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Melanocortin-1 Receptor Polymorphisms and Risk of Melanoma: Is the Association Explained Solely by Pigmentation Phenotype?

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Summary

Risk of cutaneous malignant melanoma (CMM) is increased in sun-exposed whites, particularly those with a pale complexion. This study was designed to investigate the relationship of the melanocortin-1 receptor (MC1R) genotype to CMM risk, controlled for pigmentation phenotype. We report the occurrence of five common MC1R variants in an Australian population-based sample of 460 individuals with familial and sporadic CMM and 399 control individuals—and their relationship to such other risk factors as skin, hair, and eye color; freckling; and nevus count. There was a strong relationship between MC1R variants and hair color and skin type. Moreover, MC1R variants were found in 72% of the individuals with CMM, whereas only 56% of the control individuals carried at least one variant ($P < .001$), a finding independent of strength of family history of melanoma. Three active alleles (Arg151Cys, Arg160Trp, and Asp294His), previously associated with red hair, doubled CMM risk for each additional allele carried (odds ratio 2.0; 95% confidence interval 1.6–2.6). No such independent association could be demonstrated with the Val60Leu and Asp84Glu variants. Among pale-skinned individuals alone, this association between CMM and MC1R variants was absent, but it persisted among those reporting a medium or olive/dark complexion. We conclude that the effect that MC1R variant alleles have on CMM is partly mediated via determination of pigmentation phenotype and that these alleles may also negate the protection normally afforded by darker skin coloring in some members of this white population.

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Introduction

Melanoma is a disease that presents in a number of forms (Balch et al. 1992). The most common form in whites is cutaneous malignant melanoma (CMM), which usually begins with a radial proliferation of melanocytes that is followed by a vertical growth phase in which metastasis may occur. Definition of the genes involved in the etiology of CMM (MIM 155600 and MIM 155601) has been difficult, except in cases of very strong familial risk (Hayward 1996). However, multiple phenotypic risk factors, including the number of melanocytic nevi (moles), freckling tendency, presence of dysplastic nevi, propensity to sunburn, and number of severe sunburn episodes during youth, have been identified in the etiology and pathogenesis of this disease (Tilgen 1993; Bliss et al. 1995). Evidence suggests that an individual's susceptibility to CMM may be determined through the interaction between the pigmentary system and solar UV light (Bliss et al. 1995; Breitbart et al. 1997), with lighter skin tones and red hair color significantly increasing the risk of melanoma (Garbe et al. 1994; Grange et al. 1995; Grulich et al. 1996).

That whites have a far higher incidence of CMM than do other populations suggests that deficient melanization removes the protective sun-shielding effects of pigmentation and, hence, increases the genotoxic effects of sunlight. However, the absolute amount of melanin deposited in the skin must also be viewed in light of the fact that some melanins and melanogenic precursors can be mutagenic or cytotoxic (Urabe et al. 1994). It has been noted that whites have more of the red/yellow pheomelanin, which can contribute to the generation of mutagenic photoproducts after exposure to UV light (Hill et al. 1997), and that this may lead to a higher incidence of CMM. Hence, characterization of human pigmentation genes involved in the pathway of melanin formation is needed to give a genetic explanation for the deposition of different amounts and types of melanin in the skin (Sturm et al. 1998). In the present study, we analyze known polymorphisms in the human melanocortin-1 receptor gene (MC1R [MIM 155555], located

on 16q24) involved in pigmentation, to assess their relationship to CMM risk.

The MC1R gene is remarkably polymorphic in whites (Valverde et al. 1995; Box et al. 1997; Smith et al. 1998), and there are several reasons to suggest that particular human MC1R alleles may be associated with increased CMM susceptibility. MC1R encodes the melanocyte-stimulating hormone receptor (MSHR), a member of the G-protein–coupled receptor superfamily, whose members can act as oncogenes in other systems (Coughlin 1994). However, there is no evidence that this is the mechanism of action of MC1R in CMM. During exposure of the skin to UV light, there is an increase in melanin production, through the tanning response, that may occur via an increase in tyrosinase gene transcription and enzyme activity (Gilchrest et al. 1996; Sturm 1998). It has also been shown that exposure to sunlight increases the levels of α-melanocyte-stimulating hormone and proopiomelanocortin peptides in the skin (Slominski et al. 1993), suggesting that one of the actions of solar UV may be mediated through the MSHR pathway, to stimulate cAMP (Pawelek et al. 1992) as well as other signal-transduction pathways (Romero-Graillet et al. 1996). MSHR is also known to be involved in directing the production of the two distinct melanin types, red/yellow pheomelanin and black/brown eumelanin, with particular human MC1R alleles associated with red hair and fair skin (Valverde et al. 1995; Box et al. 1997).

In addition to defining a consensus MC1R allele sequence by comparison of Chinese and white individuals encoding the 317-amino-acid MSHR protein, we found 13 variant MC1R alleles in the analysis of twins, sibs, and parents collected in southeast Queensland, Australia (Box et al. 1997; Sturm et al. 1998), for a study of the genetics of mole formation (Zhu et al. 1999). Three of these MC1R alleles—Arg151Cys, Arg160Trp, and Asp294His—were statistically associated with red hair; one allele, Val60Leu, was associated with blonde/light brown hair, and any variation from the consensus allele was associated with lighter skin tones (Box et al. 1997; Smith et al. 1998). Therefore, not only is the level of melanin pigment in the human skin dependent on the MC1R genotype, but it is also dependent on the pheomelanin/eumelanin pigment type. MC1R genotype may well be involved in determination of the tanning response of skin to UV light, which can then either ameliorate or exacerbate the genotoxic effects of sunlight, depending on the properties of melanin precursors and pigment induced.

In an earlier study, the Asp84Glu MC1R allele that was also found in our Queensland population was reported to be present, at higher frequency, in a collection of British patients with sporadic melanoma (Valverde et al. 1996). In addition, MC1R variants may modify melanoma risk in Dutch families with familial melanoma (Frants et al. 1996). Because the overall importance of MC1R gene variation in the etiology of CMM in the general population is unknown, we sought to examine the association between several major MC1R variants and melanoma risk in a population-based sample of families ascertained through probands with CMM and in unselected controls from the same population.

Subjects and Methods

Subjects

Family ascertainment and data collection of CMM case samples have been described in detail elsewhere (Aitken et al. 1996, 1999). In brief, patients with CMM were ascertained from a population-based cancer register, and family members were followed up by use of a sequential sampling scheme. Two control groups were used for comparison of MC1R allele frequencies. The first control group included a sample of 218 parents of twins who were subjects in a study of the genetics of mole formation (Box et al. 1997; McGregor et al. 1999). The second control group comprised 181 unrelated individuals, sampled at random from 3,300 participants in a twin study of the inheritance of alcohol use, who had been drawn from the Australian Twin Registry (Heath et al. 1997). This project received ethics approval from our institutional ethics committee.

Measures

CMM case samples.—The total sample of 1,897 families available for this study was divided into three strata of familial melanoma risk—low, intermediate, and high—by use of a permutation procedure described elsewhere (Aitken et al. 1994, 1999). We attempted to obtain, in each of the 91 high-risk families, blood samples from two individuals with CMM, sampled at random from all patients in the family, and from one individual with CMM in each of 200 intermediate-risk families and 200 low-risk families, selected at random. If no cases were available in a selected intermediate-risk or low-risk family, the next random number was chosen, until 200 families in each group had been recruited. Ultimately, data on patients from 482 families were obtained.

Standard risk factors for melanoma, including propensity to burn in the sun, pigmentation (skin color, hair color at 21 years, eye color, total freckling in summer, and density of melanocytic nevi) were sought by means of a mailed questionnaire. Of a total of 7,619 living relatives, 5,158 (68%) responded after intensive telephone follow-up. Index patients and other family members provided proxy reports (the quality of which we have previously reported [Aitken et al. 1993]) for an additional 4,588 relatives, providing a total of 9,746

relatives with risk-factor information. Only medically verified cases among relatives were included in the analysis. Hair color was recorded as being either black, dark brown, light brown, fair, or red/auburn, and skin color was recorded as being either fair, medium, or olive/dark. A three-point scale was also used for eye color, which included the categories "blue/gray," "green/hazel," and "brown." Total freckling in summer was self-reported as being either nil, 1–100, or >101, and density of melanocytic nevi was estimated by use of a scale including the categories "nil," "few," "moderate," or "many."

Control sample 1: parents of twin mole-study members.—Skin, hair, and eye color and mole counts have been recorded for most of the parents of twin mole-study members genotyped in this analysis, by use of measures identical to those used to record these characteristics in the individuals with CMM. However, a four-point scale was used to record freckle density, including no freckling and the presence of a few, some, and many freckles. With regard to the parents of twin mole-study members, a research nurse categorized each individual rather than relying on self-report.

Control sample 2: twin alcohol-study members.—Only hair and eye colors have been recorded for those individuals selected from the twin study of the inheritance of alcohol use, by use of measures identical to those used for both the individuals with CMM and the parents of twin mole-study members. The members of this study were phenotypically characterized by self-report.

Genotyping

DNA extraction, PCR amplification, agarose-gel electrophoresis, and filter blotting.—After extraction of DNA by standard methods (Miller et al. 1988), the nested primer strategy, described elsewhere (Box et al. 1997), was used to obtain sufficient DNA for dot blotting and for identification of MC1R alleles. Sequential *Taq* DNA polymerase amplifications were done in 96-well plates with 25–50-μl total volumes containing 10% dimethyl sulfoxide, starting with 25 ng genomic DNA and with the second reaction being initiated by use of 5 μl of the first-round reaction as template. Products from these amplifications were checked by running 2 μl on 1% agarose gels; equivalent amounts were then blotted onto dry nylon membrane (Magna; Micron Separations), and dots were allowed to air-dry. The membrane was placed on filter paper soaked in 0.5 M NaOH/1.5 M NaCl denaturation solution, 0.5 M Tris-Cl pH 7.5/1.5 M NaCl neutralization solution, and 2 × SCC rinse solution, each for 5 min. DNA was cross-linked to the filters, either by baking for 2 h at 80°C or by UV cross-linking.

Allele-specific oligonucleotide hybridization and optimization.—Probes were designed as 15-base allele-specific oligonucleotides, with the mismatch centrally positioned to provide maximal binding stability to the complementary sequence and instability of a mismatch. A total of 100 ng each oligonucleotide was radiolabeled with $\gamma[^{32}P]$-ATP, by use of polynucleotide kinase (NEB), and was fractionated from unincorporated nucleotide by use of chromatography on Sephadex G-25 (Pharmacia NAP 5 columns). Filters were prehybridized at 42°C for $\geq$2 h, in the presence of tetramethylammonium chloride (TMAC) (Wood et al. 1985) in 10 ml of 3 M TMAC, 0.1% SDS, 1 mM EDTA, 25 mM Na_3PO_4 pH 6.8, 5 × Denhardt's solution, and 0.1 mg herring-sperm DNA per ml (Shuber et al. 1993). A total of 50 μl radiolabeled probe, equating to $\geq 2.75 \times 10^5$ cpm hybridization solution per ml, was added directly to the prehybrization solution and was left to hybridize overnight at 42°C. The probe was denatured by being heated to 70°C for 5 min and was then quenched on ice before being added to the mix. Filters were rinsed in 3 M TMAC, 0.1% SDS, 1 mM EDTA, and Na_3PO_4 pH 6.8 wash solution at room temperature for 5 min; this was followed by a single 20-min wash at 50°C before autoradiographic exposure.

This method is capable of detection of single-base changes through different-temperature washes of oligonucleotides, and, with the addition of the TMAC salt in the hybridization cocktail and posthybridization washes, it can control the conditions of washing as a function of probe length and can select for exactly matching oligonucleotides (Wood et al. 1985; Shuber et al. 1993). Six MC1R variants (Val60Leu, Asp84Glu, Arg151Cys, Arg160Trp, Asp294His, and 537insC) were chosen for analysis, and oligonucleotides corresponding to the consensus or variant nucleotide substitutions and insertion were synthesized (table 1). These radiolabeled oligonucleotides were hybridized to MC1R PCR fragments that were blotted onto test filters corresponding to homozygote-consensus, heterozygote (1:1 mixture of consensus and variant), or homozygote-variant alleles. The signals generated after a washing at 50°C were visualized by autoradiography (fig. 1). Mismatched probes were effectively washed from the respective homozygote-dotted sample on each filter, with the positive and negative hybridization signal easily indicating the presence of MC1R homozygous-consensus or homozygous-variant alleles. In each case, the dotted sample representing heterozygosity of the MC1R PCR fragment showed a lower level of hybridization than did the homozygous state, with each of the consensus and variant oligonucleotides. These control experiments verified the differential hybridization-screening procedure, which was then used to determine the MC1R genotype at each of these positions in our sample collections.

Table 1

MC1R Allele-Specific Oligonucleotides and Variant Frequency in Control-Sample Subjects

Variant and Amino Acid	Oligonucleotide[a] (5′→3′)	Variant Allele Sample 1[b]	Variant Allele Sample 2[c]	Allele Frequency[d] (%)
V60L:				
Val	gctggtggtggccac			
Leu	gctggtgTtggccac	57	39	12.0
D84E:				
Asp	tgtcggacctgctgg			
Glu	tgtcggaActgctgg	7	5	1.5
R151C:				
Arg	cgcactgcgctacca			
Cys	cgcactgTgctacca	48	23	8.9
R160W:				
Arg	cctgccgcgggcgcg			
Trp	cctgccgTgggcgcg	30	31	7.6
D294H:				
Asp	catcatcgaccccct			
His	catcatcCaccccct	10	15	3.1
537insC:				
Wild type	gctcttcatcgccta			
InsC	gctcttcCatcgcct	Nil	Not done	.0

[a] Nucleotide substitutions and insertion are shown in uppercase and are underlined.
[b] Collected as parents of twins representing 436 haplotypes.
[c] Collected for a study of alcoholism representing 362 haplotypes.
[d] In the 798 haplotypes examined.

Statistical Analysis

Most analyses were done by use of SAS, version 6.11 (SAS Institute 1998), including log-linear and multinomial regression modeling done by means of SAS PROC CATMOD. Exact tests for combination of 2 × 2 tables were done by use of the program IC2X2 (Thomas and Gart 1992). Pairwise linkage disequilibrium was assessed by use of the program ASSOCIATE 2.33 (Ott 1996) and was quantified as the standardized pairwise disequilibrium coefficient (D') (Weir 1996).

Results

Phenotypes of Control Individuals and Individuals with CMM

One important step in this analysis was to ensure that the control individuals chosen were, in fact, representative of the Queensland population and were comparable to individuals with CMM. Sex ratios of those with CMM and of control individuals were not significantly different (60.0% female; $P = .16$), but control individuals were, on average, 12 years younger (age 47 years) than the individuals with CMM (age 60 years). Table 2 summarizes the sample numbers, according to phenotypic data collected for each part of the study. Preliminary analyses of the two control groups showed that they did not differ significantly in distribution of MC1R variants; ancestry; or eye, hair, or skin color; therefore, they have been combined in subsequent analyses. The distributions of eye and hair color were similar to those seen in other Australian (Mitchell et al. 1998) or white (Bliss et al. 1995) samples. With regard to ancestry, 86% of the overall sample had origins in the British Isles (32% from Ireland, Scotland, and Wales); 9%, in northern Europe; and 2%, in southern Europe.

Frequency of MC1R Variants in Control Populations and Populations with CMM

The allele frequencies of six MC1R variants (Val 60Leu, Asp84Glu, Arg151Cys, Arg160Trp, Asp294 His, and 537insC) in the control samples are shown in table 1. Individuals who were homozygous for the consensus MC1R sequence at these positions were 44.6% of the control population, and no control individual carried more than two variants (in contrast to the individuals with melanoma cases [see data that follow]). The 537insC insertion was not detected in these control samples, indicating that it is a rare variant within the southeast Queensland population. Despite the individual rarity of the Asp84Glu and Asp294His variants, five subjects carried both alleles, giving a maximum-likelihood estimate of 0.6% for the Asp84Glu/Asp294His haplotype frequency (expected frequency, under the assumption of no intragametic association 0.05%; $P < .001$).

When we examined the entire sample (table 2), we found a total of 128 (14.9%) individuals carrying two variants and 8 individuals (all of whom had CMM) carrying three variants (5 individuals had Asp84Glu,

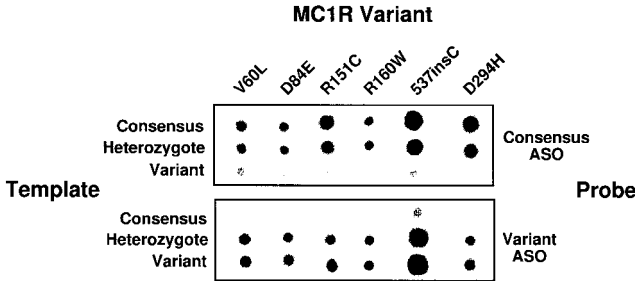

Figure 1 Use of allele-specific oligonucleotides for MC1R genotyping. A total of 100 ng each of PCR-amplified MC1R consensus and variant product were bound both independently (homozygote) and as an equimolar mixture (heterozygote) to nylon membranes and were hybridized with consensus or variant 15-base oligonucleotides, as indicated. Hybridization and washing of the [^{32}P]-labeled oligonucleotides, in the presence of TMAC, differentiates each of the six MC1R variants examined in this study.

Table 2

Phenotypic Characteristics, in Patients with CMM and in Control Subjects

Phenotype and Measure	No. of Subjects in Each Melanoma-Risk Group[a]			Total No. (%) of Subjects[b]	
	High	Intermediate	Low	With CMM[c]	Controls[d]
Hair color:					
Red/auburn	7	20	23	50 (11.6)	24 (6.2)
Fair	14	51	56	121 (28.0)	58 (15.0)
Light brown	25	64	60	149 (34.5)	138 (35.8)
Dark brown	12	45	42	99 (22.9)	144 (37.3)
Black	2	6	5	13 (3.0)	22 (5.7)
Total	60	186	186	432	386
Skin color:					
Fair	42	141	133	316 (73.5)	122 (58.9)
Medium	14	39	48	101 (23.5)	70 (33.8)
Olive/dark	4	5	4	13 (3.0)	15 (7.2)
Total	60	185	185	430	207
Eye color:					
Blue/gray	25	77	92	194 (45.4)	148 (38.0)
Green/hazel	22	81	59	162 (37.9)	145 (37.5)
Brown	12	28	31	71 (16.6)	95 (24.4)
Total	59	186	182	427	388
Freckles:					
Nil	18	49	50	117 (29.0)	66 (32.2)
Any	39	123	125	287 (71.0)	139 (67.8)
Total	57	172	175	404	205
Moles:					
Nil	2	17	19	38 (9.8)	9 (4.3)
Few	24	60	86	170 (43.8)	138 (66.8)
Moderate	25	61	57	143 (36.9)	48 (23.1)
Many	7	19	11	37 (9.5)	12 (5.8)
Total	58	157	173	388	207

[a] Melanoma-risk–group division is according to Aitken et al. (1994).

[b] Complete phenotypic data are available for only a subset of patients with melanoma and of control subjects. Data are most complete for hair and eye color. Skin and mole count are not available for one set of control subjects.

[c] Total number phenotyped = 432; total number genotyped = 460.

[d] Total number phenotyped = 389; total number genotyped = 399.

Arg160Trp, and Asp294His; 2 had Asp84Glu, Arg151 Cys, and Asp294His; and 1 had Val60Leu, Asp84Glu, and Asp294His). In one individual, the presence of parental data (not shown) allowed phase to be inferred and confirmed that the person was carrying an Asp 84Glu, Asp294His chromosome. Calculation of D′ values found statistically significant evidence of allelic association for four pairs of loci (table 3).

Association between MC1R Genotype and Skin and Hair Pigmentation

The relationship between hair color and MC1R variants is represented in figure 2. The association between total number of variants carried and red hair is striking ($P < .001$): >60% of redheads carried two or three variants, and none carried the consensus genotype. Although carriage of the Arg151Cys, Arg160Trp, and Asp294His variants increased the probability of being redheaded (odds ratio [OR] 23; 95% confidence interval [CI] 13–42; reference, consensus homozygote), the Val60Leu variant was more common among those with other hair colors ($P = .015$).

Self-reported data on constitutive skin color was available for 628 individuals (one control group was not asked about this)—433 with fair or pale skin, 167 with medium skin color, and 28 with brown or olive skin. The frequency of Arg151Cys ($P < .001$), Arg160Trp ($P = .004$), and Asp294His variants, which reflected the correlation between hair color and skin color, was increased ($P = .02$) within the fair-skinned group (fig. 3). However, the association persisted after stratification was done on the basis of ethnicity and hair color, suggesting that this was not a result of either systematic error in reporting or ethnicity. The frequency of the Val60Leu allele increased slightly, although not significantly so, with increasing pigmentation ($P = .59$). Similar trends were observed for freckling, which persisted after adjustment for hair color—for example, for the

Table 3

Standardized Pairwise Disequilibrium Coefficients (*D'*) for Each MC1R Variant

	D'				
MC1R VARIANT	V60L ($P = .82^a$)	D84E ($P = .46^a$)	R151C ($P = .31^a$)	R160W ($P = .01^a$)	D294H ($P = .25^a$)
V60L	…				
D84E	.05	…			
R151C	.15[b]	.05	…		
R160W	.12[b]	.01	.13[b]	…	
D294H	.01	.45[b]	.04	.06	…

[a] Hardy-Weinberg equilibrium.
[b] Significantly different from 0.

Arg151Cys variant versus absence or presence of freckling, the Mantel-Haenszel pooled OR was 2.1 (95% CI 1.3–3.3). By contrast, an association between eye color and MC1R genotype was not detectable in this sample. Similarly, although MC1R genotype was correlated with hair color and although hair color was significantly associated with mole count (with fair hair correlating with increased mole count and red and dark hair correlating with decreased mole count), MC1R genotype was uncorrelated with mole count in stratified analyses ($P = .30$).

Association of MC1R Genotype with CMM

Initially, we examined the known phenotypic risk factors for CMM. Individuals with CMM were significantly more likely to have fair (OR 2.0 [95% CI 1.5–2.8] reference category, black or dark brown) or red (OR 1.9 [95% CI 1.2–3.1]) hair; their eyes tended to be blue/gray (OR 1.8 [95% CI 1.2–2.7] reference category, brown) or hazel/green (OR 1.6 [95% CI 1.1–2.3]); and their constitutive skin color was lighter than that seen in controls, even after stratification was done on the basis of ethnic background (Mantel-Haenszel OR 2.0 [95% CI 1.4–2.9]; reference category, moderate or olive/dark). Finally, individuals with CMM had greater numbers of moles (46% reported a moderate number of or many moles) than did controls (29% OR 2.0 [95% CI 1.3–3.2] adjusted for hair color).

All five of the MC1R variants tested—most notably, Arg151Cys, Arg160Trp, and Asp294His—were more common in individuals with CMM than in controls (table 4; $P < .001$); however, after adjustment for both the presence of other variants and, specifically, the linkage disequilibrium between Asp84Glu and Asp294His, only the three aforementioned variants have ORs that are significantly >1. The odds of CMM occurring increased 2.2-fold (95% CI 1.6–3.0) in individuals carrying one of these three "active" variants, and it increased 4.1-fold (95% CI 2.1–7.9) if at least two variants were carried. In analyses done with adjustment for ethnicity, hair color, and skin color (table 5), we found that this risk gradient was abolished in the fair-skinned group, especially after adjustment for hair color, which was a significant risk factor. Carrying a single MC1R variant persisted in being associated with increased melanoma risk ($P = .004$) in the subgroups with a medium or olive/dark complexion (OR 2.2 [95% CI 1.2–4.1] and 10.8 [95% CI 1.2–95.6], respectively), but it had its greatest effects in individuals of non-British descent (test for interaction, $P = .02$).

Within the sample of individuals with CMM, there was no relationship between strength of family history (risk group) and MC1R genotype ($P = .27$). By contrast, there was a significant correlation between mole count and family risk index in this sample (Aitken et al. 1996).

Discussion

Two of the most prominent phenotypic risk indicators for CMM are an increased number of moles (Swerdlow and Green 1987) and the red-hair–pigmentation phenotype (Bliss et al. 1995; Grange et al. 1995). An increased number of moles exhibits a complex relationship with other pigmentary characteristics, although it is notable that mole counts have been reported to diminish in those individuals with red hair. It is clear that red hair is also associated with other pigmentary characteristics—including skin color, freckling, propensity to burn, and failure to tan—that are, in turn, indicators of CMM risk. The purpose of this investigation was to examine

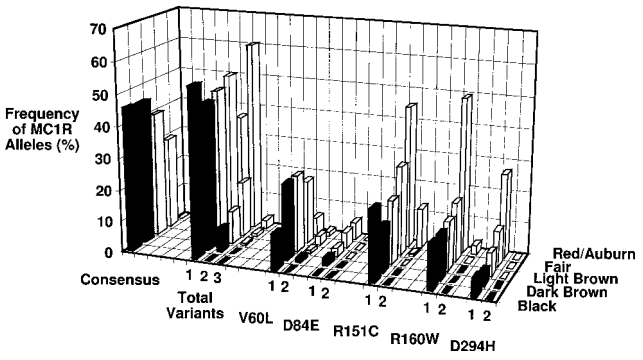

Figure 2 Association of MC1R variants with hair color. The percentage of individuals carrying a variant MC1R genotype at the five positions tested is shown, plotted against hair color. At the left-hand side of the graph are the frequencies of the MC1R consensus genotype, represented by "0," and the total for any variant genotype, represented as "1," "2," or "3" variants, in the 818 samples genotyped, for which hair-color data, which sums to 100% for each hair color, are available (table 2). The absolute percentage of each variant position considered to be independently carried on one or both alleles, represented as "1" or "2," is also plotted against hair color, which is on the right-hand side of the graph.

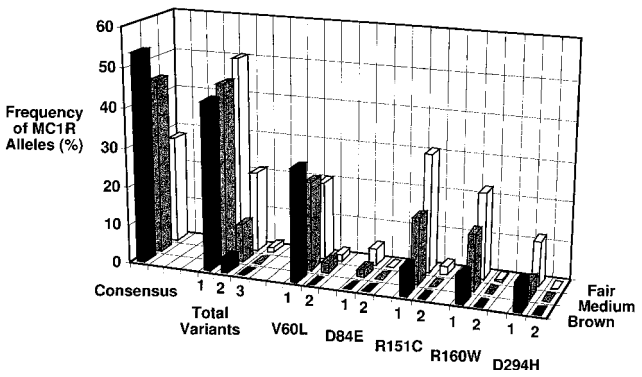

Figure 3 Association of MC1R variants with skin color. The percentage of individuals carrying a variant MC1R genotype at the five positions tested is shown, plotted against skin color. At the left-hand side of the graph are the frequencies of the MC1R consensus genotype, represented by "0," and the total for any variant genotype, represented as "1," "2," or "3" variants, in the 637 samples genotyped, for which skin-color data, which sums to 100% for each skin color, are available (table 2). The absolute percentage of each variant position considered to be independently carried on one or both alleles, represented as "1" or "2," is also plotted against skin color, which is on the right-hand side of the graph.

the underlying genetic relationship between these traits and the incidence of CMM, by assaying the frequencies of five of the more common MC1R variants found in whites in a collection of Queensland patients who had CMM.

We have used a large sample of patients with CMM and control individuals from the same population to show that an association between MC1R variants and melanoma risk exists and is mediated, in part, through known skin- and hair-pigment phenotypes, but in a complex fashion. A possible caveat of this interpretation is the degree to which the control individuals are representative of the Queensland population from which the patients with CMM were ascertained. They were, however, similar with regard to the range of pigmentation phenotypes that could be measured and compared with those found in other Australian studies (Mitchell et al. 1998). We have also examined the details of the relationship between MC1R variants, skin coloring, and hair coloring in the combined representative population sample.

Although the relationship between pigmentary phenotype and risk of melanoma has been well studied by epidemiologists, the role of the genes involved in pigment formation has yet to be fully assessed. Variants in the MC1R gene have previously been shown to be associated with red hair, pale skin, and freckling (Valverde et al. 1995; Box et al. 1997; Smith et al. 1998), and two groups have examined the relationship between particular MC1R variants and CMM. One group, led by Jonathan Rees (Valverde et al. 1996; Healy et al. 1999), genotyped 43 patients with CMM and 44 control individuals and found that carrying a variant allele increased the risk of melanoma 3.9-fold. It was reported that the Asp84Glu variant was found only among individuals with CMM, and, in contrast to the findings of that report, our results did not show a particular relationship between Asp84Glu and CMM. However, both the results of a later reanalysis (Healy et al. 1999) of the low frequency of this variant and the small sample size of the original study show that this variant is unlikely to be a major risk factor for CMM.

Another group, led by Richard Strange (Ichii-Jones et al. 1998; Strange et al. 1999), found the frequencies of the Asp84Glu and Asp294His variants to be 1.7% and 3.4%, respectively, in a series of 190 English patients with basal-cell carcinoma—very similar to the results obtained in our control group, which is largely of British descent. Asp294His was associated with red hair color (crude OR 5; exact $P = .012$; see Strange et al. [1999]) but not with skin type. Most critically, although these variants were not more common overall among 306 individuals with melanoma compared with 190 control individuals, they showed increased frequency among individuals with greater tanning ability (in Fitzpatrick skin types 3 and 4 [crude OR 3.9 {95% CI 1.7–9.3}] vs. wild-type homozygotes). Our study could detect significant association between specific variants, such as Asp294His, and CMM that the study by Ichii-Jones et al., possibly as a result of their smaller sample size, could not detect. The strong linkage disequilibrium between Asp84Glu and Asp294His in the present study's population means that it is harder to demonstrate a relationship between Asp84Glu and phenotype. Valverde et al. (1996) have pointed out that the codon 84 aspartate is highly conserved in the melanocortin receptor and in other G-protein–coupled receptor families and suggests that the glutamate substitution is likely to be functionally significant. Given its rarity relative to the other variants, it cannot explain much of the population variation in skin color and hair color.

We have found the Arg151Cys, Arg160Trp, and Asp

Table 4

Risk of CMM for Each MC1R Variant

Variant Allele	OR (95% CI) of CMM
V60L	1.3 (.9–1.7)
D84E	.8 (.3–1.9)
R151C	2.1 (1.6–3.0)
R160W	1.9 (1.3–2.7)
D294H	2.3 (1.3–4.1)

NOTE.—Logistic-regression analysis includes all variants simultaneously; the consensus sequence is the reference category.

Table 5

Association between MC1R Variants and CMM Risk, Stratified on the Basis of Skin and Hair Color

Skin Color and Hair Color	Percent (No.) Who Have MC1R Variant: Individuals with CMM		Control Individuals		Adjusted OR[a] (95% CI)
Not available:					
Red/auburn			100.0	(11)	
Fair			66.7	(27)	
Light brown			50.8	(65)	
Dark brown			47.8	(69)	
Black			62.5	(8)	
Total				(180)	
Fair:					1.2 (.7–1.9)
Red/auburn	100.0	(45)	100.0	(12)	
Fair	73.6	(110)	68.0	(25)	
Light brown	64.6	(99)	69.4	(49)	
Dark brown	71.2	(52)	61.3	(31)	
Black	62.5	(8)	42.9	(7)	
Total		(314)		(124)	
Medium:					2.2 (1.2–4.1)
Red/auburn	100.0	(4)	100.0	(2)	
Fair	87.5	(8)	16.7	(6)	
Light brown	56.8	(44)	54.5	(22)	
Dark brown	61.9	(42)	41.7	(36)	
Black	100.0	(2)	.0	(5)	
Total		(100)		(71)	
Olive/dark:					10.8 (1.2–95.6)
Red/auburn	.0	(0)	.0	(0)	
Fair	100.0	(3)	.0	(0)	
Light brown	100.0	(3)	25.0	(4)	
Dark brown	25.0	(4)	11.1	(9)	
Black	100.0	(3)	50.0	(2)	
Total		(13)		(15)	
Grand total[b]	71.7	(427)	55.9	(390)	

[a] Stratum-specific OR is adjusted for hair color, by use of the Mantel-Haenszel procedure.

[b] Total includes only those individuals with known hair color.

294His polymorphisms to be significantly associated with CMM, as well as with skin color and hair color. The most parsimonious explanation for the increased incidence of melanoma in individuals with either red or fair hair and with fair skin centers on their inability to increase melanin levels in the skin in response to high exposure to UV light. If this hypothesis is correct, then stratification done on the basis of hair color and skin color should remove any relationship between MC1R genotypes and CMM. By contrast, the alternative hypotheses are either that pheomelanin, its precursors, or photoproducts are mutagenic (Sturm 1998) or, possibly, that, as suggested by Valverde et al. (1996), this melanocortin receptor is involved in modulation of melanocyte growth and differentiation.

In the present study, we found that the first hypothesis cannot completely explain the data. Although the risk of melanoma in fair-skinned individuals varied according to hair color but not according to MC1R genotype, in individuals who reported a superior tanning ability and darker constitutive skin color, the MC1R genotype had a persisting effect on risk, even after adjustment for hair color, ethnic background, and mole count. Because the frequencies of the different MC1R variants do vary according to skin color, even when there is control for other confounders, we believe that this argues against the possibility that this effect is mediated solely by reporting bias. This explanation is made less likely by evidence relating subjective reports of skin color to objective measurements of skin reflectance and to independent clinical ratings (Green and Martin 1990; Aitken et al. 1993). Otherwise, one might suggest that light-skinned individuals who have more exposure to the sun might tend to report a darker skin color and might have more melanoma.

It is tempting to speculate that individuals who, because of their darker skin color, would normally have a decreased risk for CMM might, if carrying a variant that predisposes to production of pheomelanin, negate that protection or might, if a dose effect is present, produce more pheomelanin than would a fair-skinned individual carrying that same MC1R genotype. In keeping with this suggestion is the fact that whereas, among wild-type homozygotes, having fair skin increases the odds of CMM 2.9-fold compared with having darker skin, the odds ratio is only 1.2 for MC1R variant carriers (Breslow-Day test for difference, $P = .02$).

It is also clear, from our results, that MC1R genotype does not exert any effect on CMM risk through the intermediate variable of mole count, especially in the skin-color group in which its effect is greatest. In fact, within the pale-skinned group, red hair was associated with a decreased mole count, as has been observed in other studies (English and Armstrong 1994; Grulich et al. 1996). This lack of association tends to support such mechanisms as a direct effect of pheomelanin and is consistent with the two-hit "divergent-pathway" model suggested elsewhere (Whiteman et al. 1998). In this model, lifetime UV exposure (perhaps amplified by the presence of pheomelanin) acts to increase melanoma risk via abnormal expression of p53, and other genes, such as CDKN2A, act by inducing melanocyte proliferation and, thus, vulnerability to malignant transformation. Because known mutations of CDKN2A explain relatively little of the familial aggregation of CMM, both (*a*) the lack of a correlation between familial risk index and frequency of MC1R variants and (*b*) the presence of such a correlation between familial risk and mole count suggest that other loci, acting via this "nevus pathway," are yet to be identified, possibly close to CDKN2A itself (Zhu et al. 1999).

The precise nature of the association between MC1R genotype and hair color is best examined in individuals

carrying two variants, most of whom were compound heterozygotes. As noted, all 71 redheads in this study carried at least one variant, and 45 carried two of the five variants assayed by allele-specific oligonucleotides. Although 66% of all individuals carrying two alleles from the set including Arg151Cys, Arg160Trp, and Asp294His were redheads, the frequency of redheads dropped to 8% among those individuals in whom at least one of the two alleles was Val60Leu or Asp84Glu. In earlier work, we have shown that other MC1R variants are present in the Queensland population (Box et al. 1997). Further genotyping, plus collection of more-precise phenotypic data in a related cohort, will allow for better evaluation of the effects of particular variants—for example, particular shades of red hair and with freckling. When results of such studies are combined with data on parental coloring, it will be possible to adjust for the effects of the other putative hair- and eye-color loci (MIM 113750, MIM 227220, MIM 227240, MIM 266300, and MIM 601800).

Categorization of skin- and hair-color phenotypes is based on broad divisions that, by their generalized nature, cannot be applied as an accurate predictor of the outcome of any individual's lifetime sun exposure and resultant skin-cancer rate. It is thus necessary to seek a more direct and quantitative genotypic assessment of risk. This is relevant even among redheads, in whom significant heterogeneity of CMM risk may be present and may be regulated by MC1R variation, resulting in both visible differences in the red-hair phenotype and more-subtle differences in the eumelanin/pheomelanin ratio. It has been suggested that the eumelanin:pheomelanin ratio may differ markedly in individuals with fairly similar red-hair pigmentation and that this ratio may influence the minimal erythemal dose or UV sensitivity within these individuals (Vincensi et al. 1998). Current prevention strategies for melanoma and other skin cancers rely predominantly on campaigns that are aimed at increasing the general awareness of the damaging effects of UV radiation, as well as of encouraging those "sun-smart" behaviors that minimize levels of UV exposure within the whole of the white population, which is considered to be at high risk for skin cancer. It is clear, however, that not all whites share identical risk for skin cancers such as CMM, and better understanding of the genetic basis of UV-sensitive skin types will greatly enhance targeting of skin cancer–prevention campaigns.

In conclusion, we have shown that carrying particular MC1R variants increases CMM risk in individuals whose darker complexions would normally be considered protective. In light-skinned individuals, the association between CMM and MC1R variants disappears once skin- and hair-color phenotype are taken into account.

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Electronic-Database Information

Accession numbers and the URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CMM [MIM 155600 and MIM 155601], MC1R [MIM 155555], and hair- and eye-color loci [MIM 113750, MIM 227220, MIM 227240, MIM 266300, and MIM 601800])

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