NRAS and *BRAF* Mutations in Cutaneous Melanoma and the Association with *MC1R* Genotype: Findings from Spanish and Austrian Populations

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There is increasing epidemiologic and molecular evidence that cutaneous melanomas arise through multiple causal pathways. To further define the pathways to melanoma, we explored the relationship between germline and somatic mutations in a series of melanomas collected from 134 Spanish and 241 Austrian patients. Tumor samples were analyzed for melanocortin-1 receptor (*MC1R*) variants and mutations in the *BRAF* and *NRAS* genes. Detailed clinical data were systematically collected from patients. We found that *NRAS*-mutant melanomas were significantly more likely from older patients and *BRAF*-mutant melanomas were more frequent in melanomas from the trunk. We observed a nonsignificant association between germline *MC1R* status and somatic *BRAF* mutations in melanomas from trunk sites (odds ratio (OR) 1.8 (0.8–4.1), $P = 0.1$), whereas we observed a significant inverse association between *MC1R and BRAF* for melanomas of the head and neck (OR 0.3 (0.1–0.8), $P = 0.02$). This trend was observed in both the Spanish and Austrian populations.

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INTRODUCTION

Cutaneous melanoma, arising from melanocytes, the pigment cells of the skin, is the most lethal skin cancer. Risk factors for melanoma include large numbers of melanocytic nevi, fair skin, and sunlight exposure (Siskind *et al.*, 2005). Although UVR from sunlight is the principal environmental cause for these cancers, there is increasing evidence that the effect of UVR on pigment cells is not the same for all people. Epidemiologic data support the concept that melanomas may develop through one of the several pathways. Increasingly, it appears that the molecular profile of cutaneous melanomas (particularly for oncogenes *BRAF* and *NRAS)* reflects these causal pathways, typified by different patterns of associations with host and environmental risk factors (van 't Veer *et al.*, 1989; Maldonado *et al.*, 2003; Whiteman *et al.*, 2003, 2006; Curtin *et al.*, 2005; Landi *et al.*, 2006; Thomas *et al.*, 2007). Recent studies have 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 older people with high levels of lifetime UVR exposure are biologically distinct and appear associated with other mutation profiles (van Elsas *et al.*, 1996; Thomas *et al.*, 2007; Hacker *et al.*, 2010; Lee *et al.*, 2011).

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). The *MC1R* gene is highly polymorphic, with >60 variants documented, the nine most common variants have been classed into two groups based on the strength of their association with red hair (Sturm *et al.*, 2003; Kanetsky *et al.*, 2006). The R variants (i.e. D84E, R151K, R160W, R142H, I155T, and D294H) are most highly correlated with red hair color, whereas the r variants (V60L, V92M, and R163Q) are less strongly associated. Functional studies examining cell surface expression and intracellular activity found that the D84E, R151C, and R160W variants had reduced expression, whereas the V60L, R151C, R160W, and D294H variants had decreased cAMP activity in response to melanocortins (Schioth *et al.*, 1999; Garcia-Borron *et al.*, 2005). Recent studies by Beaumont *et al.* (2007) have found that the V92M variant showed similar activity to the wild-type receptor, and meta-analysis of 20 studies revealed that the V92M and V60L variants were not associated with melanoma or phenotype (Raimondi *et al.*, 2008). Evaluation of allele frequency for *MC1R* variants across populations including

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Abbreviations: CI, confidence interval; MC1R, melanocortin-1 receptor; OR, odds ratio

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northern European (France, Netherlands, Britain/Ireland) and southern European (Italy and Greece) countries revealed that seven common nonsynonymous *MC1R* variants (V60L, D84E, V92M, R151C, R160W, R163Q, and D294H) had significantly different allele frequencies. Comparison of these *MC1R* variants between populations showed that the allele frequencies of these variants in the Italian and Greek populations varied from the frequencies in the other Caucasian groups (Gerstenblith *et al.*, 2007).

A synergistic relationship between germline *MC1R* variants and somatic *BRAF* mutations has been suggested by Landi and co-workers (2006), whereby *MC1R* variant genotypes conferred an increased risk of developing *BRAF*-mutant melanoma in skin not damaged by sunlight. Further work by Fargnoli *et al.* (2008) in an Italian population found that melanoma patients with *MC1R* variants had a higher risk of carrying *BRAF* mutations in tumors from chronically sun-exposed sites (odds ratio (OR) 13.9; 95% confidence interval (CI) = 1.5–133.3) than intermittently sun-exposed sites (OR 3.4; 95% CI = 0.8–14.0). These findings have not been replicated in the North Carolina population (Thomas *et al.*, 2010) nor in the highly susceptible Australian population, which predominantly involves patients of northern European and Anglo-Celtic ancestry (Hacker *et al.*, 2010). Recently in a German population of melanoma cases, Scherer and co-workers (2010) observed that the frequency of somatic *BRAF* mutations was significantly lower in the carriers of *MC1R* variants. These conflicting findings across different populations highlight the complexity of gene–environment interactions for melanoma. Here we present the findings of the largest study to date to explore the relationship between germline *MC1R* status and somatic *BRAF* and *NRAS* mutations in melanomas.

RESULTS

BRAF and *NRAS* mutational frequencies

Mutually exclusive *BRAF*-mutant and *NRAS*-mutant tumors occurred at frequencies of 22% and 17%, respectively. We observed similar mutation frequencies between the Spanish (*BRAF* mutant 22%, *NRAS* mutant 15%) and Austrian (*BRAF* mutant 21%, *NRAS* mutant 17%) cohorts.

Clinical and pathological characteristics of *BRAF*- and *NRAS*-mutant lesions

The prevalence of *BRAF* mutations differed by site, with a higher frequency observed on the trunk compared with the

Table 1. Clinical characteristics of patients for *BRAF* lesions

Characteristic	Spanish $n=134$	Austrian $n=241$	*BRAF* mutation $n=30$ Spanish	*BRAF* wild-type $n=104$ Spanish	*BRAF* mutation $n=51$ Austrian	*BRAF* wild-type $n=190$ Austrian	*BRAF* mutation $n=81$ combined	*BRAF* wild-type $n=294$ combined
Age at diagnosis (years)								
<50	49 (37)	85 (35)	15 (50)	34 (33)	18 (35)	67 (35)	33 (41)	101 (34)
+50	85 (63)	156 (65)	15 (50)	70 (67)	33 (65)	123 (65)	48 (59)	193 (66)
		$P^*=0.18$		$P=0.08$		$P=0.99$		$P=0.28$
Gender, n *(%)*								
Male	72 (54)	131 (54)	18 (60)	54 (52)	24 (47)	107 (56)	42 (52)	161 (55)
Female	62 (46)	110 (46)	12 (40)	50 (48)	27 (53)	83 (44)	39 (48)	133 (45)
		$P^*=0.18$		$P=0.43$		$P=0.24$		$P=0.64$
Tumor site								
Trunk (limbs excluded)	86 (64)	129 (54)	25 (83)	61 (59)	33 (65)	96 (51)	58 (72)	157 (53)
Head and neck	48 (36)	112 (46)	5 (17)	43 (41)	18 (35)	94 (49)	23 (28)	137 (47)
		$P^*=0.28$		$P=0.43$		$P=0.07$		**$P=0.003$**
Contiguous neval remnants								
No	37 (32)	49 (20)	8 (29)	29 (32)	10 (20)	39 (21)	18 (23)	68 (24)
Yes	80 (68)	192 (80)	20 (71)	60 (68)	41 (80)	151 (79)	61 (77)	211 (76)
Not stated =17		$P^*=0.83$		$P=0.69$		$P=0.88$		$P=0.77$
Breslow thickness (mm)								
<0.75	26 (23)	130 (54)	6 (20)	20 (24)	22 (44)	108 (57)	28 (46)	128 (54)
⩾0.75	87 (77)	109 (46)	23 (77)	64 (76)	28 (56)	81 (43)	51 (50)	145 (50)
Not stated	21	2	1	20	1	1	2	21
		$P^*=66$		$P=0.73$		$P=0.10$		$P=0.07$

Value shown within parentheses is percent (%).
P-value* = test for the homogeneity of the odds ratios between Spanish and Austrian cohorts.
P-value = χ^2 analysis of *BRAF* mutant versus *BRAF* wild-type for each characteristic.
P-values shown in bold <0.05.

Table 2. Clinical characteristics of patients for *NRAS* lesions

Characteristic	*NRAS* mutation *n*=21 Spanish	*NRAS* wild-type *n*=113 Spanish	*NRAS* mutation *n*=41 Austrian	*NRAS* wild-type *n*=200 Austrian	*NRAS* mutation *n*=62 combined	*NRAS* wild-type *n*=313 combined
Age at diagnosis (years)						
<50	5 (24)	44 (39)	9 (22)	76 (38)	14 (23)	120 (38)
+50	16 (76)	69 (61)	32 (78)	124 (62)	48 (77)	193 (62)
		$P=0.19$		$P=0.05$		**$P=0.02$**
Gender, n (%)						
Male	12 (57)	60 (53)	28 (68)	103 (52)	40 (65)	163 (52)
Female	9 (43)	53 (47)	13 (32)	97 (48)	22 (35)	150 (48)
		$P=0.73$		$P=0.05$		$P=0.07$
Tumor site						
Trunk (limbs excluded)	15 (71)	71 (63)	25 (61)	104 (52)	40 (65)	175 (56)
Head and neck	6 (29)	42 (37)	16 (39)	96 (48)	22 (35)	138 (44)
		$P=0.45$		$P=0.29$		$P=0.21$
Contiguous neval remnants						
No	13 (68)	67 (68)	29 (71)	163 (82)	42 (70)	230 (77)
Yes	6 (32)	31 (32)	12 (29)	37 (18)	18 (30)	68 (23)
Not stated =17		$P=0.99$		$P=0.12$		$P=0.23$
Breslow thickness (mm)						
<0.75	4 (22)	22 (23)	18 (44)	112 (57)	22 (46)	134 (54)
⩾0.75	14 (78)	73 (77)	23 (56)	86 (43)	37 (50)	159 (50)
Not stated	3	18	0	2	3	20
		$P=0.93$		$P=0.14$		$P=0.23$

Value shown within parentheses is percent (%).
P-value = χ^2 analysis of *NRAS* mutant versus *NRAS* wild-type for each characteristic.
P-values shown in bold <0.05.

head and neck (Table 1; χ^2 $P=0.003$). When subjects were stratified by age <50 or 50+ years, we observed a higher frequency of *NRAS* mutations among those in the 50+ years group (Table 2; χ^2 $P=0.02$). There was no association between gender and either *NRAS* or *BRAF* mutation. To assess which clinical and pathological factors were most predictive of mutation status, we fitted multivariable logistic regression models in which terms for other factors were systematically added and removed from the model. The only significant predictors of *NRAS* mutation status were age and tumor site, and the only significant predictor of *BRAF* mutation status was tumor site (Table 3).

Phenotypic and environmental factors associated with *BRAF* and *NRAS* lesions in the Spanish population

Patients who presented with solar keratoses were less likely to develop *BRAF*-mutant melanoma (OR 0.1 (0.02–1.1), $P=0.01$) and more likely to develop an *NRAS*-mutant melanoma (OR 2.4 (0.7–7.8), $P=0.03$) (Supplementary Table S1 online). Patients who had many nevi (>21) tended to be more likely to develop a melanoma carrying a *BRAF* mutation than those with 0–20 nevi, although this trend did not reach statistical significance (χ^2 $P=0.12$) (Supplementary Table S1 online). We observed no association between hair or eye color, outdoor work, freckling, number of severe sunburns, and tumor mutation status (Supplementary Table S1 online).

BRAF and *MC1R*

Sixty-six percent of Spanish and 79% of Austrian melanoma patients carried *MC1R* variants ($P=0.01$). There was no association between germline *MC1R* variants and somatic *BRAF* mutations across all tumor samples; however, we did observe a modest, nonsignificant association between germline *MC1R* status and somatic *BRAF* mutations in melanomas from trunk sites (OR 1.8 (0.8–4.1), $P=0.1$). In contrast, we observed a significant inverse association between *MC1R and BRAF* for melanomas of the head and neck (OR 0.3 (0.1–0.8), $P=0.02$) (Table 4). We further investigated the relationships between *MC1R* and somatic *NRAS* mutations and observed no difference in the prevalence of *NRAS* mutations among patients carrying *MC1R* variants compared with patients with wild-type *MC1R* (Table 4).

DISCUSSION

Similar to previous studies, we observed that melanomas harboring *BRAF* mutations were more frequently found on the trunk and melanoma carrying *NRAS* mutations were

Table 3. Association between risk factors and *NRAS/BRAF* mutations in cutaneous melanoma: multivariable logistic regression model

Characteristic	OR (95% CI)[1]	*P*-value
BRAF		
Age (years)		
<50	1.0 (ref.)	0.72
50+	0.9 (0.5–1.6)	
Sex		
Male	1.0 (ref.)	0.76
Female	1.1 (0.7–1.8)	
Anatomic site		
Trunk	1.0 (ref.)	**<0.01**
Head and neck	0.5 (0.3–0.8)	
NRAS		
Age (years)		
<50	1.0 (ref.)	**<0.01**
50+	2.5 (1.3–4.9)	
Sex		
Male	1.0 (ref.)	0.06
Female	0.6 (0.3–1.0)	
Anatomic site		
Trunk	1.0 (ref.)	**0.04**
Head and neck	0.5 (0.3–1.0)	

Abbreviations: CI, confidence interval; OR, odds ratio.
P-value = Type 3 analysis of effects for each term included in the model.
[1]OR and 95% CI. The final model included terms for age stratum (<50 years, 50+ years), sex, and anatomical site of the melanoma (head and neck, trunk).
P-values shown in bold <0.05.

significantly more likely in older patients. These findings support the concept that melanomas develop through one of the several pathways and it appears that the molecular profile for oncogenes *BRAF* and *NRAS* reflects these causal pathways (Lee *et al.*, 2011). Previous studies have illustrated the heterogeneity of melanoma, whereby tumors arising on the trunk tend to occur in younger individuals and in those with numerous melanocytic nevi, and appear biologically distinct from melanomas arising on sun-exposed sites, which tend to occur on older individuals (Maldonado *et al.*, 2003; Whiteman *et al.*, 2003, 2006; Curtin *et al.*, 2005; Thomas *et al.*, 2007). There appear to be marked differences in the role of sun exposure, melanocyte susceptibility, and host characteristics along these different casual pathways to melanoma development.

Similar to earlier studies within the populations of North Carolina and Australia, we found no associations between germline *MC1R* status and somatic *NRAS* mutations. When viewed collectively, there is little to suggest from these studies that *MC1R* status determines the mutation status of *NRAS* in cutaneous melanoma. However, a relationship between germline *MC1R* variants and somatic *BRAF* mutations has been observed in the Italian population (Landi *et al.*, 2006; Fargnoli *et al.*, 2008). These findings that suggest that people carrying germline *MC1R* variants have a greater risk of developing a melanoma harboring a *BRAF* mutation have not been replicated in other populations, including cohorts from North Carolina (Thomas *et al.*, 2010), Australia (Hacker *et al.*, 2010), and Germany (Scherer *et al.*, 2010). Our analyses of Spanish and Austrian case series found that melanomas arising on the trunk more frequently gave rise to *BRAF*-mutant melanomas, and this risk was not statistically increased in people carrying *MC1R* variants. However, we found that melanomas arising on the head and neck were less likely to harbor *BRAF* mutations, and this was particularly so among the carriers of *MC1R* variants. The pattern of high UVR exposure experienced by head and neck melanocytes may induce other somatic mutations, which drive melanoma risk.

MC1R variants are associated with increased risk of melanoma and nonmelanoma skin cancer. Persons carrying *MC1R* variants produce more red/yellow pheomelanin than brown/black eumelanin. Pheomelanin is more likely than eumelanin to generate potentially damaging reactive oxygen species following UVR exposure (Hill, 1992; Takeuchi *et al.*, 2004; Baldea *et al.*, 2009). In tissue culture experiments, melanocytes harboring *MC1R* variants have less effective repair of both UVR-induced pyrimidine dimers and oxidative damage than wild-type melanocytes, and are more sensitive to UVR-induced cell death (Bohm *et al.*, 2005; Kadekaro *et al.*, 2005; Hauser *et al.*, 2006). The level of UVR exposure sufficient to induce DNA damage to melanocytes harboring *MC1R* variants and drive subsequent melanoma formation is unknown; however, the variations observed between populations and *MC1R/BRAF* association may be because of differences in environmental conditions and patterns of UVR exposure. Although the phenotypes and sun exposure histories were not measured in both populations, it would be reasonable to expect that the Spanish cases had darker pigmentation and more cumulative sun exposure than the Austrian cases.

Melanoma risk is intricately associated with pigmentation characteristics. Genome-wide 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 conflicting results across studies examining solely *MC1R* status as a determinant for developing somatic *BRAF*-mutant melanoma may be because of the confounding role of other pigmentation genes. The simple approach of studying one gene in isolation is a major limitation of all recent studies, including ours. The classification of *MC1R* across previous studies has also not taken into account the functional impact of the different variants or the different allele frequencies for *MC1R* variants across different populations. Gathering more genetic information and modeling the complex regulation of pigmentation as a factor of genetic interactions should be the focus of future studies.

The strengths of our study include the comprehensive molecular and clinical data collected on over 375 patients. Limitations of this study include the low rate of successful DNA extraction from the tumors, and the lack of phenotype

Table 4. The association between *MC1R* and *BRAF* or *NRAS*

	BRAF V600			*NRAS* Q61		
MC1R	**WT**	**Mutant**	**OR (95% CI)[1]**	**WT**	**Mutant**	**OR (95% CI)[1]**
All lesions—combined						
WT/WT	61 (76)	19 (24)	Ref.	65 (81)	15 (19)	Ref.
Any variant	185 (77)	56 (23)	1.0 (0.5–1.8)	205 (85)	36 (15)	0.8 (0.4–1.5)
Missing	48	6		43	11	
Total	294	81	$P=0.9$	313	62	$P=0.4$
Trunk—combined						
WT/WT	37 (80)	9 (20)	Ref.	34 (74)	12 (26)	Ref.
Any variant	100 (69)	44 (31)	1.8 (0.8–4.1)	119 (83)	25 (17)	0.6 (0.3–1.3)
Total	137	53	$P=0.1$	153	37	$P=0.2$
Head and neck—combined						
WT/WT	24 (71)	10 (29)	Ref.	31 (91)	3 (9)	Ref.
Any variant	85 (88)	12 (12)	0.3 (0.1–0.9)	86 (89)	11 (11)	1.3 (0.3–5.5)
Total	109	22	**$P=0.02$**	117	14	$P=0.7$
All lesions—Spanish						
WT/WT	27 (79)	7 (21)	Ref.	28 (82)	6 (18)	Ref.
Any variant	47 (70)	20 (30)	1.6 (0.6–4.3)	56 (84)	11 (46)	0.9 (0.3–2.7)
Missing	30	3		29	4	
Total	104	30	$P=0.3$	113	21	$P=0.9$
Trunk—Spanish						
WT/WT	20 (80)	5 (20)	Ref.	19 (76)	6 (24)	Ref.
Any variant	29 (62)	18 (38)	2.5 (0.8–7.8)	39 (83)	8 (17)	0.6 (0.2–2.1)
Total	49	23	$P=0.1$	58	14	$P=0.5$
Head and neck—Spanish						
WT/WT	7 (78)	2 (22)	Ref.	9 (100)	0 (0)	Ref.
Any variant	18 (90)	2 (10)	0.4 (0.05–3.2)	17 (85)	3 (15)	NV
Total	25	4	$P=0.4$	26	3	
All lesions—Austrian						
WT/WT	34 (74)	12 (26)	Ref.	37 (80)	9 (20)	Ref.
Any variant	138 (79)	36 (21)	0.7 (0.3–1.6)	149 (86)	25 (14)	0.7 (0.3–1.6)
Missing	18	3		14	7	
Total	190	51	$P=0.4$	200	41	$P=0.4$
Trunk—Austrian						
WT/WT	17 (81)	4 (19)	Ref.	15 (71)	6 (29)	Ref.
Any variant	71 (73)	26 (27)	1.6 (0.5–5.1)	80 (82)	17 (18)	0.5 (0.2–1.6)
Total	88	30	$P=0.5$	95	23	$P=0.2$
Head and neck—Austrian						
WT/WT	17 (68)	8 (32)	Ref.	22 (88)	3 (12)	Ref.
Any variant	67 (87)	10 (13)	0.3 (0.1–0.9)	69 (90)	8 (10)	0.8 (0.2–3.5)
Total	84	18	**$P=0.03$**	91	11	$P=0.8$

Abbreviations: CI, confidenc einterva; MC1R, melanocortin-1 receptor; NV, not valid because of small group numbers; OR, odds ratio; WT, wild type.
P-value = χ^2 analysis.
[1]OR and 95% CI, adjusted for age and sex.
P-values shown in bold <0.05.

data for the Austrian patients. Although it is possible that this may have introduced bias, this would only alter the findings if the strength of associations between *BRAF* or *NRAS* mutation status and, e.g., anatomical site differed between the patients for whom DNA was and was not extracted. This is possible, but considered unlikely, as the prevalence of mutations and the patterns of association that we observed accord with other published series. Should the findings here be the result of systematic bias, then this would presumably extend to other series also. Austrian cases were ascertained over a longer duration than Spanish cases, and so we cannot exclude fixation artifacts or other anomalies as an explanation for these findings. Moreover, as our sample size was relatively small, the study lacks the statistical power to test whether effect sizes for BRAF or NRAS mutation associated with clinical factors differed across study periods. We also did not perform full sequencing of the entire *MC1R* gene. Our *MC1R* genotyping platform detects more than 95% of the nonsynonymous changes observed in the population (Kanetsky *et al.*, 2006). It is therefore highly unlikely that rare *MC1R* variants not covered by our platform would markedly affect our results. Information regarding the presence or absence of solar elastosis was not available for these archived melanoma sections. As a proxy for pattern of sun exposure, we categorized melanomas arising on the head and neck as habitually sun exposed and melanomas from the trunk as intermittently sun exposed. Melanomas arising on the arms and legs were not included in this analysis, because patterns of sun exposure on the limbs are far more variable and thus cannot be assumed to represent any particular pattern of exposure based solely on their anatomic site.

Understanding the UVR dose and threshold needed to initiate melanocyte transformation and the interplay between host pigmentation are necessary steps towards shedding some light on the pathways to melanoma development. Further pooled studies, including samples across the diverse populations studied to date, are required to validate the true correlation between germline *MC1R* variants and the induction of somatic mutations in melanoma. Such studies need to be adequately powered to separate the effects of the source populations, specific *MC1R* variants, tumor site, and tumor subtypes.

MATERIALS AND METHODS

Subjects

We compared the prevalence of *BRAF* and *NRAS* mutations in formalin-fixed paraffin-embedded melanoma specimens, which were collected from the Instituto Valenciano de Oncología in Valencia, Spain (diagnosed 2000–2009) and through the Graz medical centre in Austria (diagnosed 1990–2008). 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 1154 eligible patients, we collected sufficient DNA for mutation analysis from 375 patients. The age and sex distribution of the 375 patients who were genotyped differed from the 779 patients who were not genotyped, being less likely to be >50 years ($P=0.0001$) and more likely to be male ($P=0.05$).

Approval to conduct the study was given by the Human Research Ethics Committee of the Queensland Institute of Medical Research and by the Ethics Committee of the Medical University of Graz. The study adhered to The Declaration of Helsinki Principles 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 were cut from each tumor block and deparaffinized in xylene and washed two times in absolute ethanol. DNA was isolated using Qiagen DNeasy Tissue Kit (Qiagen, Hilden, Germany), with additional proteinase K digestion at 55 °C for 48 hours. DNA was extracted from whole blood buffy coat and melanoma cell lines using the 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, San Diego, CA). An optimized multiplex assay of all nine common variants of *MC1R* (I155T, R142H, D84E, R160W, D294H, V92M, R163Q, V60L, and R151C) were used as described previously (Duffy *et al.*, 2004). Only nonsynonymous 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) previously described (Hacker *et al.*, 2010). 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. Detection of *BRAF* and *NRAS* mutations was based on cutoffs imposed using DNA from whole blood buffy coat as wild-type controls as published previously (Hacker *et al.*, 2010). The frequency of *BRAF* and *NRAS* mutations for each population are shown in Supplementary Table S2 (online).

Phenotypic characteristics and sun exposure history

Clinical, epidemiological, and histological data were collected prospectively from Spanish patients; the details of subject selection and data collection for this study have been described previously (Nagore *et al.*, 2009). Briefly, eligible patients underwent a clinical examination in which nevi >2 mm in diameter were counted. The density of freckling on the face, arm, and back was scored according to the Vancouver charts (Gallagher *et al.*, 1990). The presence or absence of actinic keratoses as well as their occupational history was recorded. Patients were also asked to report their sun exposure history recording the total number of severe and light sunburns. Hair and eye color was also recorded, as well as the skin type using the Fitzpatrick classification. This information was only collected for the Spanish case series and was not available for the Austrian cases.

Statistical analysis

We first performed simple cross-tabulations and calculated Pearson's χ^2 and/or Fischer's exact test (for cells with expected counts of <5) as a measure of statistical association between *BRAF* or *NRAS* mutation status and the range of phenotypic, histologic, and genetic variables. We tested for differences between the ORs for Spanish and Austrian

case series using the Breslow–Day homogeneity test. We then used multivariable logistic regression to calculate ORs and 95% CIs as the measure of association between patient/tumor characteristics and *BRAF* and *NRAS* mutation status, adjusting for potential confounding factors. All analyses were adjusted for age and sex. *P*-values $\leqslant 0.05$ were considered as statistically significant and all such tests were two-sided. All analyses were performed using the SAS 9.2 statistical software package (SAS institute, Cary, NC).

CONFLICT OF INTEREST

Peter Soyer is a consultant and shareholder for Molemap Australian and is the founder of e-derm-consult. All the other authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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