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MC1R Variants Increase Risk of Melanomas Harboring *BRAF* Mutations

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Abstract

Melanocortin-1 receptor (*MC1R*) variants have been associated with *BRAF* (v-raf murine sarcoma viral oncogene homolog B1) mutations in non-CSD (chronic solar-damaged) melanomas in an Italian and an American population. We studied an independent Italian population of 330 subjects (165 melanoma patients and 165 controls) to verify and estimate the magnitude of this association and to explore possible effect modifiers. We sequenced *MC1R* in all subjects and exon 15 of

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CONFLICT OF INTEREST
The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL
Table S1. Distribution of non-synonymous *MC1R* variants in melanoma patients and controls of two Italian populations.
Table S2. Comparison of patient and melanoma characteristics between two Italian populations.
Table S3. Distribution of *BRAF*-mutant melanomas by melanoma characteristics in two Italian populations.
Table S4. Risk of melanoma by *MC1R* germline variants and melanoma *BRAF* mutation status in case–control analysis in population 1.
Table S5. Comparison of demographic and phenotypic characteristics of subjects and melanoma lesions in cases analyzed and not analyzed for *BRAF* mutations in population 1.

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BRAF in 92/165 melanoma patients. Patients with *MC1R* variants had a high risk of carrying *BRAF* mutations in melanomas (odds ratio (OR) = 7.0, 95% confidence interval (CI) = 2.1–23.8) that increased with the number of *MC1R* variants and variants associated with red hair color. Combining these subjects with the originally reported Italian population (513 subjects overall), *MC1R* variant carriers had a 5- to 15-fold increased risk of *BRAF*-mutant melanomas based on carrying one or two variants ($P<0.0001$, test for trend), and regardless of signs of chronic solar damage. In contrast, no association with *BRAF*-negative melanomas was found (OR = 1.0, 95% CI = 0.6–1.6). No characteristics of subjects or melanomas, including age, nevus count, pigmentation, and melanoma thickness or location on chronically or intermittently sun-exposed body sites, substantially modified this association, although results could be affected by the small numbers in some categories. This study confirms that the known *MC1R*–melanoma risk association is confined to subjects whose melanomas harbor *BRAF* mutations.

INTRODUCTION

The melanocortin-1 receptor (*MC1R*) gene (MIM 155555) is a key determinant of human pigmentation, and is highly polymorphic in Caucasians with specific variants linked to the red hair color phenotype (Rees, 2004; Gerstenblith *et al.*, 2007). As shown in many studies worldwide, *MC1R* is also a low-penetrance melanoma susceptibility gene (Valverde *et al.*, 1996; Palmer *et al.*, 2000; Kennedy *et al.*, 2001; Dwyer *et al.*, 2004; Matichard *et al.*, 2004; Landi *et al.*, 2005; Kanetsky *et al.*, 2006; Stratigos *et al.*, 2006; Fargnoli *et al.*, 2006a; Fernandez *et al.*, 2007).

The *BRAF* oncogene (MIM 164757) encodes a serine/threonine kinase that acts in the Ras–RAF–MAPK (mitogen-activated protein kinase) pathway, and is mutated in over 60% of cutaneous melanomas, mostly in codon 600 of exon 15 (Davies *et al.*, 2002). A higher frequency of *BRAF* mutations was found in melanomas occurring on skin with absent or minor histopathological signs of chronic solar damage (CSD) (“non-CSD melanomas”) as compared to “CSD melanomas” (Maldonado *et al.*, 2003).

Variants of *MC1R* have been recently reported to be associated with *BRAF* mutation status in non-CSD melanomas in an Italian and an American population (Landi *et al.*, 2006). A few hypotheses were suggested to explain the underlying mechanism responsible for this association, but no analysis could be performed because of the small sample size and/or lack of data on phenotypic characteristics of the American study subjects.

We studied an independent Italian population from central Italy to (i) verify the association of *MC1R* variants and *BRAF*-mutant melanomas; (ii) explore possible effect modifiers of the association combining data of this population with data from the originally reported Italian population in Landi *et al.* (2006); and (iii) estimate the magnitude of the *MC1R–BRAF* association in comparison with the overall risk of melanoma associated with carrying *MC1R* variants, taking advantage of the *MC1R* data on control subjects from both Italian populations.

RESULTS AND DISCUSSION

We defined our study population as “population 1,” and the original Italian population in Landi *et al.* (2006) as “population 2,” Identified *MC1R* variants in both populations are listed in Table S1.

In population 1, melanoma patients with at least one germline *MC1R* variant had a sevenfold (95% CI = 2.1–23.8) increased risk of developing melanomas with *BRAF* mutations as compared with the individuals with two wild-type alleles ($P = 0.002$) (Table 1).

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Categorization in three finer groups showed that odds of *BRAF*-mutant melanomas increased progressively with the number of *MC1R* variants (*P*-trend < 0.01) (Table 1). Additional inclusion of D84E among the "R" variants (Duffy *et al.*, 2004) did not significantly modify the association (data not shown). For comparison, results from the Italian population in Landi *et al.* (2006), not stratified by CSD (defined as population 2), are reported in Table 1. Overall, the *MC1R–BRAF* association was similar in both Italian populations. Interestingly, in population 1, the association was stronger for multiple *MC1R* variants than for other *MC1R* classifications based on variant type ("R" or "r"), whereas in population 2, "R" variants played a major role (Table 1), possibly reflecting small numbers in some categories and different frequency of variants (Table S1).

Characteristics of patients and melanomas did not significantly differ between the two Italian populations (Table S2), with the exception of nevus count that followed different assessment and scoring criteria (population 1, ≥2mm nevi counted on the entire body and population 2, nevi of any size counted on the back only). *BRAF* mutations were present in 43.5% of melanomas in population 1 and 63.5% in population 2 ($P = 0.01$) and were not affected by anatomical location of the primary melanoma. In population 1, mutations were more frequent in thicker melanomas, whereas they were evenly distributed among *in situ* melanomas (Table S3).

Given the similarities between the two Italian populations, we combined them to increase the sample size and related statistical power to investigate possible effect modifiers of this association. In the 177 overall cases with *BRAF* data, we confirmed the *MC1R–BRAF* association (OR = 7.3, 95% CI = 2.9–18.5), with increasing risk in subjects with multiple *MC1R* variants (*P*-trend < 0.0001) (Table 1). Notably, the *MC1R–BRAF* association was not significantly affected by age, tanning ability, melanoma thickness, anatomical location of the primary tumor, or nevus count (Table 2), although differences in the estimates by tanning ability and melanoma location were observed, and significance could have been affected by the small number of subjects. We could not perform a formal statistical analysis of the *MC1R–BRAF* association in models stratified by hair or eye color (dark/light), as some categories in these analyses included no subjects. However, the distribution of subjects was similar within groups, suggesting no substantial differences in the *MC1R–BRAF* association by these pigmentation characteristics.

We then proceed with a case–control analysis to estimate the magnitude of the *MC1R–BRAF* association in comparison with the overall association between *MC1R* variant and melanoma risk, taking advantage of the *MC1R* data on all control subjects. In population 1, we found a strong association between *MC1R* variants and melanoma harboring *BRAF* mutations (Table S4). This association was confirmed in the combined cases and controls ($n = 513$) (Table 3). As found in previous studies (Palmer *et al.*, 2000;Kennedy *et al.*, 2001;Dwyer *et al.*, 2004;Matichard *et al.*, 2004;Landi *et al.*, 2005;Kanetsky *et al.*, 2006;Stratigos *et al.*, 2006;Fargnoli *et al.*, 2006a;Fernandez *et al.*, 2007), the risk of melanoma increased in subjects with MC1R variants (OR = 2.2, 95% CI = 1.4–3.4) and, particularly, in those with multiple variants (*P*-trend ≤ 0.0001). However, when we stratified the melanoma cases between those with melanomas harboring *BRAF* mutations and those with no *BRAF* mutations, the risk associated with *MC1R* variants was confined only to *BRAF*-mutant melanomas, ranging from 5- to 15-fold in carriers of one or multiple *MC1R* variants. No association was found with melanomas without *BRAF* mutations (Table 3).

This confirms that in the Italian population, *MC1R* variants are strongly associated with *BRAF*-mutant melanomas independently of the degree of solar damage in the areas adjacent to the melanoma lesions. In the original study, the association of *MC1R* with *BRAF* mutations was restricted to non-CSD-melanomas in the American population, whereas the

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number of CSD melanomas in the Italian population was too small to carry out any analysis (Landi *et al.*, 2006). The difference between the Italian and American populations with regard to CSD could be due to the age difference. In fact, both Italian populations were approximately 10 years younger than the American population in Landi *et al.* (2006), and the degree of chronic solar damage increases with age. Also, differences in sun sensitivity between populations, and the variations in tissue fixation and staining that could affect the recognition of signs of CSD adjacent to the melanoma lesions, could have played a role.

In conclusion, the original observation of an association between *MC1R* variants and *BRAF*-mutant melanomas (Landi *et al.*, 2006) is strongly confirmed in this independent population, whereas no association was observed in subjects whose melanomas had no *BRAF* mutations. Moreover, given the similarities between our population and the original Italian group in Landi *et al.* (2006), we could pool the data of two studies, and explore the effect of phenotypic characteristics of subjects and the features of the melanoma lesions on this association. No hypothesized factors modified this association. Whether the *MC1R–BRAF* link is a consequence of a direct effect of impaired *MC1R* on *BRAF* or is an epiphenomenon of alterations in other pathways is unclear and warrants further research.

MATERIALS AND METHODS

Study population

We analyzed 165 sporadic melanoma patients and 165 sex- and age-matched healthy controls (82 males and 83 females, aged 17–82 years) enrolled in central Italy (L'Aquila, Florence and Modena), from 2000 to 2002 (defined as population 1). To increase the sample size and related statistical power of possible effect modifiers of the *MC1R–BRAF* association, the results of population 1 were compared and combined with data of the Italian population in Landi *et al.* (2006) (defined as population 2), which included 183 melanoma patients (87 males and 96 females, aged 17–77 years) and 179 control subjects (89 males and 90 females), frequency-matched to cases in terms of sex and age by decade, enrolled in Northeastern Italy (Bufalini Hospital of Cesena, Italy) from 1994 to 1999. For both populations, data on characteristics of subjects were collected through standardized questionnaires (lifetime residential history, medical history, family history of cancer and other diseases, UV exposure habits, skin reaction to the first 30 minutes of sun exposure, tanning ability after prolonged sun exposure) and skin examination (skin type, hair and eye color, freckling, number of melanocytic nevi, and presence of clinically atypical nevi) are described in detail in Fargnoli *et al.* (2006b) for population 1 and Landi *et al.* (2001, 2006) for population 2, respectively. CSD in skin adjacent to melanomas was independently assessed in melanoma tissue sections by two pathologists (BCB and DEE), using a multipoint scale from 0 to 3 +, as described (Landi *et al.*, 2006). However, an unambiguous scoring in the critical moderate-to-severe range of solar elastosis could not be reached because of the variability and/or poor staining quality of some hematoxylin and eosin-stained sections. As the unequivocal CSD-positive cases were few as in the original Italian population (Landi *et al.*, 2006), we analyzed all melanomas regardless of the CSD status.

Written informed consent was obtained under Bufalini Hospital's, University of L'Aquila's, and National Cancer Institute' Institutional Review Board-approved protocols in accordance with the Declaration of Helsinki Principles.

MC1R and *BRAF* sequencing

The 951 bp *MC1R* coding region (AF153431) was directly sequenced either in its entirety or in two overlapping fragments by PCR followed by direct sequencing of the amplicon(s) in

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blood genomic DNA from all subjects. Specific primers and sequencing chemistries have been previously described (Landi *et al.*, 2005; Fargnoli *et al.*, 2006a).

Molecular analysis of *BRAF* exon 15 was carried out on somatic DNA, extracted by manual microdissection using a dissection microscope to select areas in which melanoma cells dominated over stromal cells. As in the original Italian population (Landi *et al.*, 2006), we excluded acral melanomas because *BRAF* mutations are known to be rare in these lesions (Maldonado *et al.*, 2003). Given the small size of the melanoma lesions and the necessity to use most of the lesion for diagnosis, sufficient/good quality DNA for *BRAF* analysis could be extracted only from a subgroup of tissue specimens, specifically from 92 cases in population 1 and 85 cases in population 2. Exon 15 of *BRAF* (NM_004333) was sequenced as described (Landi *et al.*, 2006). The characteristics of subjects and melanomas did not substantially differ between cases with or without data on *BRAF* mutation status (Table S5 for population 1 and Landi *et al.*, 2006 for population 2), and thus selection bias is unlikely, although cannot be excluded.

Statistical analysis

The association between *MC1R* variants and *BRAF*-mutant melanomas was explored using logistic regression models in case–case and case–control analyses adjusted for the matching variables and for other potential confounders, including pigmentation characteristics and nevus count. OR and corresponding 95% CI adjusted for age are reported (other adjustments provided similar results). All *P*-values were two-sided. For statistical analysis, *MC1R* variants were grouped as “R” (R151C, R160W, and D294H) or “r” variants (any non-R variant excluding synonymous changes), as described previously (Landi *et al.*, 2005, 2006). Patients were categorized in four groups based on *MC1R* genotype to explore possible differences of *MC1R* variants (Beaumont *et al.*, 2007).

Abbreviations

BRAF	v-raf murine sarcoma viral oncogene homolog B1
CI	confidence interval
CSD	chronic solar damage
MC1R	melanocortin-1 receptor
OR	odds ratio

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Table 1

Association between germline variants of *MC1R* and somatic *BRAF* mutations in melanoma patients from two Italian populations

	Population 1				Population 2[1]				Combined			
***MC1R* genotype[2]**	***BRAF* wt**	***BRAF* mutant**	**OR (95% CI)[3]**	***P*-value**	***BRAF* wt**	***BRAF* mutant**	**OR (95% CI)[3]**	***P*-value**	***BRAF* wt**	***BRAF* mutant**	**OR (95% CI)[3]**	***P*-value**
wt/wt	20	4	Reference		9	3	Reference		29	7	Reference	
Any variant	32	36	7.0 (2.1–23.8)	0.002	22	51	8.4 (1.9–36.9)	0.005	54	87	7.3 (2.9–18.5)	<0.0001
wt/wt	20	4	Reference		9	3	Reference		29	7	Reference	
r/wt or R/wt	24	21	5.6 (1.6–20.2)	0.008	17	33	7.4 (1.6–33.4)	0.01	41	54	6.0 (2.3–15.9)	0.0003
r/r or R/r or R/R	8	15	10.3 (2.5–42.1)	0.001	5	18	11.3 (2.1–62.1)	0.005	13	33	10.5 (3.6–31.0)	<0.0001
Total	52	40	*P*-trend	0.001	31	54	*P*-trend	0.008	83	94	*P*-trend	<0.0001
wt/wt	20	4	Reference		9	3	Reference		29	7	Reference	
r/wt	14	14	6.5 (1.7–25.3)	0.007	13	21	6.0 (1.3.28.2)	0.02	27	35	5.8 (2.1–16.1)	0.0007
r/r or R/r or R/wt or R/R	18	22	7.4 (2.0–26.9)	0.002	9	30	11.9 (2.4–57.6)	0.002	27	52	8.6 (3.2–23.2)	<0.0001
Total	52	40	*P*-trend	0.004	31	54	*P*-trend	0.003	83	94	*P*-trend	<0.0001
wt/wt	20	4	Reference		9	3	Reference		29	7	Reference	
r/wt or r/r	17	19	6.9 (1.9–25.7)	0.004	15	26	6.3 (1.4–29.0)	0.02	32	45	6.3 (2.3–16.8)	0.0003
R/wt or R/r or R/R	15	17	7.1 (1.9–26.9)	0.004	7	25	13.0 (2.5–67.0)	0.002	22	42	8.7 (3.1–24.1)	<0.0001
Total	52	40	*P*-trend	0.006	31	54	*P*-trend	0.003	83	94	*P*-trend	<0.0001

BRAF, v-raf murine sarcoma viral oncogene homolog B1; CSD, chronic solar-damaged; CI, confidence interval; *MCIR*, melanocortin-1 receptor; OR, odds ratio; wt, wild type.

[1] Data from Landi *et al.* (2006) combining cases with positive and negative signs of chronic solar damage in skin adjacent to the melanomas (non-CSD melanomas+CSD-melanomas).

[2] *MC1R* variants were grouped as “R” (R151C, R160W, and D294H) or “r” variants (any non-R variant excluding synonymous changes) (Landi *et al.*, 2005, 2006).

[3] Logistic regression models adjusted by median age (and by age and population in combined analyses).

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Table 2

Association between *MC1R* variants and *BRAF* mutations stratified by age, tanning ability, melanoma characteristics, and nevus count in the melanoma patients of the Italian populations

			Population 1 *N*=92			Population 2 *N*=85			Combined *N*=177			
Variable	**Values[1]**	***MC1R* genotype**	***BRAF* wt**	***BRAF* mutant**	**OR[2] (95% CI)**	***BRAF* wt**	***BRAF* mutant**	**OR[2] (95% CI)**	***BRAF* wt**	***BRAF* mutant**	**OR[2] (95% CI)**	***P*-value[3]**
Age (below/above median)	Below	wt/wt	9	2	Reference	5	2	Reference	13	4	Reference	
		Any variant	16	19	6.2 (1.1–34.2)	8	30	9.4 (1.5–57.6)	22	49	7.2 (2.1–24.7)	0.88
	Above	wt/wt	11	2	Reference	4	1	Reference	16	3	Reference	
		Any variant	16	17	9.7 (1.5–64.6)	14	21	6.0 (0.6–59.4)	32	38	6.3 (1.7–23.7)	
Tanning ability	Low	wt/wt	14	3	Reference	8	1	Reference	22	4	Reference	
		Any variant	18	21	11.5 (2.1–62.0)	14	32	18.1 (2.0–162.8)	32	53	13.8 (3.6–52.1)	0.23
	High	wt/wt	6	1	Reference	1	2	Reference	7	3	Reference	
		Any variant	14	15	6.1 (0.6–58.0)	8	16	2.8 (0.2–46.8)	22	31	3.4 (0.7–16.3)	
Sun exposure at tumor site[4]	Chronic	wt/wt	8	1	Reference	2	0	Reference	10	1	Reference	
		Any variant	9	17	13.9 (1.5–133.3)	8	13	NA	17	30	17.9 (2.0–157.5)	0.23
	Intermittent	wt/wt	12	3	Reference	15	3	Reference	27	6	Reference	
		Any variant	22	19	3.4 (0.8–14.0)	14	35	4.4 (0.9–22.1)	36	54	4.4 (1.6–12.2)	
Melanoma thickness[5] (below/above median)	Below	wt/wt	10	2	Reference	6	2	Reference	16	4	Reference	
		Any variant	14	8	4.8 (0.7–35.2)	6	21	11.4 (1.5–88.2)	20	29	5.6 (1.6–19.4)	0.43
	Above	wt/wt	3	2	Reference	1	1	Reference	4	3	Reference	
		Any variant	10	20	5.4 (0.4–66.9)	10	26	3.3 (0.2–64.6)	20	46	3.4 (0.7–17.3)	
Nevus count[6] (below/above median)	Below	wt/wt	8	0	Reference	4	1	Reference	8	0	Reference	
		Any variant	13	11	NA	12	27	11.5 (1.1–125.2)	17	12	NA	0.24
	Above	wt/wt	12	4	Reference	4	1	Reference	20	6	Reference	
		Any variant	19	25	5.3 (1.3–21.2)	8	20	10.0 (1.0–104.2)	35	71	6.7 (2.5–18.3)	

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BRAF, v-raf murine sarcoma viral oncogene homolog B1; CI, confidence interval; *MCIR*, melanocortin-1 receptor; OR, odds ratio; wt, wild type; NA, not available.

Numbers may vary across the strata due to missing variables.

[1]Numbers in each stratum are based on the specific values of each population (for example, medians of the same variable can vary between populations).

[2]Models adjusted by median age; the combined analyses could not be adjusted by population because of small numbers in some categories.

[3]*P*-value for interaction of the *MC1R–BRAF* association and the variable of each stratum.

[4]Chronically exposed sites: face, scalp, neck, back of hands, lower legs, and forearms; intermittently exposed sites: chest, back, upper legs, and upper arms.

Acral melanomas were excluded.

[5]*In situ* melanoma was excluded.

[6]Population 1, ≥2mm nevi counted on the entire body and population 2, nevi of any size counted on the back only.

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Table 3

Risk of melanoma in two combined Italian case–control studies by *MC1R* germline variants and *BRAF* mutation status in melanomas

MC1R genotype[1]	No. of controls[2]	No. of melanoma cases[3]			OR for melanoma risk (95% CI)[4]					
		All cases	*BRAF* wt	*BRAF* mutant	All cases	*P*-value (all cases)	*BRAF* wt	*P*-value (*BRAF* wt)	*BRAF* mutant	*P*-value (*BRAF* mutant)
wt/wt	121	36	29	7	Reference		Reference		Reference	
Any variant	214	141	54	87	2.2 (1.4–3.4)	0.0003	1.0 (0.6–1.6)	0.94	7.5 (3.3–16.8)	<0.0001
wt/wt	121	36	29	7	Reference		Reference		Reference	
r/wt or R/wt	171	95	41	54	1.9 (1.2–2.9)	0.007	1.0 (0.6–1.6)	0.85	5.8 (2.5–13.2)	<0.0001
r/r or R/r or R/R	43	46	13	33	3.7 (2.1–6.4)	<0.0001	1.1 (0.5–2.4)	0.82	15.3 (6.1–37.6)	<0.0001
Total	335	177	83	94	*P*-trend	<0.0001	*P*-trend	0.91	*P*-trend	<0.0001
wt/wt	121	36	29	7	Reference		Reference		Reference	
r/wt	129	62	27	35	1.6 (1.0–2.7)	0.05	0.8 (0.5–1.5)	0.53	5.1 (2.1–11.9)	0.0002
r/r or R/r or R/wt or R/R	85	79	27	52	3.1 (1.9–5.0)	<0.0001	1.2 (0.7–2.2)	0.54	11.0 (4.8–25.6)	<0.0001
Total	335	177	83	94	*P*-trend	<0.0001	*P*-trend	0.58	*P*-trend	<0.0001
wt/wt	121	36	29	7	Reference		Reference		Reference	
r/wt or r/r	163	77	32	45	1.6 (1.0–2.5)	0.05	0.8 (0.4–1.3)	0.34	5.1 (2.2–11.8)	0.0001
R/wt or R/r or R/R	51	64	22	42	4.2 (2.5–7.2)	<0.0001	1.8 (0.9–3.5)	0.09	14.1 (6.1–34.5)	<0.0001
Total	335	177	83	94	*P*-trend	<0.0001	*P*-trend	*0.19*	*P*-trend	<0.0001

BRAF, v-raf murine sarcoma viral oncogene homolog B1; CI, confidence interval; *MCIR*, melanocortin-1 receptor; OR, odds ratio; wt, wild type.

[1] *MC1R* variants were grouped as “R” (R151C, R160W, and D294H) or “r” variants (any non-R variant excluding synonymous changes) (Landi *et al.*, 2005, 2006).

[2] One control had missing age and was excluded from the analysis.

[3] All cases analyzed for *BRAF*.

[4] Logistic regression models adjusted by age (quartiles) and population.