAS* or *EPHB6* were more likely to carry additional mutations than melanomas without these mutations (see Supplementary Table S3 online). Although the prevalence of *BRAF* and *NRAS* mutations differed somewhat by histologic 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; however, 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 Supplementary Table S4 online), 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). The 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

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

Characteristic	Age- and sex-adjusted odds ratio (95% confidence interval)			
	BRAF V600E[1] (n = 107)	BRAF V600K[1] (n = 33)	Other BRAF mutation[1] (n = 20)	Any NRAS mutation[2] (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 no. 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 years)				
<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/gray	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)
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)

[1]Comparison group were samples wild type for BRAF.
[2]Comparison group were samples wild-type for NRAS.

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 three 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 (see Supplementary Table S5 online).

Somatic mutations and *MC1R* variants

In this series, 84% of melanoma patients carried one of the nine common *MC1R* variants, with 53% carrying red hair color (RHC) variants and 31% carrying non-red hair color (NRHC) variants (see Supplementary Tables S6 and S7

Table 4. Association between MC1R and phenotypic characteristics

	Any MC1R variant (n = 637)[1]	RHC (n = 399)	NRHC (n = 238)	Wild type (n = 120)
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-value	0.001	<0.001	0.76	Reference
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-value	0.18	0.33	0.09	Reference
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-value	<0.001	<0.001	0.18	Reference
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-value	0.02	0.004	0.28	Reference
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-value	<0.001	<0.001	0.01	Reference
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-value	<0.001	<0.001	0.05	Reference

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

Values are given as no. (%).

All *P*-values are from multivariable logistic regression models adjusted for age and sex.

[1]MC1R status missing for 9 samples cohort = 766.

online). As expected, *MC1R* status was associated with red hair color ($P < 0.001$) and skin type determinants such as susceptibility to burn ($P < 0.001$) and 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 Supplementary Table S8 online). 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 lentigo maligna melanoma subtype, but this made no material difference to our conclusions (see Supplementary Table S9 online). 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 <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 United States (Fargnoli et al., 2008; Hacker et al., 2010, 2013; Scherer et al.,

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

	Age- and sex-adjusted odds ratio (95% confidence interval)			
MC1R	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)	NA
Any variant	1.38 (0.65–2.92)	0.68 (0.25–1.88)	1.46 (0.77–2.77)	NA
RHC variant	1.20 (0.53–2.70)	0.42 (0.13–1.41)	1.24 (0.63–2.47)	NA
NRHC variant	1.67 (0.72–3.86)	1.14 (0.38–3.39)	1.80 (0.89–3.65)	NA
Head and neck melanomas				
WT/WT	1.00 (reference)	1.00 (reference)	1.00 (reference)	NA
Any variant	0.40 (0.08–1.94)	1.88 (0.18–19.42)	0.87 (0.24–3.13)	NA
RHC variant	0.51 (0.09–2.85)	0.94 (0.08–11.08)	0.87 (0.22–3.42)	NA
NRHC variant	0.16 (0.01–2.13)	5.26 (0.48–58.0)	0.61 (0.10–3.63)	NA

Abbreviations: NA, not applicable; NRHC, non-red hair color (MC1R variants: V60L, V92M, R163Q, I155T); RHC, red hair color (MC1R variants: R142H, D84E, R151C, R160W, D294H); WT, wild type.

2010; Thomas et al., 2007). In all prior series, $BRAF^{V600E}$ mutations were at least three- to fourfold 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 (Agena Bioscience), whereas *NRAS* mutant melanomas were not.

As 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 are in accordance with previous studies examining *BRAF* status and characteristics of patients with cutaneous melanoma (Fargnoli et al., 2008; Thomas et al., 2007). 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 supporting that conclusion (Jewell et al., 2012; Mar et al., 2015; Menzies et al., 2012). 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), so our study suffers from a 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 (2006), in which 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 (Hacker et al., 2013). Other studies conducted in North Carolina (Thomas et al., 2010b), Australia (Hacker et al., 2010), and Germany (Scherer et al., 2010) 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. (2010a) to explain this discordance illustrated opposing effects of MC1R status and highlighted a role for pigmentation in photoprotection and generation of oxidative stress. The allele frequencies of seven common nonsynonymous *MC1R* variants (V60L, D84E, V92M, R151C, R160W, R163Q, D294H) differ significantly between Northern European (France, Netherlands, Britain/Ireland) and Southern European populations (Italy and Greece) (Gerstenblith et al., 2007). We also observed this difference in our cancer

cohorts, with >60% of the Australian, US, and German cohorts carrying one of the *MC1R* RHC variants, whereas the Spanish, Austrian, and Italian cohorts carrying *MC1R* RHC variants only accounted for <45% (Supplementary Table S6). The downstream effects of *MC1R* on cellular function appears to vary depending on the polymorphisms, 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 UV radiation exposure.

Melanoma risk is intricately associated with pigmentation characteristics, and genomewide association studies have revealed a number of genetic variants involved in pigmentation, including *MC1R*, *ASIP*, *OCA2*, *SLC45A2*, *TYRP1*, and *TYR* (Bishop et al., 2009; Duffy et al., 2010). 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 given 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 (Agena Bioscience), 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 >95% of the nonsynonymous changes observed (Kanetsky et al., 2006). 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 because of 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 majority of patients present with thin melanomas (<1 mm). 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 histologic (contiguous neval remnants, thickness, anatomic site) characteristics as well as the age and sex among those participants with and those without tumor blocks available for analysis. We found that the participants whose tumors were not genotyped were slightly older, were more likely to have melanomas of the head and neck, and were more likely to be of the lentigo maligna subtype than those who were genotyped, but in other respects they were not significantly different. Given these features, it is possible that our sample 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 with regard to 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 genes. Taken together, these findings highlight the diversity of mutation profiles in melanoma and the heterogeneity of pathways through which these cancers arise.

MATERIALS AND 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 degrees south), Australia. Detailed descriptions of subject selection and data collection for this study have been described previously (Kvaskoff et al., 2013, 2015). 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 melanoma arising 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 1,456 eligible patients for the initial epidemiologic study, 808 (55%) completed questionnaires, 766 (53%) provided written informed consent to obtain specimens of archived melanoma tissue, and 414 (28%) patients had sufficient tissue remaining for mutation analysis (Supplementary Figure S1). The age, sex, site, and histology subtype distribution of the 414 patients who were genotyped differed from the 352 patients who were not, as described earlier.

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 written informed consent to take part in the study.

Histologic assessment

At the time of histologic 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 (Kvaskoff et al., 2013, 2015). In addition, they assessed each tumor's histologic type, tumor thickness, and presence of neval remnants adjacent to the tumor. The anatomic 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 in which melanoma cells dominated over stromal cells. Punch biopsies (2 mm) were taken from each tumor block, deparaffinized in xylene, and washed twice in absolute ethanol. DNA was isolated using Qiagen GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany), with additional proteinase K digestion at 56°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, Ontario, 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), 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 (Duffy et al., 2004). Participants with none of the *MC1R* variants listed were classified as wild type (WT) for these analyses. People carrying one or more of the RHC alleles (R142H, D84E, R160W, D294H, R151C) were classified as RHC variants, and people carrying one or more of the NRHC alleles (I155T, V92M, R163Q, V60L) were classified as NRHC variants (Supplementary Tables S6 and S7). People carrying both RHC and NRHC alleles were classified as RHC variants (Supplementary 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 (Kvaskoff et al., 2015). 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 that are darker than the surrounding skin). Using a standard international protocol (English et al., 1990), 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 χ^2 and/or Fisher exact test (for cells with expected count <5) as a measure of statistical association. We used multivariable logistic regression to calculate ORs and 95% CIs as measures 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. $P < 0.05$ was considered statistically significant, and all such tests were two 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). AG, NH, and DW are supported by fellowships from the National Health and Medical Research Council (NHMRC) of Australia.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.12.035.

REFERENCES

Bishop DT, Demenais F, Iles MM, Harland M, Taylor JC, Corda E, et al. Genome-wide association study identifies three loci associated with melanoma risk. Nat Genet 2009;41:920–5.

Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med 2005;353:2135–47.

Duffy DL, Box NF, Chen W, Palmer JS, Montgomery GW, James MR, et al. Interactive effects of MC1R and OCA2 on melanoma risk phenotypes. Hum Mol Genet 2004;13:447–61.

Duffy DL, Zhao ZZ, Sturm RA, Hayward NK, Martin NG, Montgomery GW. Multiple pigmentation gene polymorphisms account for a substantial proportion of risk of cutaneous malignant melanoma. J Invest Dermatol 2010;130:520–8.

English DR, MacLennan R, Rivers J, Kelly J, Armstrong BK. Epidemiological studies of melanocytic nevi: protocol for identifying and recording nevi. IARC internal report no90/002. Lyon, France: International Agency for Research on Cancer; 1990.

Fargnoli MC, Pike K, Pfeiffer RM, Tsang S, Rozenblum E, Munroe DJ, et al. MC1R variants increase risk of melanomas harboring BRAF mutations. J Invest Dermatol 2008;128:2485–90.

Gerstenblith MR, Goldstein AM, Fargnoli MC, Peris K, Landi MT. Comprehensive evaluation of allele frequency differences of MC1R variants across populations. Hum Mutat 2007;28:495–505.

Hacker E, Hayward NK, Dumenil T, James MR, Whiteman DC. The association between MC1R genotype and BRAF mutation status in cutaneous melanoma: findings from an Australian population. J Invest Dermatol 2010;130:241–8.

Hacker E, Nagore E, Cerroni L, Woods SL, Hayward NK, Chapman B, et al. NRAS and BRAF mutations in cutaneous melanoma and the association with MC1R genotype: findings from Spanish and Austrian populations. J Invest Dermatol 2013;133:1027–33.

Jewell R, Chambers P, Harland M, Laye J, Conway C, Mitra A, et al. Clinicopathologic features of V600E and V600K melanoma—letter. Clin Cancer Res 2012;18:6792.

Kanetsky PA, Rebbeck TR, Hummer AJ, Panossian S, Armstrong BK, Kricker A, et al. Population-based study of natural variation in the melanocortin-1 receptor gene and melanoma. Cancer Res 2006;66:9330–7.

Kvaskoff M, Pandeya N, Green AC, Perry S, Baxter C, Davis MB, et al. Site-specific determinants of cutaneous melanoma: a case-case comparison of patients with tumors arising on the head or trunk. Cancer Epidemiol Biomarkers Prev 2013;22:2222–31.

Kvaskoff M, Pandeya N, Green AC, Perry S, Baxter C, Davis MB, et al. Solar elastosis and cutaneous melanoma: a site-specific analysis. Int J Cancer 2015;136:2900–11.

Landi MT, Bauer J, Pfeiffer RM, Elder DE, Hulley B, Minghetti P, et al. MC1R germline variants confer risk for BRAF-mutant melanoma. Science 2006;313:521–2.

Lee JH, Choi JW, Kim YS. Frequencies of BRAF and NRAS mutations are different in histological types and sites of origin of cutaneous melanoma: a meta-analysis. Br J Dermatol 2011;164:776–84.

Maldonado JL, Fridlyand J, Patel H, Jain AN, Busam K, Kageshita T, et al. Determinants of BRAF mutations in primary melanomas. J Natl Cancer Inst 2003;95:1878–90.

Mar VJ, Liu W, Devitt B, Wong SQ, Dobrovic A, McArthur GA, et al. The role of BRAF mutations in primary melanoma growth rate and survival. Br J Dermatol 2015;173:76–82.

Menzies AM, Haydu LE, Visintin L, Carlino MS, Howle JR, Thompson JF, et al. Distinguishing clinicopathologic features of patients with V600E and V600K BRAF-mutant metastatic melanoma. Clin Cancer Res 2012;18:3242–9.

Palmer JS, Duffy DL, Box NF, Aitken JF, O'Gorman LE, Green AC, et al. Melanocortin-1 receptor polymorphisms and risk of melanoma: is the association explained solely by pigmentation phenotype? Am J Hum Genet 2000;66:176–86.

Scherer D, Rachakonda PS, Angelini S, Mehnert F, Sucker A, Egberts F, et al. Association between the germline MC1R variants and somatic BRAF/NRAS mutations in melanoma tumors. J Invest Dermatol 2010;130:2844–8.

Sturm RA, Duffy DL, Box NF, Newton RA, Shepherd AG, Chen W, et al. Genetic association and cellular function of MC1R variant alleles in human pigmentation. Ann NY Acad Sci 2003;994:348–58.

Thomas NE, Edmiston SN, Alexander A, Millikan RC, Groben PA, Hao H, et al. Number of nevi and early-life ambient UV exposure are associated with BRAF-mutant melanoma. Cancer Epidemiology Biomarkers Prev 2007;16:991–7.

Thomas NE, Kanetsky PA, Begg CB, Conway K, Berwick M. Melanoma molecular subtypes: unifying and paradoxical results. J Invest Dermatol 2010a;130:12–4.

Thomas NE, Kanetsky PA, Edmiston SN, Alexander A, Begg CB, Groben PA, et al. Relationship between germline MC1R variants and BRAF-mutant melanoma in a North Carolina population-based study. J Invest Dermatol 2010b;130:1463–5.

van Elsas A, Zerp SF, van der Flier S, Kruse KM, Aarnoudse C, Hayward NK, et al. Relevance of ultraviolet-induced N-ras oncogene point mutations in development of primary human cutaneous melanoma. Am J Pathol 1996;149:883–93.

Whiteman DC, Pavan WJ, Bastian BC. The melanomas: a synthesis of epidemiological, clinical, histopathological, genetic, and biological aspects, supporting distinct subtypes, causal pathways, and cells of origin. Pigment Cell Melanoma Res 2011;24:879–97.

Whiteman DC, Stickley M, Watt P, Hughes MC, Davis MB, Green AC. Anatomic site, sun exposure, and risk of cutaneous melanoma. J Clin Oncol 2006;24:3172–7.

Whiteman DC, Watt P, Purdie DM, Hughes MC, Hayward NK, Green AC. Melanocytic nevi, solar keratoses, and divergent pathways to cutaneous melanoma. J Natl Cancer Inst 2003;95:806–12.